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1 High Lytic Infection Rates but Low Abundances of Prokaryote  
2 Viruses in a Humic Lake (Vassivière, Massif Central, France)

3

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11 **Running title: High viral infection in a humic lake**

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23

## 1 Abstract

2 We explored the abundance and infection rate of viruses on time series scale in the euphotic zone of  
3 the humic mesotrophic Lake Vassivière (Massif Central, France), in comparison to non-humic lakes of  
4 contrasting trophic (i.e. the oligomesotrophic Lake Pavin and the eutrophic Lake Aydat) located in the  
5 same geographical region and sampled during the same period. In Lake Vassivière, the abundances of  
6 virus-like particles (range =  $1.7 - 2.6 \times 10^{10} \text{ l}^{-1}$ ) were significantly ( $p < 0.001$ ) lower compared to  
7 Lakes Pavin and Aydat. The percentage of viral infected prokaryotic cells (mean = 18.0%) was  
8 significantly higher ( $p < 0.001$ ) in Vassivière than in Pavin (mean = 11.5%) and Aydat (mean = 9.7%).  
9 In Vassivière, the abundance of prokaryotes was a good predictor ( $r = 0.78$ ,  $P < 0.001$ ) of the number  
10 of virus-like particles, while the potential grazing rate from heterotrophic nanoflagellates was  
11 positively correlated to the viral infection rate ( $r = 0.75$ ,  $p < 0.001$ ,  $n = 20$ ), indicating the prevalence  
12 of cycling interactions among viruses, prokaryotes and grazers, which is in agreement with past  
13 experiments. The absence of correlation between chlorophyll *a* concentrations (Chl) and viral  
14 parameters suggested that the resources for the lytic activity of viruses in Vassivière were mainly  
15 under allochthonous control, through host activity. Indeed, compilation of data obtained from several  
16 non-humic lakes in the French Massif Central revealed that Chl was positively correlated to the  
17 abundance of virus-like particles at concentration above  $0.5 \mu\text{g Chl L}^{-1}$ , and negatively at  
18 concentration below  $0.5 \mu\text{g Chl L}^{-1}$ , suggesting that phytoplankton-derived resources could force  
19 prokaryotic growth to attain a certain threshold level when the host availability is sufficient to boost  
20 the proliferation of viruses. Therefore, based on the high level of lytic infection rates in Lake  
21 Vassivière, we conclude that viruses are key agents for prokaryotic mortality and could influence the  
22 food web dynamics in humic lakes, which may ultimately depend on the internal cycling of resources  
23 and, perhaps, mainly on the allochthonous inputs and the associated humic substances.

24

25 **Key words:** Humic lakes, Microbial ecology, time series data, viruses, prokaryotes, Lytic infection

26

## INTRODUCTION

Our conceptual understanding of the function and regulation of aquatic systems, from microbial to global biogeochemical processes, has changed with the discovery of the abundance and activities of viruses (7, 44, 56). Research over the past two decades has firmly established that viruses are the most abundant and diverse biological entities (2), thereby forming an integral component of the microbial food web in the great variety of aquatic environments (10, 29, 55). Viral lysis plays fundamental roles in cycling nutrients and organic matter (57), structuring the microbial food web dynamics (15), governing microbial diversity (49) and, to a lesser extent, by being a potential food source for protists (17). The distribution of viruses is known to be determined by factors that affect the activity and density of the host populations, mainly prokaryotes (14, 33). Reports have suggested that on average 10-40% of the prokaryotic production is lysed by viruses in both marine and freshwaters (48, 55) and, at times, can match grazing by bacterivorous protists as a source of prokaryotic mortality (14, 33). This is a significant departure from the traditional view that predation and resource availability are the main factors controlling prokaryotic abundance and production in pelagic systems.

Studies on the factors that may control the distribution of virioplankton on large spatial scales are limited (13), especially for freshwater lakes. As lakes are characterized by steep changes in environmental gradients over depth, most of the studies on viral ecology have focused on the vertical spatial variability (11, 16, 37) rather than trophic gradients (13). The apparent positive relation reported between viral lytic infection and trophic state of aquatic systems is based more upon extrapolation than on direct measurements (12, 14). Few investigations conducted in Swedish lakes have revealed a very consistent relationship between lake trophic status and viral induced prokaryotic mortality. Moreover it has also been suggested that viral induced mortality may be more important in oligo-mesotrophic compared to eutrophic lakes (4, 47). Therefore, existing data on the relationship between lake trophic status and viral induced prokaryotic mortality are inconsistent.

Besides trophic status, another important characteristic of the lakes is the humic content. Food webs in humic lakes are known to function differently compared to those in clear water lakes due to the so called 'reversed microbial loop' (sensu 22), and have unusual microbial pathways (46). Humic lakes which are traditionally viewed as unproductive environments are often characterized by low levels of inorganic nutrients and photosynthetic activity (22). However, such systems are often supported by high levels of prokaryotic secondary production and biomass through increased inputs of high concentrations of dissolved organic matter from allochthonous inputs, which force the system to net heterotrophy (23). Several studies in humic lakes have focused on the nutrient limitation (22) and grazing loss of bacterioplankton (21). However, published reports on viral abundance and phage infection in such environments are limited (25, 47).

Although a body of data is now available on the significance of viruses to prokaryotic mortality in aquatic systems, it is largely unclear as to which factors will determine their importance,

1 specifically in humic lakes. In the present study, a comparison of a mesotrophic humic lake  
2 (Vassivière) with an oligomesotrophic lake (Pavin) and a eutrophic lake (Aydat), all located in the  
3 French Massif Central, was carried out on a time series scale to determine the interactions of viruses  
4 with other microbial components, together with physico-chemical parameters. In addition to the  
5 above, depth related variability in viral and prokaryotic parameters was also examined, but only in the  
6 Lake Vassivière during the stratified summer period. The main aim of the present investigation was to  
7 evaluate the dynamics of bacterioplankton, virioplankton and phage infected bacterioplankton in a  
8 humic lake on a time series scale, and discuss these findings in the light of those found in non-humic  
9 lakes of the same geographical region. As humic lakes were classified as nutrient poor environments  
10 (“dystrophic”), we hypothesize that viral abundance and infection rates will be lower compared to  
11 productive non-humic lakes (4, 37). The present study sought to uncover the environmental factor(s)  
12 for the variations in viral abundance, infection rates and burst size in humic lakes, and bring out the  
13 relative importance of viral induced versus the potential grazer induced prokaryotic mortality.

14

15

## MATERIALS AND METHODS

16 **Study sites.** Samples were collected from three freshwater lakes, namely the mesotrophic humic Lake  
17 Vassivière, the oligomesotrophic Lake Pavin, and the eutrophic Lake Aydat, which differed in  
18 watershed characteristics but were located in the same regional area, the French Massif Central. The  
19 characteristics of the studied lakes are presented in Table 1. Unlike Lake Pavin, both Lakes Vassivière  
20 and Aydat receive high input of organic matter from terrestrial sources. While the humic Lake  
21 Vassivière is surrounded by peat bog, wetland, heath lands and forests of pine, oak and beech, Lake  
22 Aydat is largely surrounded by intensive agricultural lands. There is no riverine inflow in Lake Pavin  
23 and the watershed essentially consists of beech forests.

