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Dynamics of various viral groups infecting autotrophic plankton in Lake Geneva

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Viral community structure and dynamics were investigated for the first time in surface waters (0–20 m) of Lake Geneva over a 5-month period between July and November 2011. Abundances of autotrophic picoplankton, heterotrophic bacteria and virus-like particles determined using flow cytometry revealed their predominance during the summer months followed by a slight decrease in fall. Two groups of viruses could be discriminated, referred to as virus-like particles (VLP) group 1 and 2. The abundance of VLP1 correlated significantly with the bacterial abundance, while that of VLP2 correlated with both chlorophyll *a* and picocyanobacterial abundance suggesting a tight coupling between these viral groups and bacteria or phytoplankton. The abundance of cyanomyoviruses and cyanopodoviruses varied between $7.3 \times 10^{2}\,ml^{-1}$ (July) to $1.2 \times 10^{4}\,ml^{-1}$ (November) and $5.8 \times 10^{3}\,ml^{-1}$ (July) to $2.2 \times 10^{4}\,ml^{-1}$ (September), respectively. The abundance of the picocyanobacterial hosts was in concurrence with that of the cyanophages, being higher in late summer. Polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) of viral signature genes such as *g20*, *g23*, *mcp*, *polB*, *psbA* and *psbD* revealed a relatively high richness within these genes with their diversity increasing towards the summer months. The diversity of *psbD* was found to be particularly high and correlated with picocyanobacterial abundance suggesting that cyanophages may be directly responsible for a significant proportion of carbon fixation in Lake Geneva.

Keywords: virus; cyanophages; algal viruses; PCR-DGGE; PFGE; qPCR

1. Introduction

Unicellular picophytoplankton (in particular picocyanobacteria) can be the most abundant primary producers in many aquatic environments [1,2]. Their community structure, functioning, species diversity and succession may be largely influenced by viruses directly through lysis or horizontal gene transfer and indirectly by using available nutrients following viral lysis of bacteria [3–8]. Viruses occur in high abundance in most aquatic systems, their counts ranging from 10^{6} to $10^{8}\,ml^{-1}$. Viruses of marine cyanobacteria such as *Prochlorococcus* and *Synechococcus* have been relatively well studied [9–11] and the marine field has generally received more attention than freshwaters [12,13]. Because viruses are mostly species or strain specific [14], viruses are thought to be the most diverse component in aquatic systems but temperate lakes still suffer a lack of data, especially in terms of community structure, richness and diversity [13,15].

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Unlike bacterial diversity investigations, studies on viral diversity are constrained due to the absence of universal genes such as the 16 S rRNA gene of bacteria and the 18 S rRNA gene of eukaryotes [16]. Researchers have suggested the use of family specific genes or signature genes to study different groups of viruses [17]. Thus, previous studies have used *g20* (T4 portal protein gene 20) [18–21] and *g23* genes for the detection of cyanomyoviruses and/or the T4-like myovirus community [22], *polA* gene for cyanopodoviruses [11], *psbA* and *psbD* for photosynthetic genes in cyanophages [23–25] and *polB* or *mcp* for algal viruses [26–28]. However, and to the best of our knowledge, no inclusive attempt has been made until now to incorporate the signature genes of various viral groups in the same study. Moreover freshwater European ecosystems have not been investigated yet with such a battery of viral genetic markers.

Lake Geneva (Léman) is the largest lake in Western Europe lying between Switzerland and France. Although this ecosystem has been monitored by the International Commission for the Protection of Lake Geneva Waters (CIPEL in French abbreviation) since the early 1950s, information on the microbial community (including viruses) dynamics and diversity available in the literature is still scarce [29–31]. Duhamel et al. (2006) [29] highlighted for the first time that the viral community of this lake could be important and might play a key role in microbial processes. The study of Personnic et al. (2009a) [30] confirmed a few years later host-parasite links between viruses and chlorophyll *a*, or between viruses and bacteria. Their study also revealed that *Synechococcus* spp. is the most dominant picophytoplanktonic group in Lake Geneva and the abundances of picoplankton and viruses fluctuate seasonally. At last, using a modified dilution method, these authors found a significant impact of viruses on the prokaryotic community during the most productive seasons [31]. However, the diversity in terms of genome size or about some particular groups has never been studied for the viroplankton of Lake Geneva. We thus determined for the first time the diversity of various viral groups using some signature genes using polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE), quantified them using flow cytometry (FCM) and quantitative PCR (qPCR) and obtained a whole community profiling of viral communities using pulse field gel electrophoresis (PFGE). Our aim was to propose a preliminary picture of viral gene richness in Lake Geneva and to highlight the importance and seasonal dynamics of different viral groups. In addition, we tried to relate such richness to potential autotrophic hosts and the influence of key environmental factors on the dynamics of specific viral communities in Lake Geneva.

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2. Materials and methods

2.1. *Study site and sampling strategy*

Lake Geneva which lies at an altitude of 372 m forms the border between France and Switzerland at the north of the French Alps and it is a 72 km long ecosystem with an area of 582 km^2 and maximum width of 13 km. It is a meromictic lake, never covered by ice, with temperature ranging between 4 and 22°C. It holds an approximate volume of 89×10^9 m^3 and this lake has been reported as eutrophic during the seventies and has changed following restoration programs to a mesotrophic state during the nineties in response to appropriate measurements taken to reduce phosphorus inputs to the lake. In 2011, the lake had a total phosphorus content of 27 μgP L^{-1} (CIPEL 2012).

Samples were collected at the reference station (named SHL2), corresponding to the deepest part of the lake at 46°27′ lat. N 6°32′ long, 309 m deep, once or twice every month from July to November, 2011. A 20 L water sample was collected in the 0–20 m layer using a hose and pump (developed at INRA, Thonon). Samples collected in a polycarbonate container were stored at ambient temperature, protected from light and heat, and brought to the lab, within 3 h of collection. Upon reaching the laboratory, samples were immediately pre-filtered through a 60 µm mesh filter, and then through glass fibre filter (GFF) and 1 µm filters to remove zooplankton and phytoplankton. The viruses were concentrated further using a 30KDa cut off spiral wound tangential flow filtration (TFF) cartridge (Prep scale, TFF 1 ft^2, Millipore Inc, USA). A 200 ml viral concentrate was stored at 4°C for further analysis (i.e. the PCR, PFGE etc. see below). An aliquot of this viral concentrate was filtered through a 0.22 µm filter to remove bacterial contamination and this mixture was used for PCR analysis. Alternatively, 2 L of the sample was also filtered through a polycarbonate 0.22 µ filter that was stored at −20°C for the detection of the (pico)cyanobacteria. The genomic DNA was extracted using PureLink genomic DNA extraction kit (Invitrogen, USA) following the manufacturer's instructions.

