

Viral abundance, production, decay rates and life strategies (lysogeny versus lysis) in Lake Bourget (France)

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Summary

We have investigated the ecology of viruses in Lake Bourget (France) from January to August 2008. Data were analysed for viral and bacterial abundance and production, viral decay, frequency of lysogenic cells, the contribution of bacteriophages to prokaryotic mortality and their potential influence on nutrient dynamics. Analyses and experiments were conducted on samples from the epilimnion (2 m) and the hypolimnion (50 m), taken at the reference site of the lake. The abundance of virus-like particles (VLP) varied from 3.4×10^7 to 8.2×10^7 VLP ml⁻¹; with the highest numbers and virus-to-bacterium ratio (VBR = 69) recorded in winter. Viral production varied from 3.2×10^4 VLP ml⁻¹ h⁻¹ (July) to 2×10^6 VLP ml⁻¹ h⁻¹ (February and April), and production was lower in the hypolimnion. Viral decay rate reached 0.12–0.15 day⁻¹, and this parameter varied greatly with sampling date and methodology (i.e. KCN versus filtration). Using transmission electron microscopy (TEM) analysis, viral lysis was responsible for 0% (January) to 71% (February) of bacterial mortality, while viral lysis varied between 0% (April) and 53% (January) per day when using a modified dilution approach. Calculated from viral production and burst size, the virus-induced bacterial mortality varied between 0% (January) and 68% (August). A weak relationship was

found between the two first methods (TEM versus dilution approach). Interestingly, flow cytometry analysis performed on the dilution experiment samples revealed that the viral impact was mostly on high DNA content bacterial cells whereas grazing, varying between 8.3% (June) and 75.4% (April), was reflected in both HDNA and LDNA cells equally. The lysogenic fraction varied between 0% (spring/summer) and 62% (winter) of total bacterial abundance, and increased slightly with increasing amounts of mitomycin C added. High percentages of lysogenic cells were recorded when bacterial abundance and activity were the lowest. The calculated release of carbon and phosphorus from viral lysis reached up to $56.5 \mu\text{gC l}^{-1} \text{ day}^{-1}$ (assuming 20 fgC cell^{-1}) and $1.4 \mu\text{gP l}^{-1} \text{ day}^{-1}$ (assuming $0.5 \text{ fgP cell}^{-1}$), respectively, which may represent a significant fraction of bacterioplankton nutrient demand. This study provides new evidence of the quantitative and functional importance of the viroplankton in the functioning of microbial food webs in peri-alpine lakes. It also highlights methodologically dependent results.

Introduction

Over the past 20 years, it has been realized that viruses are an important component of aquatic microbial food webs. Since they are obligatory parasites, it is assumed that they are important controlling agents of the microbial community composition, diversity and succession, and it has been shown experimentally that they play a key role in cell mortality and nutrient cycles (Fuhrman, 1999; Wommack and Colwell, 2000; Weinbauer and Rassoulzadegan, 2004). Particularly, it has been recognized that viruses are the most abundant biological particles in aquatic environments, with abundances typically ranging from 10^7 to 10^9 VLP ml⁻¹ (Bergh *et al.*, 1989; Wommack and Colwell, 2000; Sime-Ngando *et al.*, 2003) and that they can be responsible for a relevant fraction of bacterial mortality or bacterial production removal, varying typically between 10% and 60% (Fuhrman, 1999; Wommack and Colwell, 2000; Fischer and Velimirov, 2002; Bettarel *et al.*, 2003; 2004; Weinbauer, 2004; Suttle, 2007). Viral lysis, by causing the release of new viruses and host cell contents,

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can lead to a significant increase of dissolved organic carbon and phosphorus in the environment, which in turn can affect bacterial community growth and structure (Noble *et al.*, 1999; Van Hannen *et al.*, 1999; Riemann and Middelboe, 2002) and may have a large impact on bacterial carbon and/or phosphorus cycling (Dean *et al.*, 2008; Middelboe *et al.*, 2008; Riemann *et al.*, 2008).

A large majority of aquatic viral ecological studies have been carried out in seawater (Wommack and Colwell, 2000; Weinbauer, 2004; Brussaard *et al.*, 2008) and the dichotomy between marine and freshwater studies has been recently highlighted by Middelboe and colleagues (2008) in the introduction of a special issue on freshwater viruses of the journal *Freshwater Biology*. However, a variety of freshwater ecosystems has been investigated, although less often, and these include rivers (Mathias *et al.*, 1995; Farnell-Jackson and Ward, 2003), Antarctic lakes (Laybourn-Parry *et al.*, 2001; Sawstrom *et al.*, 2007), oligotrophic lakes (Hofer and Sommaruga, 2001; Bettarel *et al.*, 2004), mesotrophic lakes (Hennes and Simon, 1995; Jacquet *et al.*, 2005) and eutrophic lakes (Sommaruga *et al.*, 1995; Pradeep-Ram *et al.*, 2005), as well as lake sediments (Maranger and Bird, 1996; Filippini and Middelboe, 2007).

Recently, the largest French natural freshwater ecosystem (Lake Bourget) has been investigated concerning bacteriophage ecology. Some viral ecology data were obtained by Dorigo and colleagues (2004), while studying the cyanomyovirus diversity in this lake, detected 35 distinct cyanomyovirus *g20* genotypes in fall and winter months and a clear seasonal pattern of variation in the composition of the cyanophage community that reflects changes in its biological (e.g. *Synechococcus* spp.), chemical and/or physical environment. Researchers interested in fluctuations in the concentrations and potential activities of viruses, prokaryotes and protists in relation to environmental conditions in Lake Bourget found that variations of microbial variables were in most cases linked to biotic factors and interactions (Sime-Ngando *et al.*, 2008). Also, this study revealed that standing stocks and activities, as well as the number and levels of correlations among these microbial components, were higher in winter than in summer (Sime-Ngando *et al.*, 2008). Personnic and colleagues (2009a,b) highlighted a strong seasonal variability in viral abundance throughout the year and demonstrated viral termination of the heterotrophic bacterial blooms in autumn in this lake. Also, flow cytometry analyses allowed the discrimination of several viral groups in Lake Bourget, particularly the VLP1 (virus-like particles group 1) and VLP2 (group 2), which could be related to bacteriophages and cyanophages respectively (Personnic *et al.*, 2009b).

In the present study, we examined additional key variables and processes, which have not yet been inves-

tigated in this lake, including viral abundance, decay and production of freshwater bacteriophages at different times of the year and at two discrete depths in Lake Bourget. An accurate analysis of viral dynamics requires independent estimations of viral abundance, production, decay rates and bacterial abundance and production. The fraction of lysogenic bacterial cells (FLC) and the frequency of bacteria killed by lytic phages were also investigated, using different methods, to infer the prevalence of these two modes of infection.