24

25 **Sampling.** During all sampling occasions, integrated water samples, representative of the whole  
26 euphotic zones, were obtained from Lakes Vassivière, Pavin, and Aydat. Water samples from the lakes  
27 were collected every month from April to December 2007. All samples were collected manually at the  
28 deepest central point of the lakes, by using a flexible plastic tube (diameter, 4 cm) provided by a rope  
29 connecting the weighted bottom of the tube with a surface manipulator. Analytic samples were thus  
30 considered as integrated samples representative of the euphotic layers of the lakes. In addition, water  
31 samples from Lake Vassivière were collected with a 10L Van Dorn Bottle at different depths  
32 (including aphotic depths) of the water column (i.e. at 1, 3, 8, 10, and 19 m) during the stratified  
33 period (June, July and August 2007), to determine the depth related variability in the abundance of  
34 prokaryotes, viruses and the percentage of infected prokaryotic cells. All samples were collected in  
35 triplicates, i.e. from three independent sampling operations. Collected samples were immediately pre-

1 filtered through a 150  $\mu\text{m}$ -pore-size nylon filter (to eliminate the predatory metazoan zooplankton)  
2 when poured into clean recipients previously washed with the lake water.

3  
4 **Physicochemical analyses.** Water temperature and dissolved oxygen concentration were measured *in*  
5 *situ* with a WTW OXI 197 multiparametric probe. Secchi depth (Zs) measurements were used to  
6 estimate the euphotic depths (Zeu) in the sampled lakes, based on the general limnological assumption  
7 that Zs corresponds to the depth of approximately 10% of surface light. This assumption has been  
8 shown to be approximately correct in a variety of inland water bodies, especially during the ice-free  
9 period (cf. 34). Samples for nutrients, namely total nitrogen and total phosphorus, were analyzed  
10 spectrophotometrically (3, 45). Total organic carbon concentrations were determined by high  
11 temperature combustion in an Apollo 9000 TOC analyzer set at 700°C and calibrated with standard  
12 additions of potassium hydrogen phthalate, with a precision of 0.2  $\mu\text{M}$  (34). Chlorophyll *a*  
13 concentrations (Chl) were determined spectrophotometrically from samples (500 ml) collected on  
14 Whatman GF/F filters. Pigments were extracted in 90% acetone overnight in the dark at 4°C, and  
15 concentrations were calculated from SCOR UNESCO (40) equations. Nutrients and Chl  
16 concentrations were analyzed in the triplicate samples.

17  
18 **The abundances of prokaryotes and viral-like particles.** For the measurements of viral-like particle  
19 (VLP) and prokaryotic (PA) abundances, 50 ml of water samples were fixed with 0.02- $\mu\text{m}$  filtered  
20 buffered alkaline formalin (final concentration 2% v/v, from a 37% w/v solution of commercial  
21 formaldehyde). Subsamples (1–2 ml) were then filtered (<15 kPa vacuum) through 0.02- $\mu\text{m}$  pore size  
22 Anodisc filters (Whatman, Maidstone, England), with 1.2  $\mu\text{m}$  pore size cellulose acetate backing  
23 filters. After they were stained with SYBR Green I fluorochrome (Molecular Probes Europe, Leiden,  
24 Netherlands) (final dilution,  $2.5 \times 10^{-3}$  fold) as described by Noble and Fuhrman (32), filters were air  
25 dried on absorbent paper and mounted between slides and glass coverslips with the mountant  
26 Glycerol/PBS solution (i.e. Citifluor, London, UK) amended with a special antifading, i.e. ca. 20%  
27 (v/v) of Vecta Shield (Vector Laboratories Inc., Burlingame, CA). This amendment significantly  
28 reduced fading of the fluorochrome and gave highly stable fluorescence (36). Slides were stored at -  
29 20°C and counted within a week using a model 300F epifluorescent microscope (Leica DC).  
30 Prokaryotes were distinguished from VLP on the basis of their relative size and brightness. A blank  
31 was routinely examined to control for contamination of the equipment and reagents.

32  
33 **Lytic phage infection.** Viral lytic infection was inferred from the percentage of visibly infected cells  
34 (%VIC) according to Pradeep Ram and Sime-Ngando (36). Prokaryotic cells contained in 8 ml  
35 formalin fixed water samples were collected on copper electron microscope grids (400-mesh, carbon-  
36 coated Formvar film) by ultracentrifugation. Each grid was stained at room temperature (ca. 20°C) for

1 30 s with Uranyl acetate (2 % w/w), rinsed twice with 0.02 µm-filtered distilled water, and dried on a  
 2 filter paper. Grids were examined using a JEOL 1200Ex transmission electron microscope operated at  
 3 80kV at a magnification of 20,000 to 60,000X to distinguish between prokaryotic cells with and  
 4 without intracellular viruses. A prokaryote was considered infected when at least five viruses,  
 5 identified by their shape and size, were clearly visible inside the host cell (51). At least 600  
 6 prokaryotic cells were inspected per grid to determine % VIC. Burst size (BS, viruses prokaryote<sup>-1</sup>)  
 7 was estimated for every infected cell as average number of viral particles in a minimum of 15 visibly  
 8 infected prokaryotes. Because mature phages are visible only late in the infection cycle, % VIC counts  
 9 were converted to the percentage of infected cells (%IC) using the equation  $\%IC = 9.524 \%VIC -$   
 10  $3.256$  (53). Assuming that in steady state, infected and uninfected cells were grazed at the same rate,  
 11 and that the latent period equalled the prokaryotic generation time, %IC was converted to prokaryotic  
 12 mortality (VIBM, as percentage of prokaryotic production) using the equation:  $VIBM = (\%IC + 0.6$   
 13  $\%IC^2)/(1 - 1.2\%IC)$  (5).

14  
 15 **Contact rate.** The rate of contact ( $R$ ) between viruses and bacteria was calculated by using the  
 16 following formulae (30):  $R = (Sh2\pi wDv)VP$ , where  $Sh$  is the Sherwood number (1.06 for a prokaryotic  
 17 community with 10% motile cells) (57),  $w$  is the cell diameter (calculated from the mean prokaryotic  
 18 cell volume assuming that the cells are spheres),  $V$  and  $P$  are the abundances of viruses and  
 19 prokaryotes, respectively, and  $Dv$  is the diffusivity of viruses:  $Dv = kT/3\pi\mu dv$ , where  $k$  is the  
 20 Boltzmann constant ( $1.38 \times 10^{-23} \text{ J K}^{-1}$ ),  $T$  is the *in situ* temperature (in degrees Kelvin),  $\mu$  is the  
 21 viscosity of water (in pascals per second;  $\mu$  was calculated from values given by Schwörbel (39) for  
 22 temperatures in the range from 4 to 15°C), and  $dv$  is the mean ( $\pm$ SD) diameter of the viral capsid,  
 23 estimated at  $55 \pm 10$  nm for Lake Pavin (11), and at  $65 \pm 15$  nm for Lake Vassivière and Aydat  
 24 (Pradeep Ram *unpublished data*). The contact rate was corrected for prokaryotic abundance to  
 25 estimate the number of contacts per cell on a daily basis (50).

26  
 27 **Heterotrophic nanoflagellate abundance and grazing potential in Lake Vassivière.** Since time  
 28 series data on heterotrophic nanoflagellates (HNF) and potential flagellates grazing rates (FG) are  
 29 already available from previously published reports in Lakes Pavin and Aydat (cf. 4), the above  
 30 mentioned variables were determined only for Lake Vassivière in this study. Samples for the  
 31 measurements of HNF abundance were fixed immediately after sampling with alkaline Lugol solution  
 32 (final concentration, 0.5%) and decolorized with borate-buffered formalin (final concentration, 2%).  
 33 Primulin-stained HNF were then collected on 0.8-µm polycarbonate black filters (25 mm diameter)  
 34 and counted under UV excitation in a LEICA epifluorescent microscope (9). At least 20 microscopic  
 35 fields and 200 HNF cells were counted per slide. To estimate the rate of potential bacterivory by HNF  
 36 in Lake Vassivière, an approach to use an average flagellate clearance rate ( $1.9 \text{ nl ind}^{-1} \text{ h}^{-1}$ ) obtained

1 from published reports in freshwater lakes (see 25) was used for calculation. Potential HNF grazing  
2 rates ( $\text{cells ml}^{-1} \text{ h}^{-1}$ ) was calculated as follows: *in situ* prokaryotic abundance x *in situ* HNF abundance  
3 x the mean flagellate clearance rate ( $1.9 \text{ nl ind}^{-1} \text{ h}^{-1}$ ).