2.2. *Environmental parameters and plankton analysis*

A multiparameter probe was used to collect different parameters such as light, conductivity, and temperature while samples for chemical analysis brought back to the laboratory were analysed using standardised methods (see [32] for more details). Measurements of nitrate (N-NO_3), nitrite (N-NO_2), total nitrogen (Ntot), phosphate (PO_4), total phosphorous (Ptot) and silicate (SiO_2) were obtained. Raw water samples for the phytoplankton analysis were taken with a patented integrating instrument developed by Pelletier and Orand (1978) [33] and fixed with a few drops of Lugol's solution. For each sample analyzed a few days after sampling, 25 ml were poured into an Utermöhl room (cylinder surmounting a blade with sediment chamber; [34]) and left to form a deposit for at least 12 hours safe from light and heat. The qualitative and quantitative examination of the phytoplankton was carried out using inverted microscopy (Zeiss). For the zooplankton, vertical sampling from a depth of 50 m to the surface was carried out using a net of 212 µm mesh size. The samples were fixed with formol at 5%. The enumeration of microcrustaceae presented here was achieved by means of a standard microscope (Olympus BX40).

2.3. *Flow cytometry analysis*

Viruses were counted using a FACSCalibur flow cytometer (FCM) (Becton Dickinson) equipped with an air cooled laser providing 15 mW at 488 nm. Samples were fixed with glutaraldehyde (0.5% final concentration, grade I, Merck) for 30 min, then diluted in 0.02 µm filtered Tris ethylenediaminetetraacetic acid (EDTA) buffer (referred to as TE, 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 5 min at ambient temperature in the dark, followed by an incubation for 10 min at 75°C, and then for another 5 min at room temperature, prior to FCM analysis [30]. The analysis of heterotrophic bacteria was done as for the viruses but without heating at 75°C and by using <0.02 µm filter lake instead of TE. The picocyanobacteria and other smaller

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phytoplankters were analysed without fixing or staining by using their natural autofluorescence. 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 analyzed on a PC using the custom-designed freeware CYTOWIN [35].

2.4. *Polymerase chain reaction (PCR)*

PCR was conducted separately for the different viral groups, after optimization of the PCR conditions, as shown in Table 1. CPS1/2, CPS4GC/5 and CPS 1.1/8.1 were used for the detection of cyanomyoviruses using the *g20* gene. MZPA1B F/R was used for the detection of the *g23* gene of cyanomyoviruses. *pol* F/R was used for the detection of the DNA polymerase gene of the cyanopodoviruses. Another primer, Jason F/R, that was designed in a previous study [36], aimed at detecting the DNA polymerase gene of cyanopodovirus from lacustrine environments as we found difficulty in testing our samples with the *pol* F/R primer [11]. Double-stranded DNA algal virus (e.g. PhycoDNAviridae) detection was performed using *mcp* F/R and AVS1/2 *polB* primers. The presence of host photosynthetic genes in viral genomes was detected using *psbA* and *psbD* primers. The picocyanobacterial detection was done using CYA F/R. 1 to 2 μl of the <0.22 μm concentrate was used for the PCR and the reactions were performed in 30 μl final volume. 10 μl of the sample was used for gel electrophoresis in 1.5% agarose gel. The products were visualised in a gel documentation system and images were captured (Biorad, Germany).

2.5. *DGGE-PCR*

DGGE analysis was performed using the CBS-DGGE 2000 system (C.B.S. Scientific Co., Inc.). PCR products (20 μl) were loaded onto a 0.75-mm-thick 6% polyacrylamide gel in 1 × TAE buffer with 45 to 70% linear denaturing gradient. Electrophoresis was performed at a constant voltage of 120 V and a temperature of 60°C for the optimal duration of 16 h. Gels were stained for 45 min in the dark with SybrGold (Molecular Probes). The gels were visualized and photographed using a gel documentation system (Biorad, Germany).

2.6. *PFGE*

Pulse Field Gel Electrophoresis analysis was performed using a BioRad DR II CHEF electrophoresis system (Biorad, Germany). A 35 ml sample of viral concentrate after ultrafiltration was precipitated with polyethylene glycol 8,000 (10% w/v in final concentration) and NaCl (0.6% w/v in final concentration) for 24 h at 4°C [37]. The precipitated viral particles were concentrated by centrifugation at 15,000 g for 1 h (Beckman JA-30.50). The supernatant was discarded and the viral pellets were suspended in 100–200 μl sterile MilliQ water and stored at 4°C for further analysis. Equal volumes of the viral concentrate and molten (50°C) 1.5% InCert agarose (REF) were mixed, vortexed and dispensed into plug moulds. After solidification of the gel, plugs were treated with lysis buffer (1% SDS, 0.5 M EDTA and 20 mg/μl proteinase K) for 24 h at 37°C. Plugs were then washed 3 times with wash buffer (0.5 M EDTA, pH 8.0), loaded into 1% agarose gel and separated both large and small viral genomes using ramp conditions (i) 10 to 40 s switch time for 18 h (ii) 1 to 5 s switch time for 12 h. Gels were stained with ethidium

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Table 1. List and characteristics of the primers used in this study.

Primer set	Targeted gene and group	Primer sequence PCR conditions	Product size	Reference
CPS1/2	*g20*, capsid protein, Cyanophages	CPS1: GTAGWATTTTCTACA-TTGAYGTTGG CPS2: GGTARCCAGAAATC-YTCMAGCAT 40 cycles at 94°C 45 S 48°C 45 S, and 72°C for 45 S With 5 min of initial denaturation and final extension	165 bp	[53] Fuller et al. 1998
CPS4GC/5	*g20*, capsid protein, cyanophages	CPS4: GTAGAATTTTCTACA-YYGATGTTGG CPS5:GGTAACCAGAAA-TCTTCAAGCAT 40 cycles at 94°C 45 S 48°C 45 S, and 72°C for 45 S With 5 min of initial denaturation and final extension	165 bp	Wilson et al. 1999
CPS1.1/8.1	*g20*, capsid protein, cyanophages	CPS1.1:GTAGWATWTTY-TAYATTGAYGTWGG CPS8.1:ARTAYTTDCCDA-YRWAWGGWTC 40 cycles at 94°C 45 S 46°C 45 S, and 72°C for 45 S With 5 min of initial denaturation and final extension	592 bp	[16] Sullivan et al. 2008
MZPA1B F/R	*g23*, capsid protein, cyanomyovirus	MZPA1B: GATATTTGI GGIGTTCAGCCIATGA MZIA6: CGCGGTTGA TTTCCAGCATGATTTC 40 cycles at 94°C 30 S 48°C 30 S, and 72°C for 30 S, with 5 min of initial denaturation and final extension	550 bp	Filee et al. 2005
ProPsbA F/R	D1, photosynthetic genes in cyanophages	ProPsb AF: AACATCA TYTCWGGTGCWGT ProPsb AR: TCGTGCA TTACTTCCATACC 40 cycles at 94°C 30 S 45°C 30 S, and 72°C for 30 S, with 5 min of initial denaturation and final extension	650-800 bp	[51] Sullivan et al. 2006

(*continued*)

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Table 1. Continued.