Results

Environmental parameters

Surface water temperature increased from January (5.7°C) to August (24.3°C) while the temperature remained relatively constant and never exceeded 6.2°C at 50 m (Table 1). Chlorophyll *a* highest value was reached on mid-April (15.6 µg l⁻¹), associated with a high N-NH₄ concentration (45 µg l⁻¹). Low concentrations were recorded in January (1.9 µg l⁻¹) and in early August (1.4 µg l⁻¹). At 50 m, Chlorophyll *a* concentration was always below 3.5 µg l⁻¹. The decrease of Chlorophyll *a* from April to June was typical of the clear water phase, which is a result of metazooplankton grazing activity in mesotrophic lakes (Jacquet *et al.*, 2009). Total phosphorus ranged between 4 (early April) and 17 (mid-March) µgP l⁻¹ at 2 m while the concentration remained slightly higher, on average, at 50 m (for example in mid-May, 19 µgP l⁻¹). P-PO₄ was particularly low, especially in near surface waters (4 µgP l⁻¹).

Bacterial community

Bacterial abundance decreased steadily from winter to summer at 50 m (from *c.* 3 × 10⁶ to 1 × 10⁶ cells ml⁻¹), while the variation in bacterial abundance was more marked at 2 m. Indeed, bacterial community dynamics were significantly different (ANOVA, *P* = 0.012) between these two depths (Fig. 1A). For instance, a surface peak of bacterial abundance was recorded in spring with 5 × 10⁶ cells ml⁻¹ followed by a decline until the early summer, with another increase afterwards. This spatial and seasonal distribution of bacterial abundance followed phytoplankton distributions (data not shown). FCM allowed us to discriminate between High DNA (HDNA) and Low DNA (LDNA) content cells. The abundance of HDNA cells was generally higher than the LDNA cells (Fig. 1B). In spring, the per cent of HDNA cells decreased in the epilimnion (< 40%), whereas it remains relatively stable in the hypolimnion (about 50%). Bacterial production varied between 1 and 87 µgC l⁻¹ day⁻¹. Patterns of bacterial production observed at 2 and 50 m were rela-

Table 1. Evolution of environmental parameters.

	T°C		O ₂ (mg l ⁻¹)		Chla (µg ml ⁻¹)		N-NO ₃ (mg l ⁻¹)		SiO ₂ (mg l ⁻¹)		PTOT (mg l ⁻¹)		K-N-NH ₄ (mg l ⁻¹)		NTOT (mg l ⁻¹)		P-PO ₄ (mg l ⁻¹)	
	2 m	50 m	2 m	50 m	2 m	50 m	2 m	50 m	2 m	50 m	2 m	50 m	2 m	50 m	2 m	50 m	2 m	50 m
Unstratified	6.3	5.9	10.7	9.9	3.59	2.82	0.53	0.57	2.40	2.57	0.012	0.012	0.003	0.006	0.71	0.72	0.004	0.006
	0.8	0.2	0.9	0.3	2.08	0.59	0.01	0.05	0.19	0.30	0.006	0.005	0.003	0.007	0.07	0.07	0.001	0.002
Stratified	18.9	6.2	11.3	9.0	6.38	0.68	0.16	0.61	0.81	2.70	0.016	0.014	0.013	0.004	0.34	0.67	0.009	0.006
	6.4	0.04	1.6	0.5	6.03	0.61	0.12	0.09	0.79	0.15	0.016	0.004	0.018	0.003	0.09	0.08	0.010	0.003

Values were averaged over stratified (April to August) and unstratified (January to March) periods and are noted with standard deviations below.

tively similar except at the end of winter (Fig. 3A). Bacterial production was weakly higher at 50 m in winter, while the production was significantly greater in surface in both spring and summer.

Viral community

Electron micrographs allowed the observation of Lake Bourget viruses both inside and outside bacterial cells. Most particles had a head size < 70 nm in diameter. The most common particles belonged to the family of *Siphoviridae*, i.e. phages with long non-contractile tail appendages, morphotypes B1 and B2. There were comparatively less *Podoviridae* (morphotypes C1 and C2), or phages with a short conoid appendage with basal plate. Only a few *Myoviridae*, i.e. phages with contractile tails, were seen. We did not obtain enough data and in the same proportion at each sampling date to determine if there was a significant variation in the distribution of morphologies between 2 and 50 m or if the morphology changed with season (Fig. 2).

The highest VLP abundance was observed in winter with 8×10^7 VLP ml⁻¹ (Fig. 1C). Except for the end of winter, viral abundance was highest in surface waters. Over the course of the study, there was a slight decrease in viral abundance, with relatively similar dynamics between 2 and 50 m (ANOVA, $P = 0.6$).

The virus-to-bacterium ratio (VBR) varied between 10.8 ± 1.8 (April, 2 m) and 69 ± 38 (January, 50 m), and it was always higher at 50 m, a feature previously reported in French peri-alpine lakes (Personnic *et al.*, 2009a). On average VBR was 21.5 ± 8.7 at 2 m and 32.3 ± 13.4 at 50 m (Fig. 1D).

Viral production (VP) varied from 7×10^4 to 2×10^6 VLP ml⁻¹ h⁻¹, and the highest values were recorded at 2 m in winter and in spring (Fig. 3B) and in winter at 50 m. Viral production was always higher in the surface than at 50 m, especially during the spring period. Viral turnover time ranged between 2 and 22 days at 50 m and between 0.4 and 16 days at 2 m (Fig. 3C).

We used two different methods to assess the viral decay rate. With the method of Noble and Fuhrman (1997), we observed a two-step pattern for this parameter (Fig. 4A) with a substantial increase of the decay rate throughout the winter months (0–0.15 day⁻¹) and a regular and slight increase from the onset of spring to summer months (0–0.08 day⁻¹). Using KCN (Heldal and Bratbak, 1991), the patterns of viral decay were more confused with a maximal decay (0.12 day⁻¹) recorded at the end of spring (Fig. 4B). With both methods, it was not possible to determine if the decay rate was higher or lower in surface waters (due to non-significant differences in large temporal variations).