4  
5 **Statistical analysis.** Differences in physicochemical and biological variables between lakes and  
6 seasons (Spring: April – June, Summer: June – September, and Autumn: September – December) were  
7 tested by one-way analysis of variance (ANOVA). Interactions between sampled depths and months  
8 were tested in Lake Vassivière by two-way ANOVA. Linear regression analysis was used to test the  
9 relationship between heterotrophic nanoflagellate grazing and viral infection of prokaryotes, and  
10 between chlorophyll concentrations and viral abundance. Potential relationships among variables were  
11 tested by linear pair-wise correlations (i.e., Pearson correlation analysis) and stepwise multiple  
12 regressions. Data were log transformed to satisfy the requirements of normality and homogeneity of  
13 variance necessary for parametric statistics. All statistical analyses were performed with Minitab  
14 software for Windows (Release 12, Minitab).

## 15 16 **Results**

17 **Water chemistry.** The mean physico-chemical and microbiological characteristics of the sampled  
18 euphotic zones of the lakes under study are listed in Table 2. Water temperature showed strong  
19 ~~seasonal~~ changes ( $p < 0.01$ ) with sampled months in the euphotic zone of the three lakes, which were  
20 typical of temperate systems. The euphotic depth in Lake Vassivière ranged from 1.5 to 4.5m with a  
21 mean Secchi value of 2 m, and was generally well oxygenated (mean  $\pm$  SD =  $9.3 \pm 2.4 \text{ mg l}^{-1}$ ) during  
22 the entire study period. The three lakes differed significantly ( $p < 0.01$ ) in terms of total nitrogen,  
23 organic carbon and chlorophyll concentrations. The lowest concentration of total phosphorus ( $0.02 \pm$   
24  $0.009 \text{ mg l}^{-1}$ ) and the highest concentration of total organic carbon ( $7.6 \pm 2.1 \text{ mg l}^{-1}$ ) were recorded in  
25 Lake Vassivière where total organic carbon was significantly higher and varied significantly ( $p <$   
26  $0.001$ ) with time, compared to other lakes (Table 2). No clear trend in Chl with sampled months was  
27 observed in Lake Vassivière, with the values being significantly lower ( $p < 0.01$ ) than in Lake Aydat  
28 but higher ( $p < 0.01$ ) compared to Lake Pavin (Table 2).

29  
30 **Standing stocks of prokaryotes and virus-like particles.** We looked for evidence of time series  
31 variability in VLP and PA and the differences between the lakes under study. In Lake Vassivière, VLP  
32 and PA ranges were at  $1.7\text{-}2.6 \times 10^{10} \text{ l}^{-1}$  and  $4.3 - 6.5 \times 10^9 \text{ cells l}^{-1}$ , with the highest values noted in  
33 May and June, respectively. Similar peaks were also noted in Lake Aydat but in June and July,  
34 respectively, while in Lake Pavin both maxima were observed latter in August (Fig. 1A, B). For the  
35 three lakes, the time series variability in VLP and PA was thus generally weak and non significant,  
36 when excluding two spring peaks noted in May and June for VLP in Lake Aydat (Fig. 1A). In spite of



1 significantly ( $p < 0.001$ ) lower VLP in Lake Vassivière compared to Lakes Pavin and Aydat,  
 2 prokaryotic standing stock in Vassivière equaled that in Aydat and was even significantly higher ( $p <$   
 3  $0.001$ ) compared to Pavin (Table 2). Virus-to-prokaryote ratios in Lake Vassivière ranged from 2.6 to  
 4 4.3, with the average being significantly lower ( $p < 0.003$ ) than in Lakes Pavin and Aydat (Table 2).  
 5 VLP was significantly correlated to PA in Vassivière ( $p < 0.001$ ) and in Pavin ( $p < 0.001$ ), and to Chl  
 6 in Pavin (Table 3).

7  
 8 **Phage infection and burst size.** The time series variability in the percentage of infected cells (%IC)  
 9 in Lake Vassivière varied in the range of 9.0% and 25.3% , with a mean value ( $18.0 \pm 4.7\%$ ) that  
 10 corresponded to  $25.6 \pm 8.9\%$  of viral-induced prokaryotic mortality (i.e. VIBM). Maximum value of  
 11 %IC was observed in May, which coincided with peak in VLP (Fig. 1A, D) and corresponded to a  
 12 VIBM level of 41.9%. In contrast to VLP, %IC in Lake Vassivière was significantly ( $p < 0.007$ )  
 13 higher than in Lakes Pavin (range = 6.3 – 24.7%, mean  $\pm$  SD =  $11.5 \pm 5.4\%$ ) and Aydat (range = 3.8 –  
 14 18.0%, mean  $\pm$  SD =  $9.7 \pm 4.8\%$ ) (Table 2, Fig.1D). %IC was significantly correlated to VLP and PA  
 15 in Lake Vassivière and Pavin, and to the water temperature in the three lakes (Table 3).

16 The mean number of intracellular viruses observed per infected cell in Lake Vassivière varied  
 17 from 6 to 42 and averaged  $17 \pm 4$  viruses prokaryote<sup>-1</sup>, which was significantly lower ( $p < 0.001$ )  
 18 compared to the other sampled lakes. In Lakes Vassivière and Aydat, the variation observed for %IC  
 19 was reflected in the BS and both were significantly correlated ( $p < 0.001$ ) to each other (Table 3).

20 In Lake Vassivière, data on VLP, PA and %IC during the stratification period (i.e. June, July,  
 21 and August 2007) have been pooled and presented in Fig.2. VLP, PA and %IC values were  
 22 significantly higher ( $p < 0.001$ ) in the euphotic (< 5 m) than in the aphotic depths (5-20 m). During the  
 23 above period, 2-way ANOVA indicated that both VLP ( $F_{(2,4)} = 17.4$ ,  $p < 0.001$ ) and %IC ( $F_{(2,4)} = 17.7$ ,  
 24  $p < 0.001$ ) varied significantly with sampling month and decreased with depth. This contrasts with PA  
 25 for which the temporal (i.e. for the three summer months) and depth-related variability was low and  
 26 not significant ( $F_{(2,4)} = 0.12$ ,  $p > 0.05$ ).

27  
 28 **Contact rates.** Theoretical contact rates between viruses and their potential prokaryotic hosts, which  
 29 are necessary to quantify the rate of successful infection, were calculated according to the model of  
 30 Murray and Jackson (30). In Lake Vassivière, the specific contact rate (i.e., the number of viruses  
 31 encountering a single prokaryote per time) ranged between 84 and 253 and averaged 174 contacts cell<sup>-1</sup>  
 32 d<sup>-1</sup> (Table 2), with a peak in June. Specific contact rates in Lake Vassivière were similar to those  
 33 calculated for Lake Pavin (mean = 183 contacts cell<sup>-1</sup> d<sup>-1</sup>), but were significantly lower ( $p < 0.001$ )  
 34 compared to Lake Aydat (mean = 482 contacts cell<sup>-1</sup> d<sup>-1</sup>). In spite of this, %IC was significantly higher  
 35 ( $p < 0.002$ ) in Vassivière than in Aydat (Table 2).

