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**For publication****Utilization of interferometric light microscopy for rapid analysis of virus  
abundance in a river**

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27 **Abstract**

28           There is a constant need for direct counting of biotic nanoparticles such as viruses to  
29 unravel river functioning. We used, for the first time in freshwater, a new method based on  
30 interferometry differentiating viruses from other particles such as membrane vesicles. In the  
31 French Marne River, viruses represented between 42 and 72% of the particles. A spring  
32 monitoring in 2014 revealed their increase ( $2.1 \cdot 10^7$ - $2.1 \cdot 10^8 \text{ mL}^{-1}$ ) linked to an increase in algal  
33 biomass and diversity of bacterial plankton. Predicted virus size distributions were in  
34 agreement with transmission electron microscopy analysis suggesting a dominance of large  
35 viruses ( $\geq 60 \text{ nm}$ ).

36

37 *Keywords:* Virus; Membrane vesicle; Interferometric light microscopy; River

38

## 39 1. Introduction

40 Water from the Marne River, one of the major tributaries of the Seine River, is used  
41 for the production of drinking water for most of the inhabitants of Paris and surroundings.  
42 The Marne River used to be subjected to high levels of nutrients (nitrogen and phosphorus),  
43 originating from intensive agriculture and urban impact which entailed uneven and recurrent  
44 development of algae. These algal blooms were mostly due to diatoms, and secondarily to  
45 Chlorophyceae, cyanobacteria forming only low populations in lotic systems [1-3]. Since the  
46 2000s, with the EU Water Framework Directive (2000/60/CE), phosphorus has been  
47 considerably reduced [4, 5]. Algal blooms have become scarce, except in dry years. These  
48 blooms are mainly observed in spring, when the dilution rate by the discharge becomes lower  
49 than algal growth rate. These algal blooms constitute a significant inconvenience for the  
50 production of drinking water as, in addition to clogging filters, they increase the water pH [2].  
51 Algal dynamics have been extensively studied for 25 years in order to improve their growing  
52 prediction by modeling [6], without explicitly considering the ecological effect of viruses.

53 In any aquatic environment, viruses, about ten times more abundant than bacteria,  
54 control the algal and microbial diversity by lysis and may influence biogeochemical cycles.  
55 The abundance of aquatic viruses fluctuates over time, especially with the seasons. A peak of  
56 concentration is usually observed during spring-summer periods in surface waters;  
57 conversely, a significant decline occurs in autumn-winter [7]. The most common aquatic  
58 viruses of phytoplankton are double-stranded DNA-tailed viruses (Caudovirales) such as the  
59 following families: Myovirus (head size: 60-145 nm), Podovirus (60-70 nm) and Siphovirus  
60 (40-80 nm) which infect prokaryotes while PhycoDNA viruses (large DNA viruses, 100-220  
61 nm) or picorna-like viruses (RNA viruses, 35nm) infect eukaryotic algae [7].

62 Viral direct counts in any environments remain a critical step requiring a reliable and  
63 accurate method [8]. Transmission electron microscopy (TEM) is one of the best techniques

64 since it allows virus morphotypes to be counted and characterized [9], but is highly time-  
65 consuming. Optical methods based on epifluorescence microscopy to detect double-stranded  
66 DNA binding fluorophores such as DAPI or SYBR are used extensively. However, these  
67 methods are adapted neither for detection of single-stranded DNA, nor RNA viruses [10].  
68 Flow cytometry is also frequently used to enumerate viruses in natural viral assemblages [11].  
69 Most previous methods need expensive equipment and do not distinguish viruses from  
70 extracellular membrane vesicles. Extracellular membrane vesicles are another type of aquatic  
71 environmental nanoparticle. Produced by organisms from the three domains of life, they are  
72 made of lipids and proteins and sometimes contain genetic material, and could thus be  
73 mistaken for viruses [12].

74 We used a new method suitable for all genetic material of viruses and able to  
75 differentiate viruses from membrane vesicles. The interferometric light microscope (ILM)  
76 combines two measurements for a single nanoparticle (30 to 100nm): its scattered signal and  
77 its Brownian motion. The scattered signal is a function of the size and refractive index  
78 (related to density) of the particle, while the Brownian motion is a function only of the size of  
79 the particle [13]. ILM was utilized to distinguish virus from other particles and then tested to  
80 explore the variations of viral communities in the Marne River in spring 2014, expected to be  
81 the algal bloom period. The discharge averaging  $180 \text{ m}^3 \text{ s}^{-1}$  in January-March decreased to  $60$   
82  $\text{m}^3 \text{ s}^{-1}$  during the studied period (April-May). The diversity of eukaryotic and bacterial  
83 communities was determined simultaneously.