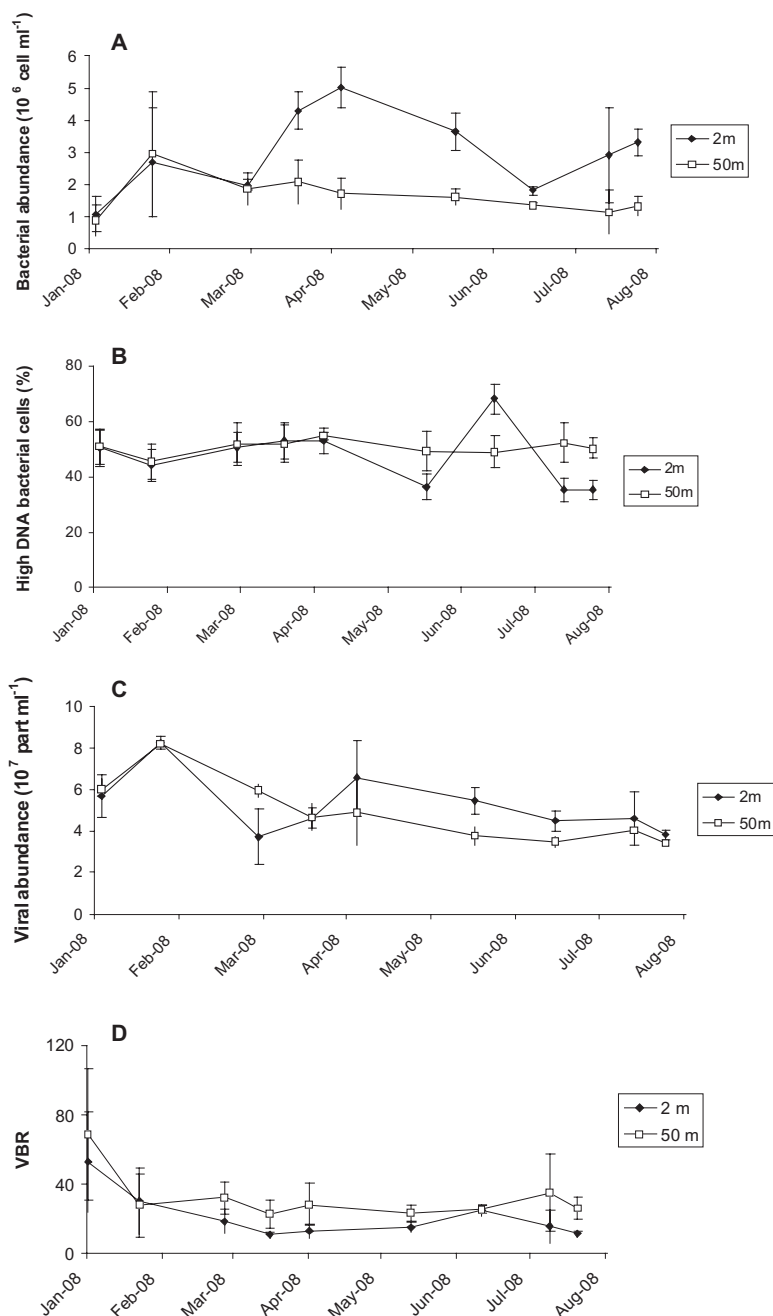


Fig. 1. Temporal variations of bacterial abundance (A), High DNA content cells (B), viral abundance (C) and VBR ratio (D) at 2 and 50 m in Lake Bourget, from January to August 2008. Error bars were calculated thanks to triplicates samples measurements except for VBR. For VBR, the error was calculated as follows: First, we calculated a per cent error for each measurement; then values were squared, added together, and finally we took the square root. Second, this per cent was multiplied by the VBR value to get the absolute error used for error bars.

Lysogeny versus lysis

From the transmission electron microscopy (TEM) analysis, we estimated that the burst size (BS) ranged from 4 to 36, the largest concentration being observed in spring at 2 m. The maximal percentage of infected heterotrophic bacteria was 40% in April at 2 and 50 m, and the virus-induced bacterial mortality (VIBM) was thus 65%. Overall, virus-induced bacterial mortality varied between 0% and 65%, with highest mortality found in June. Most of the time, the VIBM (obtained by microscopy) was significantly

higher at 50 than at 2 m (Table 2). Using another proxy of viral-mediated bacterial mortality, i.e. the ratio between VP and BS, similar values of between 0% and 62% were obtained, with the highest values recorded also at the end of spring. A weak positive relationship was found between the two methods (TEM versus ratio between VP and BS). However, these values were different from the results obtained with the dilution-FCM based method where highest VIBM were recorded in January and June (53.3% and 50.3% respectively). Thus, although the magnitude of VIBM is similar to previous two methods, the seasonal

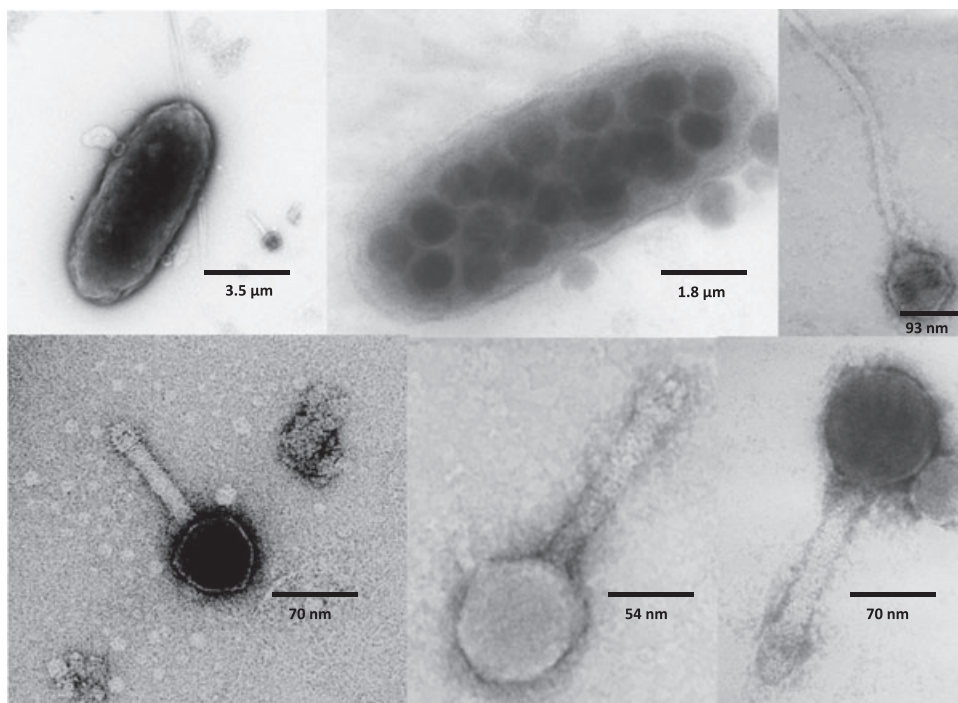


Fig. 2. Electronic micrographs of infected bacteria and some viruses.

trend was different (Table 2). FCM analysis of viruses and bacteria samples issued from the dilution experiment revealed that viruses had an effect mainly on HDNA bacterial cells because population at this size (DNA content) decreased over time while population at LDNA cell size was more stable.

The proportion of lysogenic cells was inferred from incubations with two different concentrations of mitomycin C. Using the $1 \mu\text{g ml}^{-1}$ concentration (Fig. 5A), we observed between 0% and 60% of bacterial cells were lysogenic, with highest values in winter. This frequency then decreased regularly until May and increased again during summer. The patterns were almost identical at 2 and 50 m. The same kind of temporal pattern was recorded

Table 2. Virus-induced bacterial mortality inferred from different methods: the TEM empirical equation, the viral production derivate method and the dilution approach.