36

1 **Heterotrophic nanoflagellates in Lake Vassivière.** The abundance of heterotrophic nanoflagellate  
 2 (HNF) in Lake Vassivière was marked by two similar peaks of about  $3.0 \times 10^6$  cells  $l^{-1}$  in August and  
 3 October (Fig. 3A), and varied significantly with seasons ( $p < 0.003$ ). Flagellate grazing potential on  
 4 prokaryotes, which was measured only in the Lake Vassivière in the present study showed large (CV =  
 5 77%) and significant ( $p < 0.03$ ) variability with sampled months, ranging from 4.3 to  $35.3 \times 10^6$   
 6 prokaryote  $l^{-1} h^{-1}$  (mean =  $16.8 \times 10^6$  prokaryote  $l^{-1} h^{-1}$ ), with a maximum in August (Fig. 3A).  
 7 Flagellate grazing potential  $FG$  was significantly correlated to %IC, which could be best described by  
 8 a linear function ( $\log y = 0.4x + 0.8$ ,  $r = 0.75$ ,  $p < 0.001$ ) (Fig. 3B). Flagellate grazing potential  $FG$   
 9 was also significantly correlated with the water temperature (Table 3).

10  
 11 **Regression analyses.** Forward stepwise multiple regression analysis using all the environmental  
 12 variables as provided in Table 2 was conducted using the time series data obtained, in order to select  
 13 the variables that significantly accounted for the variability in VLP and %IC in the three lakes. Results  
 14 indicated that PA was the lone significant predictor of VLP in Lake Vassivière (i.e.,  $VLP = 0.50x^2 -$   
 15  $5.16x + 14.8$ ,  $r^2 = 0.61$ ,  $n = 18$ ). In Lake Pavin, Chl and PA were strong predictors for VLP ( $VLP = -$   
 16  $0.212 + 0.0965Chl + 0.96PA$ ,  $r^2 = 0.90$ ,  $n = 18$ ). In Lake Aydat, none of the measured variables  
 17 accounted significantly in the variability in VLP. For %IC, VLP along with temperature, BS and PA  
 18 were selected as significant ( $p < 0.05$ ) predictor variables in Lake Vassivière ( $\%IC = -9.7 +$   
 19  $0.122Temp + 2.50 VLP + 2.53PA + 0.417BS$ ,  $r^2 = 0.68$ ,  $n = 18$ ). Similar variables was also found to  
 20 predict %IC in Lake Aydat, with the exception of temperature ( $\%IC = -21.9 + 2.96PA + 0.644BS$ ,  $r^2 =$   
 21  $0.95$ ,  $n = 18$ ). In Lake Pavin, the measured variables failed to significantly predict %IC.

22 Relationship of VA with Chl, as an indicator of trophic status of an ecosystem was determined  
 23 for both humic and non-humic lakes using the data from the present and previously published studies  
 24 of lakes in the same geographical region. Scatter plots suggested that VLP was strongly positively  
 25 correlated ( $p < 0.001$ ) to Chl in non-humic lakes only, with an upper threshold Chl concentration of  
 26  $0.5 \mu g ChlL^{-1}$  (Fig. 4). The inverse was observed when Chl concentration was  $< 0.5 \mu g ChlL^{-1}$ . No  
 27 significant correlation was observed between Chl and VLP in Lake Vassivière.

## 28 29 DISCUSSION

30 **Time series abundances.** Among freshwater systems, our knowledge on viral activities in humic  
 31 lakes are limited and the present investigation is one of the few that documents the time series  
 32 standing stock of viruses and phage infection in relation to environmental parameters in the pelagic  
 33 realm. Our time series on viral and prokaryotic variables collected in the upper euphotic zone of Lake  
 34 Vassivière revealed known and new features regarding the potential links between viruses and other  
 35 microbial components, i.e. in the general context of aquatic viral ecology. The virioplankton  
 36 abundances in Lake Vassivière were within the previously reported ranges of values for temperate

1 lakes (48, 55), including those from humic lakes in Sweden (1, 47). A prominent feature was the  
2 significant correlation between PA and VLP, which so far has not been established in humic lakes (25,  
3 47). PA alone explained 78% of the variance in VLP, which suggested that PA is a good predictor of  
4 VLP in Lake Vassivière, as prokaryotes are known to be the main hosts for pelagic viruses (32, 38).  
5 With the exception of temperature, none of the measured abiotic variables was able to explain the  
6 variations in viral parameters in Lake Vassivière (Table 3). Although, the abundances of both viruses  
7 and bacteria in Lake Vassivière were rather homeostatic as they did not vary more than 2 fold (Fig.  
8 1A,B), similar to those reported by Mathias et al. (27) in the backwater systems of the River Danube,  
9 by Hennes and Simon (19) in the mesotrophic Lake Constance (Germany), by DeBruyn et al. (13) in  
10 the Lake Erie (USA/Canada), and by ourselves in the eutrophic Lake Grangent, France (37). Unlike in  
11 Lakes Pavin and Aydat, the virus-to-prokaryote ratio in Lake Vassivière were relatively constant and  
12 was at the lower end of the range (i.e. 3 to 10 viruses prokaryote<sup>-1</sup>) reported in other pelagic  
13 environments (48). This corroborates similar findings by Vrede et al. (47) in a comparative study of  
14 Swedish humic lakes and clear water lakes.

15         The brown water colour of Lake Vassivière indicated its DOM was mainly composed of  
16 terrestrial derived humics, which are intrinsically refractory and therefore less prone to rapid  
17 prokaryotic incorporation. Such substances have high capacity to absorb light energy required for  
18 photosynthesis (42). This explains the general low primary production in Lake Vassivière (< 15 mg m<sup>-3</sup>  
19 h<sup>-1</sup>), where the incident light energy in the surface waters as low as < 20 μmol s<sup>-1</sup> m<sup>-2</sup> has been  
20 recorded (28). Under such conditions, prokaryotes have a competitive advantage over light-limited  
21 phytoplankton to harness inorganic nutrients, which could thus help to explain the high prokaryotic  
22 abundance in Lake Vassivière. Moreover, the inflow of labile organic carbon from terrestrial inputs  
23 could also equally help to sustain high prokaryotic activity. Although studies pertaining to the effect of  
24 dissolved humic substances on prokaryotic activity and its impact on prokaryotic community structure  
25 has been carried out in freshwater systems (8, 20), the influence of humic substances on viral infection  
26 and production have received less attention. Earlier reports have suggested that viruses can be  
27 negatively influenced by binding to humic substances (1). We believe such inactivation could have  
28 likely affect more the numerical abundance of viruses rather than their lytic activity in Lake  
29 Vassivière, which is one of the original findings of the present study.