Primer set	Targeted gene and group	Primer sequence PCR conditions	Product size	Reference
psbD F/R	D2, photosynthetic genes in cyanophages	40 cycles at 94°C 30 S 45°C 30 S, and 72°C for 30 S With 5 min of initial denaturation and final extension	383 bp	[23] Clokie et al. 2006
Jason F/R	DNA polymerase gene, Cyanopodovirus	Jason F: ACTGCA ACGCCTGGGATGGTG Jason R: AGCAAT GCGGCGACCGTCAA 40 cycles at 94°C 30 S 50°C 30 S, and 72°C for 30 S With 5 min of initial denaturation and final extension	380-480 bp	Present study [36] Jacquet et al. 2012
Mcp F/R	Major capsid protein, phycodnaviridae	MCP F: GGYGGYCAR CGYATT MCP R: TGIARYTGY TCRAYIAGGTA 40 cycles at 94°C 30 S 48°C 30 S, and 72°C for 30 S, with 5 min of initial denaturation and final extension	347-518 bp	[28] Larsen et al. 2008
AVS 1/2	DNA polymerase gene, Phycodnaviridae	AVS F: GARGGIGCIA CIGTIYTIGAYGC AVS R: GCIGCRTAI CKYTTYTTISWRTA 40 cycles at 94°C 30 S 48°C 30 S, and 72°C for 30 S, with 5 min of initial denaturation and final extension	700 bp	Culley et al., 2008
Pol F/R	DNA polymerase gene, Cyanopodovirus	CP-DNAP-349 F: CCAAA YCTYGCMCARGT CP-DNAP-533Ra: CTCG TCRTGSACRAASGC CP-DNAP-533RB:CTCG TCRTGDATRAASGC 40 cycles at 94°C 30 S 50°C 30 S, and 72°C for 30 S, with 5 min of initial denaturation and final extension	650-750 bp	[11] Chen et al., 2009, Huang et al., 2010

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bromide for 24 h, visualized and photographed using a gel documentation system (Biorad, Germany).

2.7. *Quantitative PCR (qPCR)*

qPCR analysis was performed in the Rotor gene system (Stratagene) to give estimates of cyanopodovirus and cyanomyovirus abundances. The primers were added at a concentration of 5 pm to Qiagen SYBR Green mix and the reaction was performed in 20 µl volume in triplicate. A 2 µl of the <0.2 µm viral concentrate was used for PCR. qPCR conditions were the same as for normal PCR for the respective genes. A standard curve was created for each of these genes using 10^7 to 10^{-1} copies of respective cloned genes from known viral cultures. qPCR efficiency was tested (not shown). We assumed that there was only 1 gene copy per cyanoviral particle [38]. After the run, the samples were quantified by importing the standard curves and the viruses are reported as number of gene copies/ml of the water sample (taking into account and using appropriate conversion and dilution factors).

2.8. *Gel comparison*

The PFGE and DGGE banding patterns were analyzed by considering both the presence and relative abundance of bands using the GelCompare II software package (Applied Maths, Kortrijk, Belgium) and the Paleontological Statistics Software Package for education and Software analysis (PAST version 2.14) [39]. Briefly, fingerprinting patterns were first standardized with a reference pattern. Each band was described by its position and its relative intensity in the profiles, which could be described as the ratio between the surface of the peak and the sum of the surfaces for all the peaks within the profile. A similarity matrix between densitometric curves of the band patterns was calculated based on the Bray-Curtis index and used to perform unweighted pair group cluster (UPGMA).

2.9. *Statistical analysis*

Statistical analysis was only performed using Spearman's correlation (with ExcelStat) to highlight the relationships between the different variables and with viral diversity through the number of DGGE bands.

3. Results

3.1. *Environmental parameters*

The values for the main environmental parameters are provided in Table 2. Temperature was relatively warm and evolved on average between 14.2 ± 4.6°C (July) and 18.9 ± 4.3°C (November) when integrated over the 0–20 m layer. Near the surface, it could reach up to 21.5°C in August and dropped to around 7.7°C near 20 m in November. The highest oxygen concentration was recorded in early July with approximately 10 ± 1.01 µg O_2 L^{-1} and values remained relatively high, i.e. between 9 and 10 µg O_2 L^{-1}, between July and October, while a significant decrease occurred in November with 7.5 ± 1.5 µg O_2 L^{-1}. Total phosphorus and orthophosphates varied between 6.6 ± 0.8 (November) and 13.8 ± 3.9 (July) µgP L^{-1} and between 3 ± 0.5 (October) and 6.6 ± 0.5 (August) µgP L^{-1}, respectively. Comparatively

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Table 2. Evolution of the main environmental parameters in Lake Geneva in the 0–20 m layer.

Parameters	July	August	September	October	November
Temperature °C	14.19 ±4.6	16.67 ±6.2	18.19 ±6.9	17.22 ±5.87	17.65 ±4.3
Oxygen mg/L	9.96 ±1.01	9.25 ±0.37	9.56 ±0.35	8.90 ±1.04	7.46 ±1.5
Chlorophyll a µg/L	4.89 ±1.3	4.55 ±1.52	2.99 ±0.95	2.89 ±0.58	4.46 ±1.53
pH no unit	8.35 ±0.33	8.47 ±0.41	8.37 ±0.27	8.24 ±0.26	7.81 ±0.13
Light µE	187.57 ±189.44	144.39 ±61.95	73.00 ±4.6	196.23 ±301.52	100.21 ±152.4
Transp m	5.6 ±1.6	10.5 ±2.1	11.2 ±1.8	10.6 ±2.7	11.6 ±1.7
Ptot mg/L	0.0138 ±0.0039	0.0106 ±0.011	0.0166 ±0.0011	0.0106 ±0.0017	0.0066 ±0.0008
P-PO_4 mg/L	0.0038 ±0.0019	0.0066 ±0.0005	0.0032 ±0.0050	0.003 ±0.0005	0.0036 ±0.0005
Ntot mg/L	0.579 ±0.858	0.436 ±0.172	0.474 ±0.0718	0.28 ±0.288	0.666 ±0.07
N-NO_3 mg/L	0.354 ±0.087	0.2 ±0.24	0.156 ±0.195	0.096 ±0.22	0.522 ±0.078
N-NH_4 mg/L	0.008 ±0.0067	0.011 ±0.002	0.0068 ±0.002	0.0132 ±0.006	0.0102 ±0.002
SiO_2 mg/L	0.262 ±0.33	0.256 ±0.51	0.204 ±0.58	0.368 ±0.38	1.108 ±0.25
COT mg/L	1.296 ±0.289	1.086 ±0.618	1.198 ±0.27	1.308 ±0.24	0.834 ±0.064

N-NO_3 was relatively high varying between 96 ± 22 (October) and 522 ± 78 (November) $\mu gN\,L^{-1}$ while N-NH_4 ranged between 8 ± 6.7 (July) and 13 ± 6 (October) $\mu gP\,L^{-1}$. The ratio between total N and P (from 23 to 100) supported the idea that P was probably limiting but it has also been reported recently that N can be limiting for phytoplankton growth in Lake Geneva [40]. Finally, SiO_2 was the lowest in September with $204 \pm 58\ \mu g\,L^{-1}$, while the highest value recorded was $1.1 \pm 2.5\ mg\,L^{-1}$ in November. This period was very sunny and the transparency varied between 5.6 ± 1.6 (July) and 11.2 ± 1.8 (November) m. Chlorophyll *a* concentrations ranged from 2.9 ± 0.58 in October to $4.9 \pm 1.3\ \mu g\,L^{-1}$ in July. Heavy winds were recorded in October-November (data not shown).