	VIBM (%)		VP/BS (%)		Dilution (%)
	2 m	50 m	2 m	50 m	2 m
January	0	ND	0	2	53.3
February	ND	71.0	4	9	26.5
March	21.6	21.8	ND	ND	18.0
April	11.2	35.3	ND	55	0
May	43.2	23.1	57	17	10.7
June	64.6	ND	62	3	50.3
July	3.2	2.5	ND	ND	11.5
August	1.9	7.7	ND	68	41.9

ND, not determined.

using $0.5 \mu\text{g ml}^{-1}$ mitomycin C at 2 m except on the first sampling date and also it looks quite different at 50 m in April (Fig. 5B).

The ratio between infected cells (FIC) and mortality (VIBM) was higher in March and the FLC was lowest at the beginning of April. These two parameters were not inversely correlated ($P = 0.24$).

From the dilution experiments, we could also infer the grazing impact (by flagellates) and deduce the situations when viral lysis did or did not prevail over this grazing impact in controlling bacterial abundance at 2 m. We found that small grazers (i.e. cells $< 11 \mu\text{m}$) were responsible for 8.3–75.4% of bacterial removal, and this pressure was also for HDNA and LDNA content cells (Fig. 6). The grazing pressure was low in January (~20%) and highest in April (~75%). The grazing impact clearly exceeded viral lysis in April, but viral lysis equalled or exceeded by two- to threefold the grazing impact for the other sampling dates.

Viral-mediated nutrient release and bacterial demand

There were two maximum estimated carbon release rates from viral lysis. There were at the end of spring in the epilimnion (with $40 \mu\text{gC l}^{-1} \text{day}^{-1}$) and also in February ($56.5 \mu\text{gC l}^{-1} \text{day}^{-1}$) and could fulfil between 64% and 175% of the bacterial carbon demand assuming only 20 fgC cell^{-1} and a BGE of 0.1 in winter and 0.5 in spring.

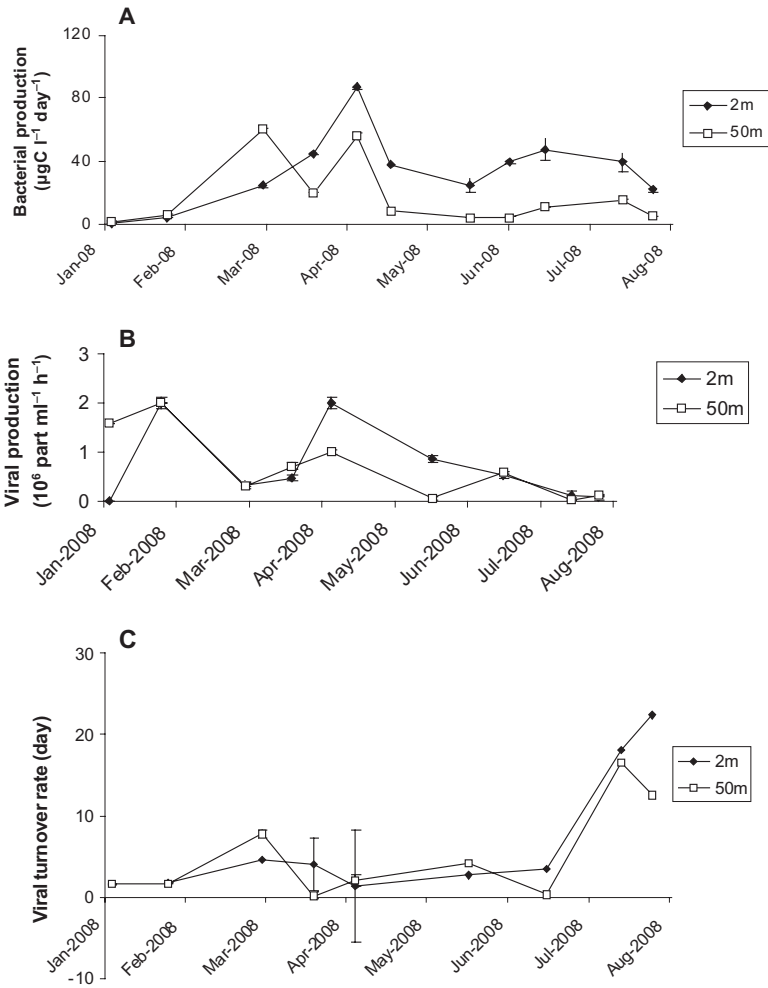


Fig. 3. Temporal variations of bacterial production (A), viral production (B) and viral turnover (C) at 2 and 50 m from January to August 2008. (A) Error bars were calculated thanks to triplicates samples measurements. (B) Error bars were calculated with confidence intervals of the slopes.

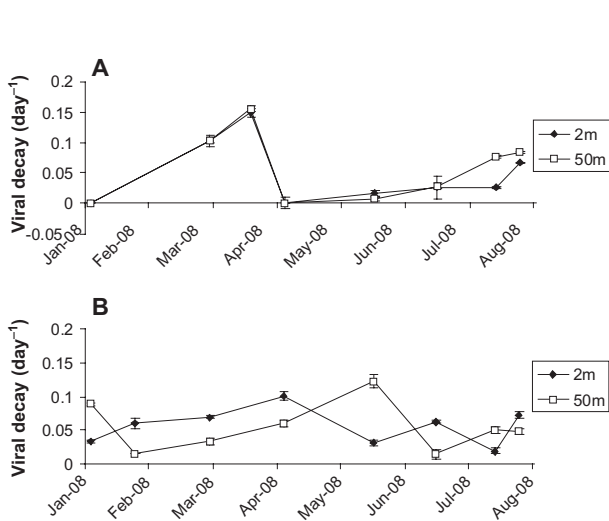


Fig. 4. Temporal variations of viral decay rates using (A) the method of Noble and Fuhrman (1997) or (B) the method of Haldal and Bratbak (1991) at 2 and 50 m from January to August 2008. Error bars were calculated with confidence intervals of the slopes.

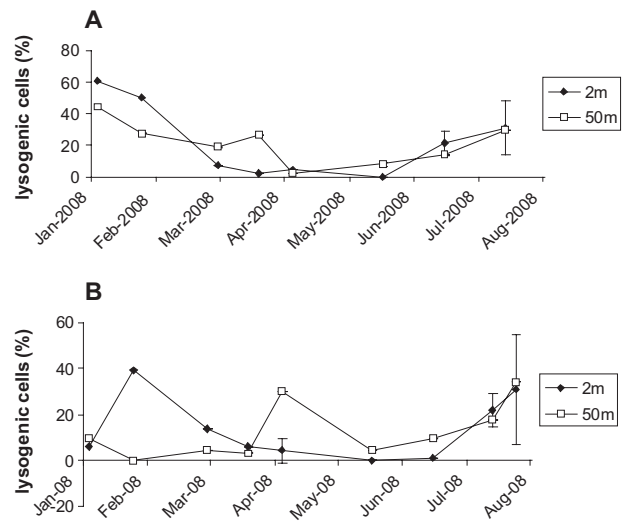


Fig. 5. Percentage of lysogenic cells, using mitomycin C 1 $\mu\text{g ml}^{-1}$ (A) or 0.5 $\mu\text{g ml}^{-1}$ (B) final concentrations, at 2 and 50 m from January to August 2008. Error bars were calculated thanks to triplicates samples measurements.