30  
31 **Phage infection and burst size.** Recent studies have suggested that the viral lytic infection contribute  
32 significantly to the bulk of prokaryotic mortality in aquatic ecosystems, and %VIC (expressed as % of  
33 total prokaryotic cells) is a measure of the magnitude of this process (16, 38). We used the  
34 transmission electron microscopy (TEM) method (i.e. whole cell approach) for the determination of  
35 %VIC, which provides direct evidence of phage infection. As most of the literature data to date on  
36 %VIC and burst size are derived from TEM-based estimates (18, 48), comparison among aquatic

1 systems are relatively easy. In our study, a minimum of 500 - 800 cells were examined for 1.3 to 3.0%  
2 visibly-infected cells in Lake Vassivière, which are comparable to the typical range of %VIC (i.e. <  
3 5%) reported for limnetic systems (48, 55), including humic environments (30, 52). However, %VIC  
4 in Lake Vassivière was significantly higher than in Lake Pavin and Aydat, contrasting with the virus-  
5 like particle abundance (Table 2). We consider that this was not an artifact due to methodological  
6 problems because the same approaches were applied in the different lakes tested. Comparatively high  
7 infection rate in Lake Vassivière agreed well with the high prokaryotic standing stock, similar to those  
8 observed in Swedish humic lakes (47). Lymer et al. (25) also emphasized the relatively higher  
9 importance of viruses as agents of prokaryotic mortality in humic than in clear water lakes, when  
10 investigating a set of 21 Swedish lakes with differing trophic statuses. Such trends occurring in humic  
11 lakes suggest that when the labile substrates are in short supply for prokaryotic production, viral lysis  
12 might represent an important source of dissolved organic substrates and inorganic nutrients. This is  
13 supported by the coupling between prokaryotes and both virus-like particle abundance and infection  
14 rate in Lake Vassivière (Table 3). It is also likely that lytic infection is prevalent over lysogeny in Lake  
15 Vassivière, based on the finding that the two viral lifestyles often are mutually exclusive (i.e. from  
16 negative correlations) in pelagic systems where high host abundances generally favor lytic infection  
17 (33).

18 BS reported across lakes in this study was relatively stable, irrespective of trophic status or  
19 humic content. This contrasts with other studies where BS estimates were found to be higher in  
20 productive systems where both cell size and growth are generally greater than in oligotrophic  
21 environments (35). TEM observations revealed that, in Lake Vassivière, BS estimates were indeed  
22 lower than the values (mean around 34 viruses prokaryote<sup>-1</sup>) reported for freshwater systems (48),  
23 which could be explained when lytic phages have short latent period due to short generation time of  
24 hosts.

25  
26 **Infection paradox.** The high viral lytic production in Lake Vassivière was not reflected in the low  
27 VLP and the related contact rates as well (Table 3), which is a paradox. One of the possible reasons for  
28 the low ambient viruses is the consistently low viral burst size in Lake Vassivière, as discussed above.  
29 In addition, viral particles are good candidates for absorption to humic substances (24), well known as  
30 complex natural heterogenous substances with acidic functional groups (COOH) that are reaction sites  
31 on the molecule (42). The trend of low VLP and virus-prokaryote ratio arising due to high prokaryotic  
32 standing stock can also be the result of the growth of phage resistant populations within prokaryotic  
33 communities (31, 58). It is important to give to the readers that even if specific contact rate (SCR) in  
34 Lake Vassivière (mean = 174 contacts cell<sup>-1</sup> d<sup>-1</sup>) was lower than in Lake Aydat (mean = 482 contacts  
35 cell<sup>-1</sup> d<sup>-1</sup>), the rates still were considerably high to represent a major factor for prokaryotic mortality.

36

1 **Depth related variability in Lake Vassivière.** The significant decrease in both VLP and %IC with  
2 depth during the stratified summer period in Lake Vassivière is similar to a recent finding in the  
3 eutrophic Lake Grangent, located in the same regional area (37). This contrasts with deep stratified  
4 lakes such as the meromictic Lake Pavin, France (11) and the moderately hypersaline Mono Lake,  
5 USA (8), where VLP abundance and activity are higher in deeper than in surface waters, due to  
6 dramatic differences in environmental gradients, with persistent anoxic bottom waters. VLP  
7 abundance and infection rates were clearly higher in the photic than in the aphotic zones of Lake  
8 Vassivière, contrasting with the vertical distribution in PA which also decreased with depth but this  
9 was low and non significant. However, these variables were tightly coupled to each other on a vertical  
10 basis ( $r > 0.70$ ,  $p < 0.05$ ), suggesting that the viral attack in the two regimes could be rather dependent  
11 on the density of the susceptible host populations, rather than to the density of the whole host  
12 community.

13  
14 **Heterotrophic nanoflagellates and potential grazing estimates in Lake Vassivière.** The potential  
15 flagellate grazing rates (FG) observed in Lake Vassivière (mean =  $16.8 \times 10^6$  bacteria  $l^{-1} h^{-1}$ ) was  
16 higher compared to previous reports in oligomesotrophic Lake Pavin (mean =  $3.8 \times 10^6$  bacteria  $l^{-1} h^{-1}$ )  
17 and eutrophic Lake Aydat (mean =  $10.4 \times 10^6$  bacteria  $l^{-1} h^{-1}$ ) (5). Among the sampled lakes, data from  
18 Lake Vassivière indicated a strong correlation between %IC and FG, suggestive of synergistic  
19 interactions between lysis and grazing activity relative to their prokaryotic resources, which agrees  
20 with our recent experiments in nutrient limited freshwater microcosms from the same geographical  
21 area (36). In addition, consistent with our results, the %IC tended to be high in the Rimov Reservoir  
22 (South Bohemia, Czech Republic) when FG rates was high (41). These data might suggest that viral  
23 infectivity increases with increasing grazing activity of HNF, because grazers provide substrates to  
24 uninfected prokaryotes (36). Such trophic cascading cycling of substrates and nutrients was suggested  
25 to be of major importance in oligotrophic conditions (36), likely including light-limited humic lakes.  
26 Under high grazing pressure, grazing-resistant forms of bacteria (e.g., filamentous and floc-forming  
27 prokaryotes) become abundant in freshwater lake communities (41). Some studies have reported that  
28 these grazing-resistant forms of prokaryotes are more susceptible to viral infection, presumably  
29 because there is a trade-off between grazing resistance and viral resistance (36, 52). This hypothesis  
30 needs to be tested in future studies. Comparison of prokaryotic losses resulting from protistan  
31 predation and viral lysis in aquatic systems is often calculated and expressed in terms of percentage of  
32 prokaryotic production (4). Since prokaryotic production was not measured in our study, we could not  
33 directly estimate and compare prokaryotic mortality between the two sources. Given the fact that  
34 VIBM and FG were high, especially during the summer period, both viruses and predators could have  
35 contributed to the bulk of prokaryotic mortality rates. Lymer et al. (25) suggested that flagellate  
36 grazing appeared to be more important for prokaryotic mortality than viral contribution, as inferred

1 from survey conducted in a set of 21 boreal lakes in Sweden along the trophic gradient, including  
2 humic lakes such as Vassivière.