84

## 85 2. Material and methods

### 86 2.1. Sample preparation

87 Water was collected at Saint Maurice (5 km upstream from Paris, 48°48'58.16" North,  
88 002°25'27.35" East), the outlet of the Marne River, at three dates in 2014: April 1<sup>st</sup> and 22<sup>nd</sup>  
89 and May 14<sup>th</sup>. Samples were kept in an ice cooler during transport and filtered as soon as they  
90 arrived at the laboratory. Two liters of water were first filtered through 0.22 µm filters  
91 (PolyVinylidene Fluoride, Millipore) to collect eukaryotic and prokaryotic plankton. The  
92 filters were replaced every 0.5 L to avoid clogging. Filtrates (nanometric fraction) were then  
93 concentrated (10,000x) using a 30 KDa cut off filter (Amicon, Millipore).

94

### 95 2.2. Virioplankton characterization and interferometric data processing

96 We analyzed 5 µL of each filtrate (diluted in PBS to be 50x-concentrated) to collect a  
97 stack of 200 images (CMOS camera). The particles were first localized on each image and the  
98 average number per frame determined (volume = 10<sup>-8</sup> mL, concentration in particles of the  
99 sample). The maximum scattering intensity of each particle and its trajectory was computed  
100 [13]. With these data, we calculated the diameter of each particle from its scattering signal  
101 (diameter<sub>scat</sub>) and from its Brownian diffusion (diameter<sub>BM</sub>). Viral particles are the particles  
102 for which the two diameter measurements are close (refractive index n = 1.5). The  
103 nanoparticle populations were resolved using the Mclust package of R software with default  
104 parameters. Particles for which the diameter<sub>BM</sub> was over 20% larger than the diameter<sub>scat</sub>  
105 were considered as empty capsids or vesicles.

### 106 2.3. Transmission electron microscopy

107 Samples (10 µl, 10,000x-concentrated filtrates using Amicon 30 KDa cut off filters)  
108 were applied to electron microscope hydrophilic grids coated with carbon film. The specimen  
109 was then negatively stained with 2% uranyl acetate. The preparations were examined using a

110 Jeol 2100 electron microscope equipped with an LaB6 filament, with an acceleration voltage  
111 of 200 kV and a 2K x 2K Gatan CCD camera. The nominal magnification was 12.5K. Taking  
112 into account the position of the camera, this gives a final magnification at the specimen level  
113 of 17.8K. Therefore, the pixel size was 0.8 nm. The pictures (>20) were analyzed to count and  
114 sort viruses. Viral capsid diameters were measured using ImageJ software (US National  
115 Institutes of Health, Bethesda, MD, USA; [14]).

116

#### 117 2.4. Algal biomass

118 Chlorophyll-a concentrations (*Chl-a*) used as a proxy of the biomass of all  
119 photosynthetic organisms were determined by spectrophotometry according to [15].

120

### 121 3. Results and discussion

122

#### 123 3.1. Virus-like particle direct counting from river water

124 Using interferometric light microscopy, we counted particles in water samples  
125 previously filtered ( $<0.2 \mu\text{m}$ ) and concentrated (50x). We observed a sixfold increase in the  
126 total number of particles between the beginning ( $5 \cdot 10^7 \text{ mL}^{-1}$ , April 1<sup>st</sup>) and the end of the  
127 sampling ( $3 \cdot 10^8 \text{ mL}^{-1}$ , May 14<sup>th</sup>). We then analyzed the samples from a more qualitative point  
128 of view, calculating and plotting for each particle  $\text{diameter}_{\text{scat}}$  and  $\text{diameter}_{\text{BM}}$ . (Fig. 1).  
129 Indeed, any particle differing from a virus showed a similar movement, but a lower scattering  
130 signal [13]. We observed other dispersed particles that we predicted to be membrane vesicles  
131 [12]. Viruses therefore represented between 42% ( $2.1 \cdot 10^7 \text{ mL}^{-1}$ , April 1<sup>st</sup>) and 72% ( $2.1 \cdot 10^8$   
132  $\text{mL}^{-1}$ , May 14<sup>th</sup>) of the particles, in light of the first and last sampling date (Table 1). This  
133 analysis revealed that, in the studied river samples, the number of membrane vesicles could  
134 represent (by difference) 28-58 % of the detected particles. Estimating the number of  
135 membrane vesicles is a novelty which can provide new insight into the dynamics of viruses  
136 and their ecological importance.