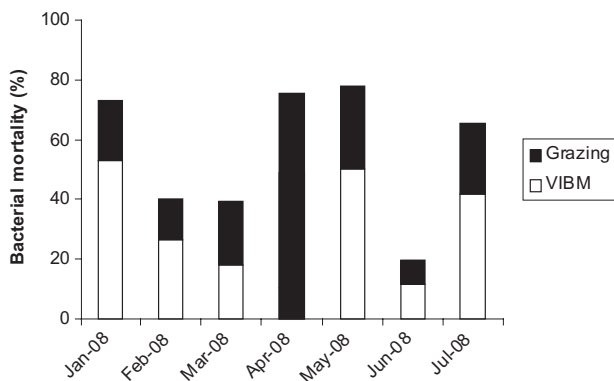


Fig. 6. Percentage of bacterial mortality due to grazing and virus (VIBM), among all sampling dates at both depth.

Viral carbon release was clearly lower in the hypolimnion ($15.1 \mu\text{gC l}^{-1} \text{ day}^{-1}$ on average). Phosphorus released through cell lysis in the epilimnion was also maximal at the end of spring and in February and was about 1.6 times lower in the hypolimnion (Table 3).

Relationships between variables

Pooling all data together (Table 4), bacterial abundance was significantly correlated to temperature and to viral decay. Bacterial production was related to the FLC and to VP at 50 m.

Discussion

This study provides new data on a variety of viral parameters in the largest French natural lake during three successive seasons. The dynamics of the microbial communities in Lake Bourget were similar to those found in many other freshwater ecosystems in which, after a winter period characterized by relatively low concentrations of the different biological groups, there is a spring bloom of several components of the food web in the epilimnion, supported by increases in temperature and light, and sustained by the presence of nutrients in surface waters (Weisse, 1993; Bettarel *et al.*, 2003). Thus, bacterial and viral abundances in Lake Bourget followed a classical seasonal pattern as observed by past during several years (Personnic *et al.*, 2009a,b). Viral abundance and production were likely influenced by host metabolic status, with higher host growth rates associated with higher temperatures, which in turn supported higher VP, abundance and turnover rates (Lenski, 1988; Corinaldesi *et al.*, 2003).

Dynamics of bacterial populations

The general dynamics of bacterial abundance and production with the significant decrease observed in May to June matched previous observations (Comte *et al.*, 2006; Personnic *et al.*, 2009a). We reported indeed that, in early

Table 3. Temporal variations of viral-induced carbon and phosphorus release by bacterial cells, from January to August 2008 and percentage of bacterial carbon demand met by viral lysis assuming 20 fg cell^{-1} .

	C released $\mu\text{g l}^{-1} \text{ day}^{-1}$ (20 fg cell^{-1})		C released $\mu\text{g l}^{-1} \text{ day}^{-1}$ (200 fg cell^{-1})		P released $\mu\text{g l}^{-1} \text{ day}^{-1}$ (0.5 fg cell^{-1})		P released $\mu\text{g l}^{-1} \text{ day}^{-1}$ (4.5 fg cell^{-1})	
	2 m	50 m	2 m	50 m	2 m	50 m	2 m	50 m
17-Jan	ND	6.1	0	61	0	0.2	ND	1.4
07-Feb	56.5	41.7	565	417	1.4	1	12.7	9.4
13-Mar	8.5	8	85	80	0.2	0.2	1.9	1.8
01-Apr	9	14.5	90	145	0.2	0.4	2	3.2
17-Apr	40	20.9	400	209	1	0.5	9	4.7
29-May	31.3	7.4	313	74	0.8	0.2	7	1.7
27-Jun	23.3	34.7	233	347	0.6	0.9	5.2	7.8
25-Jul	1.90	0.60	19	6	0.005	0.01	0.42	0.13
05-Aug	1.30	2	13	20	0.03	0.05	0.28	0.46

ND, not determined.

Bacterial carbon demand (%)	2 m	50 m
17-Jan	12	20
07-Feb	41	65
13-Mar	49	120
01-Apr	90	39
17-Apr	175	111
30-Apr	75	18
29-May	49	8
13-Jun	80	9
26-Jun	95	22
25-Jul	79	30
05-Aug	44	10

Table 4. Spearman's correlation coefficients.

	BA	BP	VA	VP	Decay	VBR	CR
2 m							
BA							
BP	0.68						
VA	0.1	0.63					
VP	0.5	0.24	0.13				
Decay	0.3	0.3	0.18	0.63			
VBR	0.04*	0.09	0.51	0.02*	0.016*		
CR	0.02*	0.29	0.09	0.017*	0.51	0.22	
FLC	0.6	0.017*	0.22	0.13	0.07	0.02*	0.002**
T°C	0.05*	0.24	0.18	0.31	0.012*	0.3	0.54
50 m							
BA							
BP	0.16						
VA	0.3	0.8					
VP	0.14	0.05*	0.09				
Decay	0.18	0.18	0.29	0.17			
VBR	0.19	0.02*	0.17	0.69	0.32		
CR	0.002**	0.26	0.02*	0.09	0.34	0.62	
FLC	0.05*	0.52	0.21	0.03*	0.17	0.04*	0.27
T°C	0.04*	0.72	0.01*	0.04*	0.04*	0.002**	0.48

* $P < 0.05$, ** $P < 0.01$.

BA, bacterial abundance; BP, bacterial production; VA, viral abundance; VP, viral production; Decay, viral decay rates; VBR, virus bacteria ratio; CR, contact rate.

summer, ciliate and flagellate predation pressure increased along with bacterial abundance and activity. Very interestingly, we observed that lysis affects mainly or even exclusively HDNA-content cells in winter while in spring and summer both LDNA and HDNA bacteria were hit by viruses. In contrast, grazers seemed to operate mainly on HDNA bacteria in March and April while the effect of these predators was more divided for the other months.

Dynamics of viral populations

As in previous studies, we could discriminate two to four subgroups of viruses (Marie *et al.*, 1999; Duhamel and Jacquet, 2006) using FCM. VLP1 was the most abundant and tightly coupled with bacteria, especially the HDNA cells. Moreover, as said above, when applying the dilution approach to assess virus-induced bacterial mortality, the viral impact was mainly observed on the HDNA bacterial cells, suggesting that HDNA cells may support a higher rate of lytic viral infection. As the dilution approach measures only lytic production, it is likely indeed that the relevant effect on HDNA cells indicates that most viruses of this group were lytic.