3  
4 **Humic content versus trophic status.** Studies examining larger data set (e.g. studies based on  
5 regression analysis of reported values) have revealed that viral and prokaryotic abundances are  
6 significantly correlated to each other, which is in turn ultimately dependent on the levels of primary  
7 production (4). Investigation of this possibility is also important for elucidating the link between  
8 trophic conditions, humic content, and viral parameters in humic lakes. It has been proposed that  
9 trophic status is a possible driving force in controlling the spatial distribution of viruses, the rationale  
10 being that eutrophic environments support higher standing stock of prokaryotes and consequently of  
11 viruses, compared to oligotrophic systems. In this study, the investigated lakes displayed clear  
12 gradients in chemical and biological parameters. In order to draw comparisons between humic versus  
13 non-humic lakes with respect to the trophic state control hypothesis (12), data on VLPs and infection  
14 rates from the present study and from the previous published reports in regional lakes located in the  
15 French Massif Central, were plotted against chlorophyll *a* concentration (Chl), considered an index of  
16 trophy. Scatter plots indicated that both VLP and infection were not correlated to Chl in the humic  
17 Lake Vassivière, which suggest that prokaryote-viral interactions could largely be forced by  
18 exogenous supplies of organic carbon. In non-humic systems, VLP was positively correlated to Chl.  
19 This followed an apparent trend with a clear increase in VLP along trophic gradient only from 0.5  $\mu\text{g}$   
20  $\text{ChlL}^{-1}$ . Below this value, the relationship was negative (Fig. 4), suggesting that phytoplankton-derived  
21 resources could force prokaryotic growth to attain a certain threshold level where the host availability  
22 is sufficient to boost the lytic proliferation of viruses. Similar positive virus versus trophy pattern has  
23 been previously observed in the northern Adriatic Sea (52) and in non-humic Swedish lakes (25).  
24 However, contrasting reports exist where VLP does not seem to be related to the trophic status, such  
25 as in Quebec lakes (Canada) (26) and in the Adriatic Basin (Mediterranean) (12). %VIC in Lake  
26 Vassivière were within the range which covers almost the entire span of previously published data  
27 obtained from a number of systems of different trophic status. Although studies have stressed the  
28 importance of viral infection in oligo versus eutrophic systems (4, 47), such apparent trend in viral  
29 infection from oligo- to eutrophic systems, derived from estimates from different temperate lakes in  
30 the same regional location (Table 3), was not observed in our comparative analysis for non-humic  
31 lakes. We therefore conclude that in our study systems, humic content prevailed over the ecosystem  
32 productivity which, alone, appears as a poor predictor of the level of viral infection in freshwater lake  
33 ecosystems.

34  
35 **Conclusions.** This study provides original data on viruses with focus on a site (i.e. the humic Lake  
36 Vassivière) that offers unique peculiarities, with few available data in the literature. The

1 methodological approaches used were those commonly applied in aquatic viral ecology, providing a  
2 good basis for comparative ecology. Although VLP abundance and virus-to-prokaryote ratio in Lake  
3 Vassivière were lower compared to the productive Lake Aydat, data from visibly infected cells  
4 provided concrete evidence that viruses were important agents for prokaryotic mortality. This  
5 indicated that the impact of viruses on the food web dynamics of humic lakes might be substantial,  
6 which may ultimately depend on internal cycling of resources and/or on allochthonous inputs. The  
7 paradox between low occurring viruses and high infection rates in Lake Vassivière was mainly related  
8 to low burst size estimates, which was characteristic of this ecosystem where humic content apparently  
9 prevailed over the trophic status in constraining microbial communities. Based on the substantially  
10 high level of viral infection rates in Lake Vassivière, we reject our initial hypothesis that viral  
11 infection could be of minor importance in humic lakes which are traditionally viewed as unproductive  
12 environments, often characterized by low levels of inorganic nutrients and photosynthetic activities.  
13 Clearly, because Lake Vassivière may not be fully representative of the world humic lake, additional  
14 data on viral ecology are needed for humic lakes where, for example, the nature of the association  
15 between viruses and humic substances indeed deserves further investigations, to precisely determine  
16 the interactions between viruses and prokaryotic diversity.

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18

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4

1 **Figure legends**

2

3 **Figure 1.** Time series variations in the abundances of viruses (A), prokaryotes (B), virus-to-prokaryote  
4 ratio (C) and the percentage of infected prokaryotic cells (D) in the euphotic zones of Lakes  
5 Vassivière, Pavin and Aydat. Error bars indicate standard errors of the mean ( $n = 3$ ).

6 **Figure 2.** Depth-related variability in the abundances of viruses (dark bars), prokaryotes (light bars)  
7 and the percentage of infected cells (dotted lines) during the stratified period in Lake Vassivière.  
8 Values correspond to mean  $\pm$  standard error ( $n = 3$ ), for samples collected in June, July, and August  
9 2007.

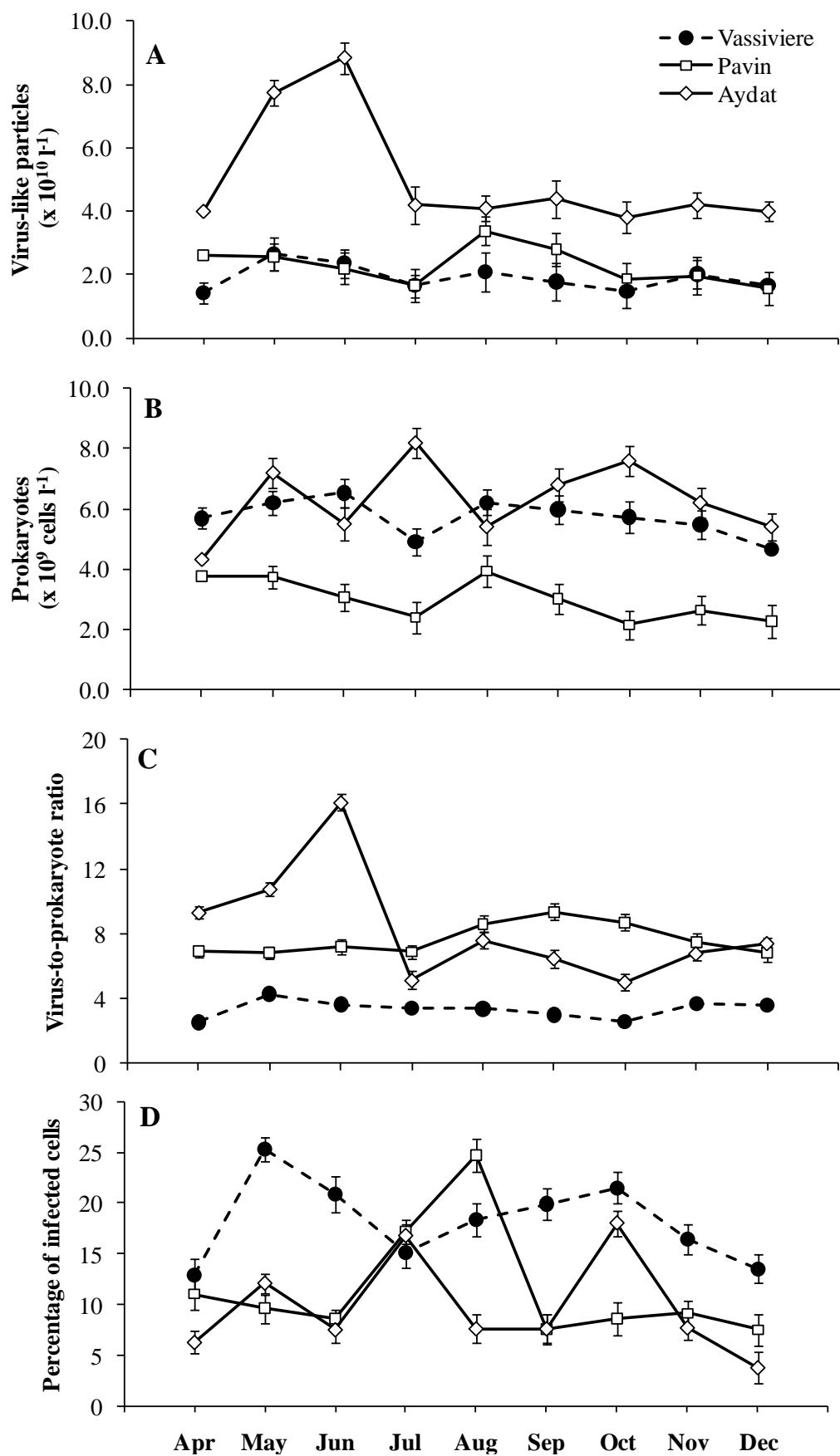
10 **Figure 3.** Time series variability in (A) the abundance (HNF) and grazing rate (FG) of heterotrophic  
11 nanoflagellates, and (B) the relationship between FG and the percentage of prokaryotic cells infected  
12 with viruses (%IC) in Lake Vassivière.