3.2. *Abundances of the microbial, phytoplankton and zooplankton communities*

The abundance of the heterotrophic bacteria was on average about 2.5×10^6 cells ml^{-1} but concentrations varied between $9.35 \times 10^5 \pm 2.93 \times 10^5$ cells ml^{-1} (November) and $4.3 \times$

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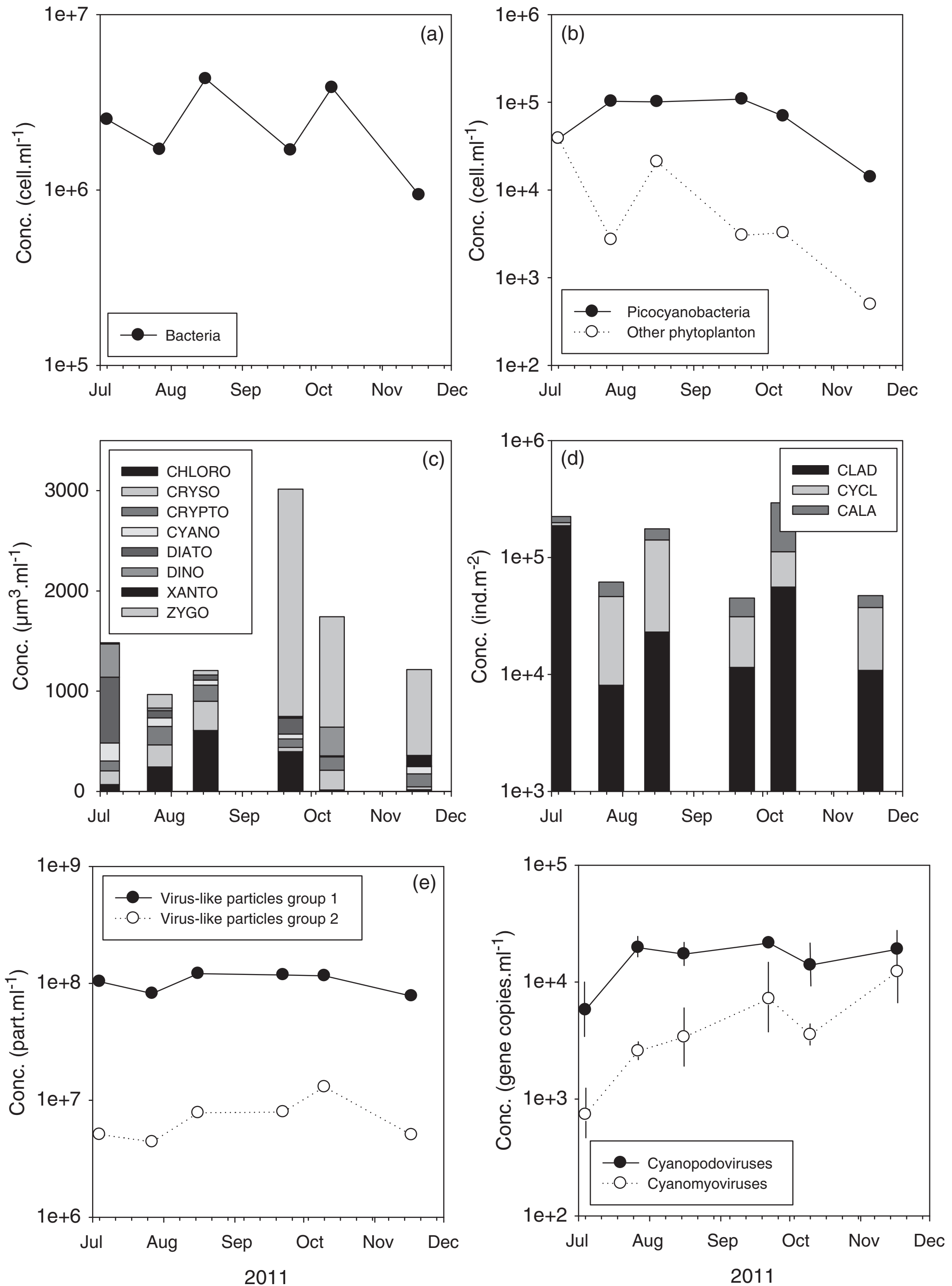

Figure 1. Abundances of the heterotrophic bacteria (A) and the picocyanobacteria and all other phytoplankters (B) obtained using flow cytometry. Biomass of phytoplanktonic (C) and zooplanktonic groups (D) counted by microscopy. Abundances of virus-like particles groups 1 and 2 (E) and of the cyanomyo- and cyanopodo-viruses (F).

$10^6 \pm 2.91 \times 10^6$ cells ml^{-1} (August) when integrated over the 0–20 m layer (Figure 1a). There were significant variations in the picocyanobacterial and other phytoplanktonic abundances (Figure 1b), as observed using FCM, during the period of study. The lowest picocyanobacterial abundance was recorded in November with $1.4 \times 10^4 \pm 2.09 \times 10^4$ cells ml^{-1} and the highest concentrations were recorded between the end of July and the end of September with approximately $1.1 \times 10^5 \pm 6.65 \times 10^4$ cells ml^{-1}. For the other (grouped) phytoplankters we could detect using FCM, there was a clear decrease from early summer (3.9×10^4 cells ml^{-1}) to the end of fall (4.9×10^2 cells ml^{-1}) but the proportion of the different populations changed throughout the period of study as already mentioned elsewhere [41]. The phytoplankton analysis was made complete using microscopy. Between July and November the biomass and the dominance of the phytoplankton species counted by microscopy changed dramatically (Figure 1c). The biomass varied between 380 (November) and 3000 (September) μg L^{-1}. Diatoms (mainly *Fragilaria crotonensis*) dominated the phytoplankton biomass in early July, while it was a mixture of diatoms, chlorophyceae, crysophyceae, cryptophyceae, zygophyceae (i.e. *Mougeotia gracillima*) and cyanobacteria at the end of the same month. In August, chlorophyceae (*Stichococcus bacillaris*) was the dominant group followed in almost equivalent proportion by crysophyceae (*Dinobryon* spp.) and cryptophyceae (*Rhodomonas*, *Cryptomonas*). In September, the chlorophyceae remained an important group but zygophyceae (*M. gracillima*) were clearly the most populated. The same trend was recorded in October and November with an increasing proportion of the cryptophyceae. The microscopic analysis of the micro-crustacean community revealed significant changes over the different groups throughout the period of study, for both the cladocerans, cyclopoids, and calanoids (Figure 1d) and the proportion between the herbivorous and carnivorous forms also changed greatly (not shown). Briefly, cladocerans (i.e. *Daphnia*) were the dominant group in July, representing 85% of the total planktonic micro-crustaceae. The following months, this community represented between 13% (August) and 25% (September) of the microcrustacean community. The cyclopoids (e.g. *Cyclops prealpinus*) became the dominant community during summer months, i.e. between the end of July and the end of September, representing between 43 and 67% of the total zooplanktonic community. The calanoids (represented only by *Eudiaptomus gracilis*) were the dominant group only in October (62%).