Logically, high viral abundances have been often coupled with high BS. In the present study, the BS was positively correlated with bacterial production, which agrees with various previous reports (Bettarel *et al.*, 2004; Parada *et al.*, 2006). Burst size increases with productivity due to faster growth rate and larger cell size, likely fuelled

by higher nutrient availability (Weinbauer and Suttle, 1999; Weinbauer *et al.*, 2003; Bongiorno *et al.*, 2005).

The high rates of viral-induced bacterial mortality suggested that viruses are the main bacterial predators in Lake Bourget mostly on each sampling dates. This has also been observed in many other aquatic ecosystems (reviewed by Weinbauer and Rassoulzadegan, 2004). This prokaryotic phage production depends on several factors, such as the contact rate between viruses and hosts, contact success or infection rate, the growth rate of the host and the BS (Weinbauer, 2004; Parada *et al.*, 2006). Viral production is counterbalanced by viral losses through viral decay. The filtration-based method for estimating viral decay (Noble and Fuhrman, 1997) is based on the removal of bacteria, suspended particles, and organic molecules or aggregates, and may result in a lower rate of virus removal from the system. Hence, viral decay rates could be partially underestimated with this method. The extent of the potential bias caused by this latter method is difficult to quantify. In sunlit waters, the decay is generally about 1–2 days (Heldal and Bratbak, 1991), and the main factors determining viral decay are UV, adsorption to particles, temperature, capsid thickness, and density of the packaged genome (Weinbauer, 2004). Our results indicate throughout this study that VP exceeded viral decay (the decay represented on average 30% of VP), causing a positive net balance of viral abundance. In deep waters, the decay was slower than in surface waters. Decay rates of viruses often increase with temperature and/or light (Bongiorno *et al.*, 2005). Thus the

low irradiance level and/or temperature of the deep water (< 7°C) could result in decay rates lower than those seen for warmer surface waters (Parada *et al.*, 2007).

Prevalence and patterns of lysogeny

Although lytic activity is generally prevalent, lysogeny can be also very important in aquatic environments (Wommack and Colwell, 2000; Weinbauer, 2004). The molecular mechanisms and environmental controls on lysogeny are still poorly understood (Weinbauer and Ras-soulzadegan, 2004; Paul, 2008). A variety of agents have been used to induce indigenous lysogenic bacteria, such as UV radiation, sunlight, temperature, pressure, aromatic and aliphatic hydrocarbons and mitomycin C. Although the latest is an artificial inducing agent, it is also the most used and allows comparison between the large majority of studies. However, we should keep in mind that mitomycin C used at the same concentration at different periods of the year may not be the best strategy to make reliable comparison. The concentration used may either kill the natural bacterial community (or a part of it) before induction occurs or not be sufficient enough to cause the induction. Additionally, different populations of bacteria in the natural community may respond very differently to treatments, and this diversity aspect has been rarely taken into account (Hewson and Fuhrman, 2007). For further studies, it would be better to consider the potential variation of lysogeny and bacterial community composition among seasons to adapt mitomycin c concentration in order to avoid the potential toxicity of this product.

In Lake Bourget, the percentage of lysogenic cells could reach 60%. The FLC was the highest in January and decreased to null at 2 m in spring. A preliminary work on lysogeny by Tapper and Hicks (1998) in Lake Superior revealed that less than 10% of the bacterioplankton contained temperate prophages, similar to our low percentages at certain periods of the year. Weinbauer and colleagues (2003) reported that the FLC was negatively related to bacterial abundance and production, and our results showed that the FLC was weakly correlated to VP ($P = 0.03$). This is consistent with seasonal studies from Tampa Bay in which lysogeny was primarily detected in winter months during periods of low host cell density and activity (Williamson *et al.*, 2002; McDaniel *et al.*, 2006). Lysogenic infection is considered the most favourable survival strategy during episodes of low energy sources of generally unfavourable conditions, particularly in waters characterized by low bacterial and primary production (Jiang and Paul, 1996; Weinbauer and Suttle, 1999; Williamson *et al.*, 2002). Indeed, there was an inverse relationship between lysogeny and the frequency of visible infected cells (FVIC), indicative of lysogeny being favoured in waters of low host abundance and productiv-

ity. A lack of induction suggests that the samples had low levels of lysogens (Jiang and Paul, 1996). Our findings support previous studies indicating that unfavourable conditions, like those encountered in winter, drive viral life strategies towards lysogenic rather than lytic infection. The seasonal distribution of lysogens was inversely related to temperature, host abundance and bacterial production as shown several times before (Williamson *et al.*, 2002; Weinbauer *et al.*, 2003; McDaniel *et al.*, 2006). The greatest occurrence of lysogeny was indeed in winter months when bacterial production was the lowest. Similar observations were made in polar oligotrophic lakes where lysogeny was mainly detected in winter and early spring (Laybourn-Parry *et al.*, 2001; Sawstrom *et al.*, 2007). It is possible that in summer, bacteria were nutrient limited and this favoured lysogeny as shown for *Synechococcus* in P-depleted conditions by Wilson and colleagues (1996) or McDaniel and Paul (2005). Lysogeny occurring mainly in winter and in summer was also observed in Blanes Bay (Boras *et al.*, 2009), but these authors could not conclude unambiguously that lysogeny was influenced by the trophic status (in summer typically). It is noteworthy, however, that other studies sustained such a hypothesis (Weinbauer *et al.*, 2003).

Interestingly, at 50 m where temperature remains relatively constant and low, FLC remained relatively high in April to May and bacterial abundance and production did not increase as at 2 m. At this depth, FLC increased again in summer to reach values up to 20–30%, but since a similar pattern was also recorded at 50 m, temperature was likely not responsible for this FLC increase. Other reasons for increased FLC include nutrient limitation or solar effect in surface although some authors reported the ability of viruses to resist to highly irradiated environment (Jacquet and Bratbak, 2003; Prigent *et al.*, 2005; Joux *et al.*, 2006), and no clear link could be detected with either bacterial abundance or production or with nutrients at 50 m. Finally, a shift from *Myoviridae*, which are mainly lytic viruses, to *Podo-* or *Siphoviridae*, which are more often related to lysogenic processes and low proliferation rates (Suttle, 2005; 2007), could explain higher FLC observed in summer. The relatively low VP recorded in summer could indeed support such a hypothesis.

Moreover, lysogeny confers immunity to infection by related viruses, resulting in resistance to viral infection, as often observed in *Synechococcus* (reviewed in Lennon *et al.*, 2007). Lysogeny has also been shown to benefit the host for a variety of reasons, including homoimmunity, conversion by phage genomes and increased general fitness (Levin and Lenski, 1983). For instance, lysogens of *Escherichia coli* have been shown to reproduce more rapidly than non-lysogens in nutrient limited chemostats (Lin *et al.*, 1977). However, this may not be always the case and Paul (2008) reported that prophage

maintenance resulted principally in a reduction in substrate utilization capability for the *Listonella pelagia* bacterial strain. Thus, the increase in fitness was due to lysogenic repressors which serve to 'throttle down' bacterial metabolism, conserving energy by shutting down unnecessary metabolic pathways and ensuring survival under unfavourable conditions. Paul (2008) proposed that marine prophages serve to repress host growth in times of resource partitioning. So, prophages repress not only their own lytic genes but also unnecessary and wasteful host metabolic process genes. When we recorded increased FLC in winter along with low bacterial production, the two may be inter-linked: low host productivity favours lysogeny, which may then enhance low host production (by suppression of host metabolic processes). This would deserve to be highlighted in forthcoming studies.