13

14 **Figure 4.** Scatter plot of chlorophyll *a* concentration vs the abundance of virus-like particles in the  
15 humic Lake Vassivière (white circles) and in several other non-humic lakes (dark circles) located in  
16 the same geographical areas. Data for the non-humic lakes were from this study for Lakes Pavin and  
17 Aydat, and our recent previous studies in Lakes Sep and Grangent (cf. 41).

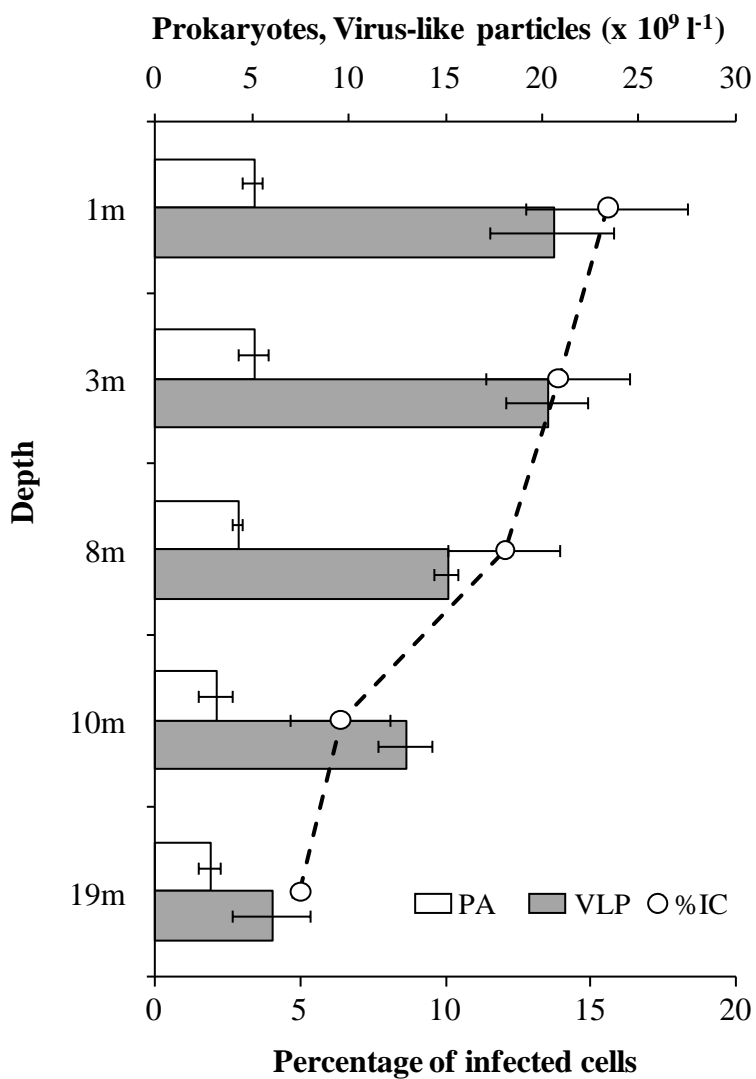
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**Fig. 1**  
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1



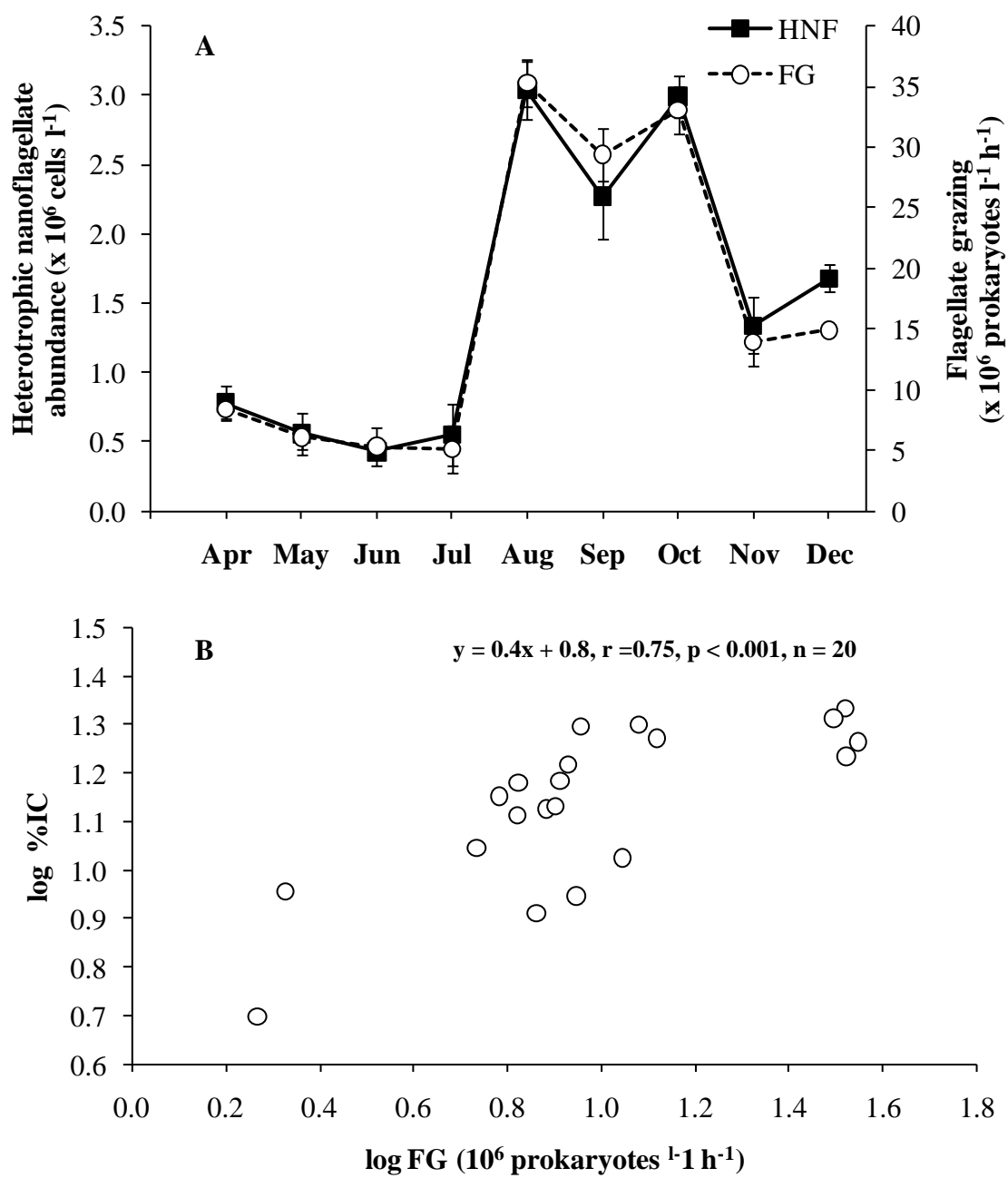
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5 **Fig. 2**6 **Pradeep Ram et al.**

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**Fig. 3**  
Pradeep Ram et al.