3.3. *Abundances of the viruses*

In Lake Geneva, high concentrations of virus-like particles (VLPs) have already been reported [29–31] and this study confirms also that these VLPs could be easily discriminated into two groups referred to as VLP1 and VLP2. It was previously reported that these two groups could be mainly associated with bacteriophages and cyanophages, respectively [30]. Over the 0–20 m layer, the concentration of the VLP1 varied in a relatively short range, between $7.8 \times 10^7 \pm 1.84 \times 10^7$ (November) and $1.2 \times 10^8 \pm 3.36 \times 10^7$ (August) part mL^{-1} while the abundance of VLP2 ranged from $4.42 \times 10^6 \pm 2.59 \times 10^6$ (August) to $1.3 \times 10^7 \pm 2.71 \times 10^7$ (October) part mL^{-1} (Figure 1e). Viral to bacterial ratio (VBR), i.e. the ratio of the viral abundance to the bacterial abundance which can be considered as a proxy of the relationships between these two communities, was in the range of 15 to 36 during the summer months. This ratio slowly increased to about 170 during the month of July (not shown). Among the viruses, we could quantify two groups of viruses belonging to

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the cyanophages, i.e. the cyanomyoviruses and the cyanopodoviruses throughout the copy numbers of the *g20* and *polA* genes counted by qPCR (Figure 1f). Cyanopodovirus concentration ranged between 5.8×10^3 (July) and 2.2×10^4 (September). The cyanomyoviral concentration as revealed by the CPS4/5 primer set ranged from 7.4×10^2 (July) to 1.2×10^4 (November). The same trend was recorded for CPS1/2 and we only showed the former (the correlation between the CPS1/2 and CPS4/5 abundances was $r = 0.95$, not shown). The abundances of all the viral groups were thus relatively high from the end of July to September, representing the summer months. It was also evident from the qPCR analysis that changes in viral abundances were in concurrence with the abundance of the picocyanobacteria (see Discussion).

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3.4. *Diversity of the different viral and prokaryotic groups*

The structure of the different targeted genes or groups revealed clear changes throughout the period of study, highlighting for most of the populations marked transitions, especially between summer and fall.

Firstly, the analysis using PFGE revealed the existence of 28 different viral bands corresponding to genome sizes ranging from 30 to 300 kb (Figure 2). The most abundant populations detected in each sample ranged from 10–17 with the lowest number of populations in July and the maximum at the summer-fall period. The most abundant groups were in the sizes 150–200 kb and 30–100 kb with bands of 35, 38, 48, 75, 95, 160, 170, 190, 200, 230 and 300 kb in common. The cluster analysis of PFGE patterns revealed two groups with a clear separation between early summer and the rest of the samples, i.e. early July *vs*. the other months. There was $>70\%$ and 75% similarity between samples taken at the end of July and mid-August and for October and November months, respectively. September seemed to differ from the two previous clusters.

The PCR-DGGE analysis for the picocyanobacteria gave 9–18 bands, with nine bands detected in early summer (Figure 3). The maximum number of bands was recorded in September and then the amount decreased during fall. Only four bands were common to all samples. The cluster analysis revealed that the samples belonged to three clusters: July, August-September and October-November (Figure 4a) with a clear transition between summer and fall.

For the *g20* gene (Figures 3 and 4b), the number of bands ranged from 7 (July) to 14 (August and September). There were four common bands in all the samples. There were seven common bands in September to November and five in July and August. The cluster analysis revealed the existence of two main clusters with fall (October and November) samples clustering separately against the summer months. The similarity between the fall and the summer samples were however larger ($>40\%$) compared to the previous 16 S gene ($<10\%$). Interestingly, it seems that there was no difference in the structure of the cyanomyophages between the end of July and mid-August.

For the *g23* gene (Figures 3 and 4c), the number of bands ranged from 16 to 24. Sixteen bands were observed during fall and 24 in early summer. Twelve bands were common from July to October. The intensities of the bands were lower when compared to PCR-DGGE with other genes even though the PCR amplification was good. The highest diversity was observed in August and October. The cluster analysis indicated that October differed from the other months, a period for which we also previously found marked changes in viral and

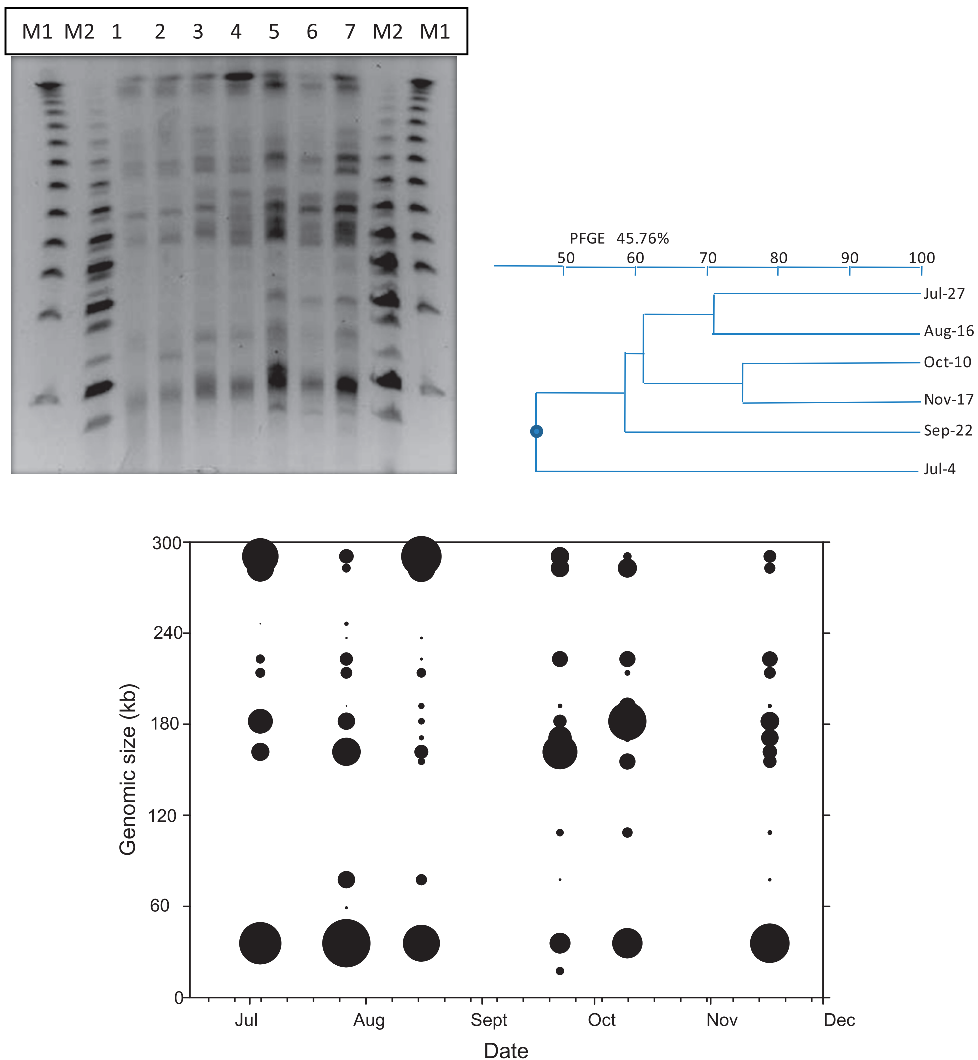

Figure 2. Gel and schematic outline of the relative abundance of viral populations determined by PFGE between July and November 2011, and output of the clustering analyses. M1 and M2 are Lambda and Midrange markers, 1–3 correspond to July samples, 4-August, 5-September, 6-October and 7-November.