137

#### 138 3.2. Viral population composition

139 After excluding membrane vesicles from the analysis, viral populations were further  
140 characterized according to their size. Throughout the study, we observed two major peaks  
141 centered at 45 and 60 nm diameters (Fig. 2). We classified the predicted viruses into two  
142 groups (Table 1): medium-size diameters (40-59 nm) and larger diameters ( $\geq 60 \text{ nm}$ ). In  
143 addition to a general increase in the abundance of 45 nm viruses, the proportion of  $\geq 60 \text{ nm}$   
144 viruses increased over time (from 7, 19% to 42%, for April 1<sup>st</sup> and 22<sup>nd</sup> and May 14<sup>th</sup>,  
145 respectively). The viral population of  $\geq 60 \text{ nm}$  in size would correspond to myocyanophages  
146 and PhycoDNAviruses [16]. However, we detected neither small viruses (30 nm range,

147 known to infect diatoms) nor very large viruses (>100nm, infecting freshwater green algae).  
148 In fact, for particles larger than 100 nm, there was no simple proportionality between the  
149 scattering signal and the diameter with ILM [13].

### 150 *3.3. Comparison of interferometric light and transmission electron microscopy*

151 Using TEM, viruses were observed and counted by major family and sized  
152 (diameters). Viruses were classified as Myoviruses, Podoviruses, Siphoviruses or  
153 PhycoDNAviruses based on their morphology [16] or spherical particles (the last category  
154 corresponds to membrane vesicles, untailed viruses and cellular debris). The percentages of  
155 Siphoviruses and Podoviruses were stable between samples, while Myoviruses increased  
156 gradually (Fig. S2, S3). We were also able to detect Podoviruses and Myoviruses by DNA  
157 amplification (see supplementary information), only in the most virus-concentrated sample  
158 from May 14<sup>th</sup> (data not shown). However the lack of reproducible amplification for  
159 Siphoviruses or PhycoDNAviruses suggested either that the amount of template was too small  
160 for proper amplification or that the degenerated primers were not suitable. Overall, while the  
161 proportion of prokaryotic viruses (Myoviruses and Siphoviruses and Podoviruses) increased  
162 (from 24 to 34%), that of eukaryotic viruses (PhycoDNAviruses) decreased (from 9 to 5%).

163 To compare the distribution of virus diameters between the TEM measurements with  
164 those obtained with ILM, we divided the samples into two groups: above and below 60 nm in  
165 diameter (Table 1). Although TEM is not a quantitative method, we observed the same  
166 distribution patterns for the two latter samples. However, we noticed a discrepancy for the  
167 first sample with a low viral concentration, suggesting that we need a threshold concentration  
168 of particles (of about  $10^9$  particles mL<sup>-1</sup>, corresponding to at least an average number of 10  
169 particles per frame) to reliably analyze samples by ILM.

170 3.4. Dynamics of photosynthetic populations during spring 2014 at Saint Maurice

171 We observed a fivefold increase in *Chl-a* content between April 1<sup>st</sup> (0.27  $\mu\text{g L}^{-1}$ ) and  
172 May 14<sup>th</sup> (1.35  $\mu\text{g L}^{-1}$ ), suggesting slight algal growth, but not true algal bloom. Indeed,  
173 during a spring algal bloom in the Marne River basin, especially composed of diatoms, the  
174 *Chl-a* concentration at St Maurice tended to reach 100  $\mu\text{g L}^{-1}$  or more [1]. The last algal bloom  
175 was observed in May 2011 and produced 144  $\mu\text{g L}^{-1}$  of total chlorophyll concentration perri  
176 (*Chl-a* and Pheopigments). On May 14, 2014, diatoms were responsible for 67% of the total  
177 algal biomass, with Cyanobacteria representing less than 0.1%.

178 DNA fingerprinting methods such as PCR-DGGE enable diversity investigation from  
179 natural microbial communities by studying the pattern of operational taxonomic units  
180 (OTUs). Concerning the aquatic eukaryotic communities, we noted a similar number of OTU  
181 (i. e. an approximation of the species richness) between the beginning (April 1<sup>st</sup>,  $21 \pm 2.8$  OTU)  
182 and end of sampling (May 14<sup>th</sup>,  $18 \pm 0$  OTU) (Fig. S1A). However, community  
183 composition showed a net modification, as the patterns of May 14<sup>th</sup> showed a weak similarity  
184 percentage (43.2%) with those of the other two dates.

185 In contrast, the species richness of aquatic bacterial communities (Fig. S1B) increased  
186 between early April and mid-May (from  $13 \pm 0.7$  to  $24 \pm 1.4$  OTU). Subsequently, the bacterial  
187 composition varied between dates: communities from April 1<sup>st</sup> and May 14<sup>th</sup> shared 43.1% of  
188 their OTU, whereas those of April 22<sup>nd</sup> shared 33.2% with the other two dates.

189 Thus, in the studied station of the Marne River, over the short sampling period,  
190 prokaryotic viruses proliferated. This suggests that their hosts (whose the number of OTUs  
191 increased) would also be numerically dominant in the microbial community. In contrast,  
192 eukaryotic phytoplankton viruses became a minor part of the viral population, while their  
193 hosts harbored less richness (cf. OTU numbers).