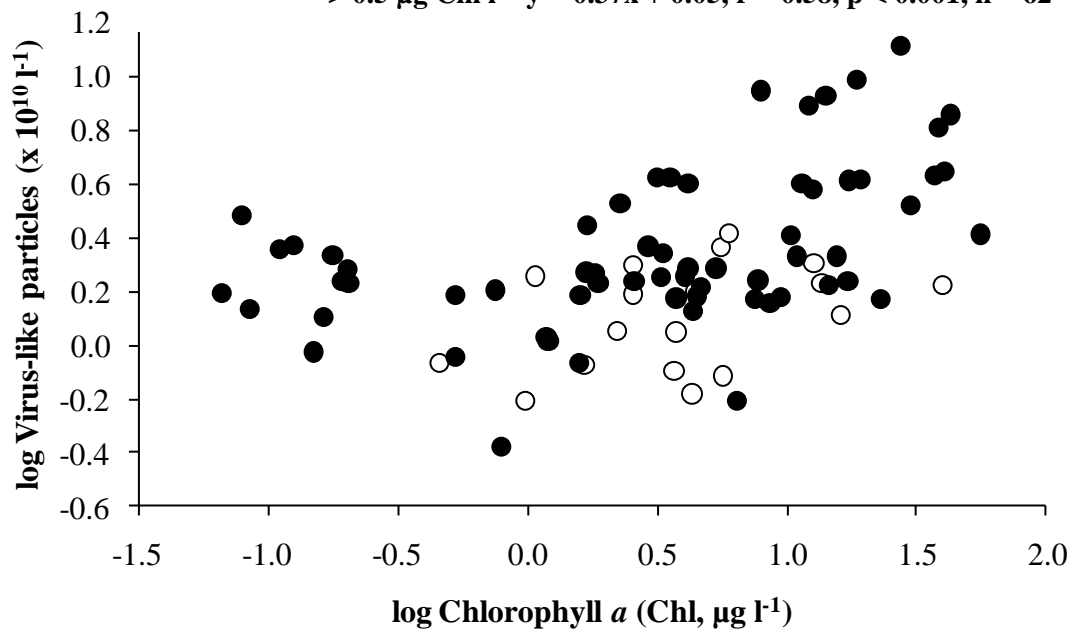


**Humic: not significant**

**Non-humic : Overall**  $y = 0.18x + 0.21, r = 0.44, p < 0.001, n = 78$

**< 0.5  $\mu\text{g Chl l}^{-1}$**   $y = -0.43x - 0.15, r = -0.69, p < 0.01, n = 16$

**> 0.5  $\mu\text{g Chl l}^{-1}$**   $y = 0.37x + 0.05, r = 0.58, p < 0.001, n = 62$



**Fig. 4**  
**Pradeep Ram et al.**

1 Table 1. Location and morphometric characteristics of the studied lakes

	Vassivière	Pavin	Aydat
Location	45°48'51"N, 01°51'09"E	45°29'41"N, 02°53'12"E	45°39'48"N, 02°59'04"E
Altitude (m)	650	1197	825
Origin	Man made	Volcanic	Volcanic
Trophic status	Mesotrophic	Oligomesotrophic	Eutrophic
Humicity	Humic	Non-humic	Non-humic
pH	Moderately acidic (6.5)	Alkaline (9.1)	Alkaline (9.2)
Maximum depth (m)	25	92	15
Water circulation	holomictic	meromictic	holomictic
Lake surface area (ha)	1000	44	60
Lake storage capacity (10 <sup>6</sup> m <sup>3</sup> )	106	23	4.1
Watershed area (ha)	7600	50	3000
Catchment : lake area ratio	7.6	0.8	49.8

2

3

- 1 Table 2. Mean (% coefficient of variation) physico-chemical characteristics, chlorophyll *a* concentrations, prokaryotic and viral parameters in  
 2 Lakes Vassivière, Pavin and Aydat for the euphotic depth integrated samples during the study period. ND = not determined.

Parameters	Vassivière	Pavin	Aydat
Temperature (°C)	13.2 (44)	11.6 (43)	13.7 (39)
Dissolved oxygen (mg l <sup>-1</sup> )	9.1 (25)	9.6 (20)	8.9 (18)
Total nitrogen (mg l <sup>-1</sup> )	0.6 (53)	0.2 (25)	0.9 (51)
Total phosphorous (mg l <sup>-1</sup> )	0.02 (54)	0.03 (35)	0.03 (45)
Total organic carbon (mg l <sup>-1</sup> )	7.6 (35)	2.8 (28)	5.1 (41)
Chlorophyll <i>a</i> (µg l <sup>-1</sup> )	10.8 (33)	3.9 (71)	17.9 (59)
Virus-like particle abundance (10 <sup>10</sup> l <sup>-1</sup> )	1.9 (26)	2.3 (26)	5.0 (32)
Prokaryote abundance (10 <sup>9</sup> cells l <sup>-1</sup> )	5.7 (11)	3.0 (23)	5.9 (28)
Virus-to-prokaryote ratio	3.3 (19)	7.6 (13)	8.5 (37)
Percentage of infected cells (%)	17.6 (27)	11.2 (49)	8.3 (42)
Viral induced prokaryotic mortality (%)	25.6 (23)	15.3 (70)	12.2 (61)
Burst size (viruses prokaryote <sup>-1</sup> )	17.0 (10)	34.5 (27)	37.2 (38)
Specific contact rate (contacts cell <sup>-1</sup> d <sup>-1</sup> )	174 (29)	183 (26)	482 (37)
Heterotrophic nanoflagellates (10 <sup>6</sup> cells l <sup>-1</sup> )	1.5 (81)	ND	ND
Flagellate grazing (10 <sup>6</sup> prokaryote l <sup>-1</sup> h <sup>-1</sup> )	16.8 (77)	ND	ND

- 1 Table 3. Pearson's correlation coefficients (r) between different variables in the euphotic zone of the studied lakes.  
 2 Values are indicated in bold for Lake Vassivière and in italics for Lakes Pavin/Aydat. Flagellate grazing was compared with other variables in  
 3 Lake Vassivière only.

Parameters	Temperature	Chlorophyll <i>a</i>	Prokaryote abundance	Virus-like particle abundance	Percentage of infected cells	Flagellate grazing	Specific contact rates
Chlorophyll <i>a</i>	<b>0.71***</b> <i>NS/NS</i>						
Prokaryote abundance	<b>0.71***</b> <i>0.74***/0.52*</i>	<b>NS</b> <i>0.69***/NS</i>					
Virus-like particle abundance	<b>0.66***</b> <i>0.61**/NS</i>	<b>NS</b> <i>0.74***/NS</i>	<b>0.78***</b> <i>0.87***/NS</i>				
Percentage of infected cells	<b>0.80***</b> <i>0.49*/0.48*</i>	<b>NS</b> <i>NS/NS</i>	<b>0.49*</b> <i>0.49*/NS</i>	<b>0.71***</b> <i>0.53*/NS</i>			
Flagellate grazing	<b>0.57**</b>	<b>NS</b>	<b>NS</b>	<b>NS</b>	<b>0.80***</b>		
Specific contact rates	<b>0.71***</b> <i>0.66***/NS</i>	<b>NS</b> <i>0.51*/NS</i>	<b>0.83***</b> <i>0.88***/NS</i>	<b>NA</b>	<b>0.72***</b> <i>0.60**/0.78***</i>	<b>NS</b>	
Burst size	<b>0.57**</b> <i>NS/NS</i>	<b>NS</b> <i>0.59**/NS</i>	<b>NS</b> <i>NS/NS</i>	<b>0.69***</b> <i>NS/NS</i>	<b>0.69***</b> <i>NS/0.61**</i>	<b>NS</b>	<b>0.50*</b> <i>NS/0.83***</i>

- 4  
 5 Levels of significance: \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ . NS = not significant, NA = not applicable (autocorrelation)