bacterial abundances for peri-alpine lakes [30]. The similarity between all the samples (except for October) remained relatively large (between 65 and 80%).

For the *psbA* gene (Figures 3 and 4d), the number of bands ranged from 6 to 13 with a minimum during spring and a maximum during summer. The *psbA* gene had the minimum number of bands during July and a maximum diversity during September. There was just one common band among the samples. The cluster analysis revealed two main clusters separating July-August to the rest of the period studied, the difference between these two groups of samples being relatively important (approximately 90%). *psbA* diversity was $>50\%$ similar during both summer and fall months.

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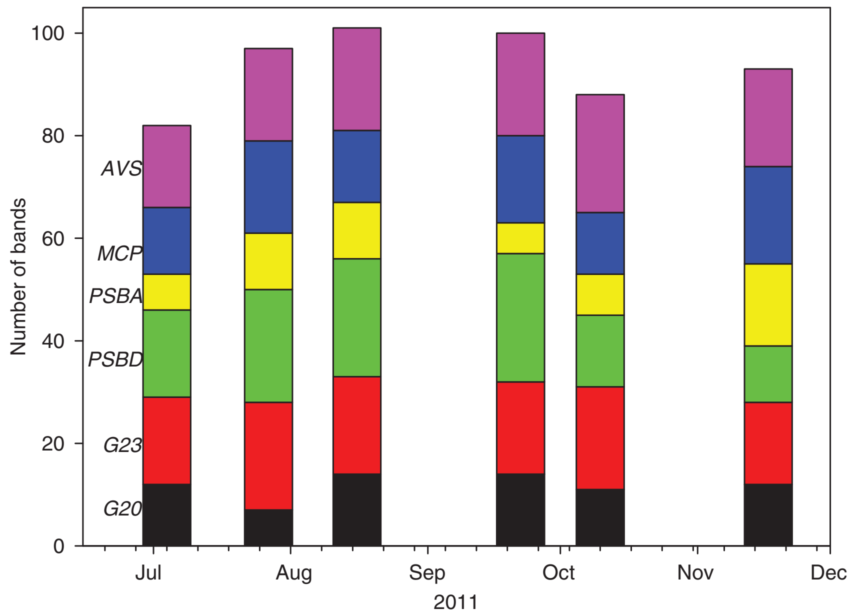

Figure 3. Evolution throughout the period of study of the DGGE bands numbers for viral signature genes.

psbD analysis showed the maximum diversity with the number of bands ranging from 11 (October and November) to 30 (in September, as for picocyanobacteria and *psbA*). However, the number of common bands was only three. There were nine common bands from September to November. The cluster analysis separated the samples into three clusters: one in early July, a second in end July to mid-August, and a third for the fall months (Figures 3 and 4e) with a high similarity (>90%) between October and November. Differently said, it seems that this gene behaved differently than *psbA*.

For the *mcp* gene, the number of bands ranged from 12 to 19. There were nine common bands among the samples. The number of bands was more important during the summer-fall transition period (September to November) and the peak of diversity was recorded in October. The cluster analysis showed a separation between October and the other months (Figures 3 and 4f) as previously reported for *g23*. However, the separation between the other months were different for these two genes. Typically, for *mcp*, September and November seemed more related to each other compared to the other months (July–August).

For the *polB* gene, the number of bands varied between 16 (early July) and 23 (October), and there were eight common bands among the samples. The diversity increased from summer till October and decreased in November. The cluster analysis revealed first a clear separation between August and the other months and then between July and the rest (i.e. September to November). The highest similarity (approximately 80%) was between October and November (Figures 3 and 4g).

Finally, the DGGE analysis of the bacteria revealed a number of bands ranging from 43 to 52. The cluster analysis revealed a separation between July–August (that were very similar, i.e. >90%) and the other months (that displayed lower similarity

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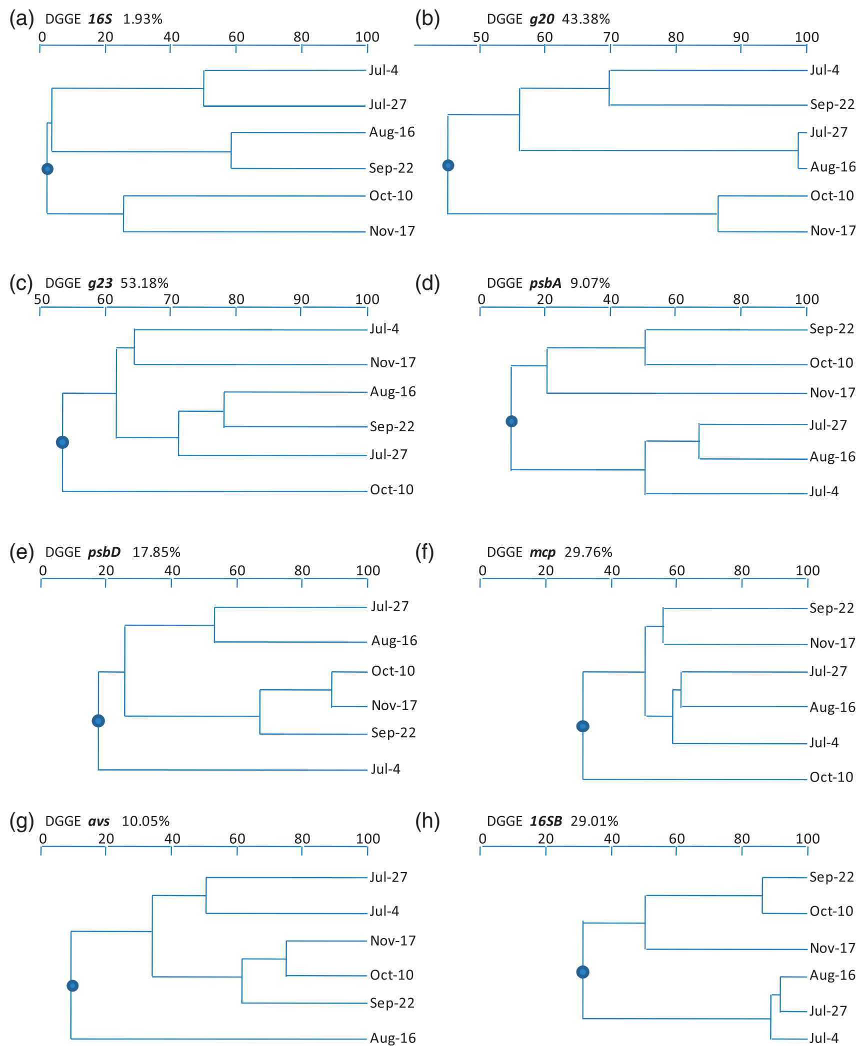

Figure 4. Cluster analyses of the different viral and cellular gene populations. The dendograms are constructed from a binary matrix of similarity values, using a distance calculation algorithm based on the absence/presence and relative fluorescence of the bands.