Nutrients and bacterial demand

After lysis, the high carbon, nitrogen and phosphorus content of bacteria is released in the environment and it is not surprising that it stimulates growth of non-infected bacterioplankton (Middelboe and Jorgensen, 2006; Riemann *et al.*, 2008). However, the nutritional value of lysates may depend on the prevailing nutrient limitation. We observed higher carbon and phosphorus releases in the epilimnion, especially in spring, but there was an important variability (from 1.9 to 56.5 $\mu\text{gC l}^{-1} \text{ day}^{-1}$ and from 0 and 1.4 $\mu\text{gP l}^{-1} \text{ day}^{-1}$ assuming 20 fgC cell^{-1} and 0.5 fgP cell^{-1} respectively). Using the empirical relation established by Wilhelm and Suttle (1999), we estimated how important carbon was on some occasions for bacterial growth, reaching potentially a high per cent of the C bacterial demand and indicating a significant recycling through the viral loop.

In contrast, viral mediated phosphorus release seemed to be low compared with bacterial demand, about 10-fold lower than reported in literature (Smith and Prairie, 2004). This discrepancy could be explained by basing estimations on average BGE. Indeed, the use of BGE measurements has been questioned on the basis of potentially large uncertainties resulting from methodological concerns (Goldman and Dennett, 2000).

Concluding remarks

Our data provide new evidence for the importance of considering bacteriophages for a more complete understanding of the microbial ecology of peri-alpine lakes. Clearly, this compartment and more particularly its role (as mortality agents of the bacteria versus protistan grazing or the potential significance of viral life strategies such as lysogeny as opposed to lytic in biogeochemical cycles)

revealed that these processes are crucial if one is interested in the functioning of such ecosystems. Many questions remain unanswered such as the comparison of depth to (i) infer the effect of vertical gradients on abundance; (ii) to assess variations in the diversity of prokaryotes and viruses along with variations in bacteriophage production and life strategies, with special emphasis on lysogeny (that seems very important in this lake) versus lytic activity; (iii) to determine relationships between diversity and production; and (iv) to sample on shorter time scales to understand how viral associated processes operate and vary over diurnal cycles. Finally, the comparison between two lakes of various trophic status in a same eco-region, i.e. Annecy (oligotrophic) versus Bourget (mesotrophic), is promising.

Experimental procedures

Study site and sample collection

Sampling was carried out in Lake Bourget in western Alps (45°44'N, 05°51'W, 231 m altitude). This lake has maximum and average depths of 147 and 80 m, respectively, an area of 42 km², a total volume of $3.5 \times 10^9 \text{ m}^3$, and a water residence time of approximately 8.5 years. The lake suffered from eutrophication until the mid 1980s when an important policy program helped to restore this ecosystem, whose status is now mesotrophic according to OECD criteria (Jacquet *et al.*, 2005; 2009). Polycarbonate bottles, previously sterilized using HCl and rinsed several times with milliQ water, were used to collect water samples (a total of 18) at 2 and 50 m depths, at the reference site of the lake, during nine sampling dates between January and August 2008. It is noteworthy here that the reference site, situated in the middle and deepest part of the lake (45°44'750"N, 5°51'650"E), was previously shown to be representative of the whole pelagic ecosystem (Dorigo *et al.*, 2004). Lake water was transported within a few hours to the laboratory in refrigerated boxes, and processed without delay for subsequent experiments and analyses.

Environmental variables

A conductivity-temperature-depth measuring device (SBE 19 Seacat profiler, Seabird) was used to obtain vertical profiles of water temperature, dissolved oxygen (DO sensor SBE43, Seabird) and chlorophyll *a* (using MiniBackScat Fluorometer model 1010P, Dr Haart). Nutrient concentrations (total phosphorus and nitrogen, P-PO₄, N-NO₃, N-NH₄ and Si-SiO₂) were measured at the Institut National de la Recherche Agronomique Hydrobiological Station Chemistry laboratory (Thonon-les-Bains, France). Meteorological data were obtained from a reference station based at Voglans in the southern part of the lake.

Flow cytometry analysis

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. Viruses were fixed with glutaraldehyde (0.5% final concentration, grade I, Merck) for 15 min, 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 10⁻⁴ dilution of the commercial stock solution; Molecular Probes), for 30 min at ambient temperature in the dark, followed by 10 min at 75°C, and then another 5 min at room temperature, prior to FCM analysis (Duhamel and Jacquet, 2006). Heterotrophic bacterial counts were performed on samples that were also fixed with glutaraldehyde (0.5% final concentration) for 15 min, and incubated with SYBR Green I (10⁻⁴ dilution of the commercial stock solution) for 30 min. Analysis was made on samples to which a suspension of 1 µm beads had been added (Molecular probes). Flow cytometer listmode files obtained were then transferred and analysed on a PC using the custom-designed freeware CYTOWIN (Vaulot, 1989). More details about the FCM analysis and data treatment can be obtained elsewhere (Marie *et al.*, 1999; Brussaard, 2004).

Viral production and turnover rates

In order to estimate VP, we used the dilution technique of Wilhelm and colleagues (2002). Briefly, 300 ml sample was filtered through 0.2 µm pore size polycarbonate filter. With vacuum on, the sample was mixed with 250 ml of 0.02 µm ultrafiltered water (virus-free) of the same initial sample, maintaining a final volume of 300 ml. This resulted in viruses being diluted to approximately 20% of their initial abundance. Triplicates of 100 ml were made and incubated at *in situ* temperature in the dark. Two-millilitre subsamples were collected at t₀, 3, 6, 12, 18 and 24 h. Therefore, the VP is determined from the production of new viral particles after the dilution of the initial viral abundance. Then VP rates were determined from first-order regressions of viral abundance versus time after correcting for the loss of the bacterial hosts between the experimental samples and the natural lake water community. Viral production was calculated as $VP = m \times (B/b)$ (Hewson and Fuhrman, 2007), where *m* is the slope of the regression line, *b* the concentration of bacteria after dilution, and *B* the concentration of bacteria prior to dilution. Viral turnover rates were estimated by dividing viral abundance by VP rates.