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between them, especially between September–October and November, i.e. around 50%, see Figure 4h).

3.5. *Relationships between variables*

The relationships between all the parameters were calculated using the Spearman's correlation. Typically, positive relationships were found between heterotrophic bacteria and the calanoid *E. gracilis* ($r = 0.94$, $n = 6$), and between phytoplankton and *Daphnia*

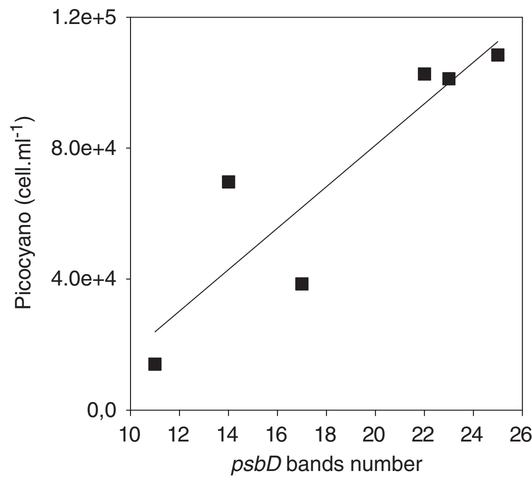

Figure 5. An example of relationships between variables: the number of the *psbD* number of bands in PCR-DGGE and the picocyanobacterial abundance.

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($r = 0.89$, $n = 6$) and O_2 ($r = 0.94$, $n = 6$) while there was a negative relationship between the picocyanobacterial abundance and SiO_2 ($r = -0.88$, $n = 6$). There were other relationships but with lower significance. Heterotrophic bacterial abundance was significantly correlated to crysophyceae (*Dinobryon* spp) and NH_4 ($r = 0.79$ and 0.88, $n = 6$). It was also found that the VLP2 had a significant positive correlation with chlorophyll *a* ($r = 0.83$, $n = 6$), cyanobacterial abundance ($r = 0.81$, $n = 6$) and NH_4 ($r = 0.80$) and VLP1 had a significant positive correlation only with bacterial abundance ($r = 0.76$, $n = 6$). In addition to these biological factors, the VLPs were found to be negatively related to N-NO_3 ($r = -0.82$, $n = 6$) and SiO_2 ($r = -0.75$, $n = 6$). The bacterial abundance had only a significant positive correlation with Ptot and Ntot while we did not find any significant relationship between the picocyanobacteria and the other biological or chemical factors. Phytoplankton abundance correlated with diatoms and this group correlated with Ptot, cladocerans and the number of PFGE bands. Cyanopodoviruses correlated with PFGE bands while cyanomyoviruses did not correlate with any biological variable.

For the gene band numbers, there was no significant relationship between any variable and the *g20* specific richness. However, we did observe significant relationships between *mcp* and heterotrophic bacterial abundance ($r = -0.85$, $n = 6$) and between *g23* and Ntot ($r = -0.83$, $n = 6$). There were also significant relationships between *psbD* and picocyanobacterial abundance ($r = 0.90$, $n = 6$, Figure 5), chlorophyceae ($r = 0.86$, $n = 6$) and SiO_2 ($r = -0.90$, $n = 6$). *psbA* presented significant relationships with crysophyceae ($r = 0.91$, $n = 6$), cryptophyceae ($r = 0.97$, $n = 6$) and xanthophyceae ($r = -0.83$, $n = 6$).

4. Discussion

Lake Geneva, despite being the largest lake in Western Europe, has been poorly investigated in terms of viral ecology. To the best of our knowledge, only three studies have been conducted so far [29–31] and none of them encompassed the viral 'diversity'.

Here, we could propose for the first time a survey over a short period of time (5 months) of viral group relative diversity and abundances in relation to the main environmental parameters. This study is particularly original since (i) six different signature genes were used to assess viral richness at the same time in parallel with PFGE and PCR-DGGE also applied to cyanobacteria and bacteria, (ii) cyanophages were enumerated using real-time PCR, (iii) cyanopodoviruses were enumerated using quantitative PCR.

As already reported elsewhere, two groups of viruses could be discriminated using flow cytometry and VLP1 was correlated to bacterial abundances while VLP2 was correlated with both picocyanobacterial abundance and chlorophyll *a*. We already observed such relationships in peri-alpine lakes [30] suggesting that VLP1 and VLP2 could be more associated with bacteriophages and cyanophages, respectively. To go further, we used the flow cytometric sorting capacity of our instrument at different occasions and the different primers used in this study were tested on the sorted groups with/without reconcentrating them. We could not, however, confirm that algal and cyanobacterial virus primers only amplify viruses sorted from the VLP2 signature. On the other hand, in most cases AVS and *mcp* primers were (90%) unable to amplify both VLP1 and VLP2, suggesting that algal viruses were either not sufficiently concentrated or that they were elsewhere (i.e. not in these FCM signatures, see [29]). In contrast, VLP1 and VLP2 responded often positively to CPS4/5 but more to VLP2 than to VLP1, supporting the previous assumption that VLP2 could be mainly related to cyanophages [31].

Unlike the 16 S rRNA gene which is used as a universal marker for assessing bacterial diversity, only partial virus diversity can be assessed using smaller taxonomic group specific markers that are available [42]. In our study, we used and targeted genes which encode the portal protein, the DNA polymerase, the major capsid protein and photosynthetic proteins, providing for the first time in an aquatic ecosystem a synoptic view of phytoplanktonic viral richness and structure evolution. Although we did not cut bands or use PCR products for cloning/sequencing to verify the identity of bands, the simple use of the fingerprinting methods (i.e. PFGE and DGGE) was very useful to assess viral richness in a lake where such methods have not been applied so far.