Viral decay

The viroplankton decay rates were assessed according to either the method of Noble and Fuhrman (1997) or the method of Heldal and Bratbak (1991). For the first method, filtration was performed through 0.2 µm pore size polycarbonate filters with 150 ml samples to remove bacteria and > 0.2 µm particles. Triplicates (50 ml) were made and incubated at *in situ* temperature in the dark. Viral decay rates were estimated over a 24 h period from subsamples (2 ml) collected at t₀, 3, 6, 12, 18 and 24 h. The second method consisted of using potassium cyanide (KCN, 2 mM) to inhibit the production of new viruses in natural raw samples. Viral decay rates were also estimated from triplicate samples over a 24 h period, with subsampling done at the same six time points as given above. The viral decay rate was calculated

from the log-linear part of the decay curves using linear regression. We also used the ratio of the VP to viral decay as a proxy of viral dynamics.

Bacterial lysogenic fraction

The fraction of bacteria that were lysogenic was estimated by adding mitomycin C (Paul, 2008) to three replicates of 50 ml samples of lake water at a final concentration of 0.5 and 1 µg ml⁻¹. Samples were incubated in the dark at *in situ* temperature. Two-millilitre subsamples were collected as above (six time points over a 24 h period) and analysed for bacterial and viral abundances. The fraction of lysogenic bacterial cells (FLC) was calculated using the formula of Weinbauer and colleagues (2003), i.e. $FLC(\%) = 100 \times [(V_{mc_{24}} - V_{c_{24}})/(BS \times BA_{t_0})]$ using BS (the number of viruses liberated from a bacterium as a result of lytic infection) obtained from transmission electronic microscopy analyses. Burst sizes ranged from 4 (50 m, May) to 34 (50 m, January).

Phage morphology, phage-infected bacteria, burst size and bacterial mortality induced by viral lysis from TEM analysis

Bacteria contained in 15 ml aliquots of glutaraldehyde-fixed samples were harvested by ultracentrifugation (Centrikon Beckman) at 116 000 *g* for 120 min at 4°C onto 400-mesh electron microscope grids with carbon-coated formvar film. Each grid was then stained for 30 s with uranyl acetate (2% wt/wt) and examined at ×13 000 to ×40 000 by using a Philips CM12 TEM to distinguish between virus-infected and uninfected bacteria. A bacterium was considered infected when at least three phages, identified by shape and size, were clearly visible inside the host cell. At least 100 bacterial cells were inspected per grid. To estimate the VIBM, the frequency of infected cells (FIC) was calculated from the FVIC (as a per cent) using the formula $FIC = 9.524FVIC - 3.256$ (Weinbauer, 2002). FIC was then converted to VIBM according to the method of Binder (1999): $VIBM = (FIC + 0.6FIC^2)/(1 - 1.2FIC)$.

Phage morphology was recorded through the observation of about 100 particles per month at each depth from January to June. Phage particles were classified according to Ackermann and Krisch (1997).

Bacterial mortality induced by viral lysis from dilution experiments

Briefly, 15 l of water samples from only the 2 m depth was serially filtered through 11 µm mesh filters, then 0.7 µm glass fibre filters (Whatman) and 0.45 µm polycarbonate filters (Whatman) to eliminate zooplankton, phytoplankton and bacteria respectively (Evans *et al.*, 2003). We used the 0.45 µm filtrate rather than 0.2 µm filtrate to insure that all viruses remained in the filtrate (less than 1% bacteria passed through). For the second parallel dilution series, another filtration step was added by subjecting 10 l of the filtrate to tangential ultra-filtration using a mini-ultrasette with a 30 kDa

cut-off membrane to remove all particles (including viruses). Filtration efficiency was verified subsequently using FCM (always > 95%, Personnic *et al.*, 2009b). The two parallel dilution series (i.e. using < 0.2 and < 0.02 µm lake water as diluents) of natural freshwater were then prepared to contain 1 l of 20%, 40%, 60%, 80% and 100% of the < 11 µm lake water. Two control bottles contained only diluents. All dilutions were prepared in acid-washed and water-rinsed 2 l polycarbonate bottles which were incubated for 48 h at equivalent ambient temperature. Bacterial net growth rates were calculated from the change in abundance at t₀, 24 and 48 h time points. The slope of the regression lines gives the percentage of mortality due to grazing (diluent 0.2 µm) or grazing plus lysis (diluent 0.02 µm) following the equation (Suzuki *et al.*, 2002): % of mortality = [1 - EXP(Slope)] × 100. All coefficients of correlation R^2 were statistically tested (Pearson comparison) to verify that the slope value could be used (Personnic *et al.*, 2009b).

Another way of calculating the viral-mediated mortality of bacteria involves calculating the ratio between VP and BS (Helton *et al.*, 2005; Winget *et al.*, 2005). The fraction of bacterial production lysed by viruses per day could thus be estimated by dividing the average bacterial production (cells l⁻¹ day⁻¹) by the viral-mediated mortality of bacteria determined previously.

In addition, we calculated the ratio of VP to bacterial production as an index of the viral lytic pressure as proposed by Motegi and colleagues (2009).

Bacterial production

Heterotrophic bacterial production was estimated from the rate of [³H]Leucine incorporation. For each sample, three replicates and one formaldehyde (2% final concentration)-killed control were incubated in Pyrex glass bottles containing 80 nM [³H]Leucine (specific activity, 73 Ci mmol⁻¹; Amersham) and held *in situ* temperature. Incubations were stopped after 2 h by the addition of formaldehyde to a 2% final concentration. Samples were then filtered onto cellulose nitrate filters (pore size, 0.22 µm; Millipore) and extracted with 5% ice-cold trichloroacetic acid for 5 min. Three millilitres of scintillation cocktail was added to each vial, and radioactivity was determined using a liquid scintillation counter. The quantity of [³H]leucine incorporated into protein was converted into bacterial production by using the formula of Kirchman and colleagues (1985).

Virus-induced nutrient release from bacteria

We determined the rate of phosphorus remobilization from bacteria to the DOM/POM pool by viral lytic activity. Virus-mediated mortality rates were calculated by dividing VP rate by BS (measured by TEM), resulting in an estimate of the bacterial cells lysed per ml and per hour (Wilhelm *et al.*, 2002). From this inferred mortality, the potential amounts of P release as a result of viral lysis was estimated from quotas of P from heterotrophic bacterial cells, which can range from 0.5 fg cell⁻¹ (Heldal *et al.*, 1996) to 4.5 fg cell⁻¹ (Makino and Cotner, 2004; Dean *et al.*, 2008). For carbon, we used a range of 20–200 fgC cell⁻¹ (reviewed in Fukuda

et al., 1998). Bacterial carbon demand was calculated using a growth efficiency varying according to season between 0.1 and 0.5 (Middelboe *et al.*, 1992; Del Giorgio and Cole, 2000).

Statistical analyses

Differences in biological variables compared with qualitative variables were tested using the non-parametric Kruskal–Wallis test (5% and 1%). We used one-way ANOVA when data were modelled with the normal distribution. Relationships between data sets were tested by Pearson correlation analysis. All statistical analyses were performed using SigmaStat 3.1 software.

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