At first sight, PFGE analysis suggested that total viral richness increased along summer until early fall. The increase of the number of bands in the 30–100 kb range from September to November was accompanied by the increase of the concentration of heterotrophic bacteria while it was the picocyanobacteria for the 150–200 kb. Such a result could be expected since such size genome ranges suit what has been reported elsewhere for both bacteriophages and cyanophages [43]. Also a tight coupling could be observed between 16 S rRNA PCR-DGGE and PFGE ($r = 0.72$) suggesting co-variation between virus and prokaryotic host diversity in Lake Geneva. Such data indicating that phage and bacteria may co-vary remain, however, scarce and almost no data exist for freshwaters [44]. The phage community was prevalent in all the samples with five core genome sizes being common in all the samples (35, 38, 48, 75, 95 kb). It is noteworthy that the 95 kb band could also be a cyanophage [45]. The bands possibly representing cyanophage genomes [46], i.e. the 160, 170, 190 and 200 kb were present in all the samples. Finally, the 195, 250, 270 290, 310 and 320 kb size populations appeared from September to November and the presence of the 200, 230 and 300 kb were clearly linked to the higher abundance of some eukaryotic algae at fall. Indeed, these bands were either less prominent or absent in July and August when the abundance of the eukaryotic algae was lower. Viral populations with genome sizes above 300 kb (likely algal viruses) were less visible either due to lower abundance or loss during the ultrafiltration process. It appears clear that the variability

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observed in the total viral community structure was likely a result of the net production and loss of viral populations, which was directly influenced by host abundance and susceptibility [15,47]. In turn, changes in the viral community structure could strongly influence shifts in the prokaryotic and eukaryotic communities through the interaction of the viruses with their potential hosts.

The PCR-DGGE of the employed signature genes revealed marked seasonal differences. Indeed, we could observe that the structure changed in various ways, in July, August and sometimes September clustering together or at least revealing a clear separation with the following fall months (e.g. for the picocyanobacteria, the cyanomyoviruses, *psbA*). For some other genes, a transition could also be marked with a single month, e.g. early July for *psbD*, August for AVS and October for *g23* and *mcp*. Thus, there were clear differences in the seasonal patterns of the genes investigated and such changes in the structure of the different signature genes were likely to be attributed to the changes in host structure. The detailed analysis of the phytoplankton database revealed that important changes occurred in the abundance, dynamics and diversity of the phytoplankton but it was not possible from these data to discriminate between bottom up (light, nutrients, etc.) and top down effects (including possible viral lysis effect but also and merely zooplanktonic grazing). Though no association could be observed between the Shannon diversity index and any of the genes examined, a significant and negative relationship was recorded between *mcp* and the Brettum index applied to the phytoplankton ($r = 0.65$, $n = 6$, not shown), suggesting, as expected, a tight coupling between phycoDNA viruses and eukaryotic algal hosts. The difficulty in presenting obvious and clear associations between viruses and hosts in general can be associated with sampling strategies. In our case, sampling approximately every month over a (too) short period of time (e.g. 5 months) and from an integrated water volume (0–20 m) could have masked relationships between viruses and hosts both in terms of abundance and structure [48].

Among targeted viral genes, *psbD* seem to be an excellent marker, providing the highest number of bands and discriminating early summer, end of summer and fall samples. Firstly, this result reinforces the idea that *psbD* may be widely distributed in cyanophage isolates and through a diversity of environments [45,49–51]. Secondly, our results could suggest that *psbD* is much more diverse than previously reported and could be found in many groups affecting the cyanobacteria of Lake Geneva. This is supported by the observation that the diversity in *psbD* was significantly and positively correlated with picocyanobacterial abundance reinforcing the previous assumption and the tight coupling already reported in the marine field between this gene and picocyanobacteria. In addition, we did not detect any clear link with other (colonial and/or filamentous) cyanobacteria. On the other hand, we found that *psbD* diversity was linked to chlorophyceae and *psbA* also displayed a significant correlation with both cryptophyceae and crysophyceae. Even though no proof of the existence of such photosynthetic genes has been provided yet in eukaryotic algal viruses, we believe that such transfers may not be impossible. A pertinent question here is: Could viral photosynthetic genes be exchanged not only between phages of *Synechococcus* but also with viruses of green algae? Of course, such relationships have to be interpreted with caution since they could just be indirect. The finding that viruses carry such photosynthetic genes could finally suggest that they may also be directly responsible for a significant proportion of carbon fixation in Lake Geneva.

Another important assay was the qPCR which allowed us to assess the abundance (i.e. gene copies) of both cyanomyoviruses and cyanopodoviruses. Until now, only a couple of studies have provided such estimates (see below). Total cyanophages were relatively

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abundant but they did not represent a substantial proportion of the total viral community (largely $< 1\%$). Their variations with time suggested possible interactions with some hosts but this could also be a response to some environmental parameters (see below). The cyanopodoviruses could be present in similar or sometimes even higher abundance than cyanomyoviruses. This is in contrast to earlier reports that reported the dominance of cyanomyoviruses over cyanopodoviruses [16,52]. It is noteworthy however that this previous study was conducted in a marine environment and it is assumed that in marine environments, genetic exchanges occur among viruses infecting more distantly related hosts [53]. It is possible that cyanopodoviruses are present with equal significance as cyanomyoviruses in lacustrine environments and this could be easily explained through the highest diversity of cyanobacteria present in freshwaters. Also, the previous studies that reported on the higher abundance of cyanomyoviruses used degenerate primers for the detection of the *g20* gene [53] but reported to be specific for marine cyanomyoviruses. Hence it is important to re-evaluate the use of such primers specific for marine cyanomyoviruses in studies pertaining to lacustrine environments. Besides, the burst size of the cyanopodoviruses could be higher than] for the cyanomyoviruses explaining higher abundances for this T7-like cyanophage community. Our estimates of cyanomyoviruses could only be compared to the two available studies existing so far: that of [54] for Lake Erie and that of [38] for a coastal area in Norwegian waters. Our estimates were clearly lower than those found in Lake Erie but comparable and even slightly higher than that of Raunefjorden. Finally, the cyanomyoviral density displayed significant positive correlations with NO_3 and PO_4 whereas the cyanopodovirus showed a negative relationship with the diatoms, dinoflagellates and PFGE banding patterns. This could suggest very different dynamics, role and influence for these two groups of cyanophages but further investigations are needed to determine the time scales of maximal abundances of these cyanophages and their ecological role in Lake Geneva.

5. Conclusion

In the assessment of viral richness and dynamics in Lake Geneva, PCR-DGGE proved to be very useful and provided a lot of information on the potential diversity of viruses infecting phytoplankton and seasonality of the main groups in relation to possible hosts and environmental parameters. Though our data clearly suggested that viral diversity changed over the period of study, revealing a marked transition for many groups between summer and fall, the sampling strategy adopted here could also be responsible for the difficulty encountered in interpreting some relationships between the different parameters. Obtaining, in such an ecosystem survey, a longer time series with a better sampling strategy (i.e. at discrete depths and with a higher frequency) is thus critical to highlight more precisely both diversity and algal-virus relationships. Finally, we are also aware of the limitations of such fingerprinting methods and, in the future, PCR assays using specific primers to follow the dynamics of abundances of specific viral groups infecting key phytoplanktonic groups or species such as diatoms, chlorophyceae or zygophyceae, together with pyrosequencing and metagenomics will prove invaluable in understanding of the diversity and importance of viruses in Lake Geneva. This was recently proposed for a neighbouring lake (i.e. Lake Bourget) to Lake Geneva [55].

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