194

195           In conclusion, we have presented an analysis of virus abundance and diversity in a  
196 lotic freshwater environment, the Marne River, during a growth phase of phytoplankton. To  
197 analyze this viral abundance, we used an optical method based on interferometric light  
198 microscopy. This method proved its efficacy even at a low algal level. With this method, we  
199 observed an increase in virus particles and membrane vesicles which has not been previously  
200 described in freshwater.

201           New ecological studies on viral dynamics in freshwater are needed to understand  
202 how algal and bacterial dynamics are linked to viral communities and to possibly integrate  
203 them into existing ecological models.

204

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209

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- 249

250 **Legends to figure**

251

252

253 **Fig. 1. Analysis of particles present at the Saint Maurice station, an outlet of the Marne**

254 **River.**

255 Plot of the diameters of particles computed with their scattered signals (Diameter\_scatter)

256 and Brownian motion (Diameter<sub>BM</sub>). Lines indicate refractive indexes of the different

257 particles (viruses and vesicles). The colored bar corresponds to the relative number of

258 characterized particles: the different colors correspond to the Matlab histogram plotted in

259 normalized linear scale. The viruses that possess a refractive index of 1.5 are likely close to

260 this line, while the rest of the particles could be vesicles.

261

262 **Fig. 2. Analysis of predicted viral particles from the Saint Maurice station.**

263 The scattered signals of individual particles were resolved using the Mclust package of

264 R software in two populations (curves: 1 red, 2 blue and in black, the sum of all curves).

265 The number of particles, mean diameters and variances in populations 1 and 2 are noted in the

266 inset.

267 **Table 1: Comparison of analyzed viral particles by interferometric light (ILM) and transmission electron microscopy (TEM).**

	Interferometric light microscopy <sup>‡</sup>				Transmission electron microscopy			
	Particle concentrations in the water column (mL <sup>-1</sup> )	Number of particles <sup>#</sup>	Number of predicted viruses (% vs. particles)	ILM		Number of particles <sup>*</sup>	TEM	
<60 nm (%)				≥60 nm (%)	<60 nm (%)		≥60 nm (%)	
<b>April 1</b>	5 10 <sup>7</sup>	331	141 (42)	93	7	174	74	26
<b>April 22</b>	8 10 <sup>7</sup>	830	535 (64)	81	19	172	67	33
<b>May 14</b>	3 10 <sup>8</sup>	2243	1625 (72)	58	42	308	74	26

268

269 <sup>‡</sup>The same samples were concentrated 50 or 10,000 times for ILM and TEM, respectively270 <sup>#</sup>Number of particles for which we measured the diameter by scattering signal and by Brownian motion.271 <sup>\*</sup>Number of particles analyzed from about 20 TEM images.

272

273  
274 **Supplementary material for on-line submission**

275 *PCR-DGGE*

276 Genomic DNA was extracted in duplicate from PVDF filters (Millipore) using the  
277 PowerSoil DNA isolation kit (MO BIO Laboratories, Inc). Amplification was performed with  
278 specific primers for DGGE (Table S1) to amplify fragments from 16S and 18S ribosomal  
279 DNA to study bacterial and eukaryotic diversity, respectively. Denaturing gradient gel  
280 electrophoresis (DGGE) was performed using the Phor-U system (Ingenu) as described in [1].  
281 All PCR products were run on a single gel for the 16S and another for the 18S. After  
282 development of the DGGE polyacrylamide gel, each image was analyzed to determine  
283 similarities between profiles using the software GelCompar II 6.6 (Applied, Math). Each of  
284 the two gels was normalized based on an external reference pattern (the 100 bp ladder,  
285 Invitrogen) required by the software to compare lanes. Each band of a lane was automatically  
286 assigned to a band class, and all band classes were manually checked and eventually  
287 overridden to make them consistent between lanes. The Jaccard coefficient was calculated  
288 considering only the presence/absence of bands, each representing an operational taxonomic  
289 unit (OTU). Aquatic prokaryotic and eukaryotic communities could be compared thanks to  
290 their similarity percentages.

291

292 *PCR amplification of viral fragments*

293 We first established amplification conditions directly with known viruses of  
294 Siphovirus, Myovirus, Podoviruses and PhycodNAvirus. Then, each concentrated filtrate (50x)  
295 from water samples was used as a template with the following program: after initial  
296 denaturation at 95°C, 1 min annealing at 50°C and 1.5 min of extension at 72°C for 35

297 cycles. PCR products were electrophoresed in 1% agarose gels in TAE buffer. Primers are  
298 listed in Table S1.

299

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300 **References**

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320

321 *Legends to figures*

322

323 **Fig. S1.** DGGE analysis for eukaryotic (A) and prokaryotic (B) plankton from the Marne  
324 River at St Maurice screened via their 18S and 16S rRNA gene, respectively. From the  
325 bottom to the top of the figure, processed images by Gelcompar II, resulting similarity  
326 dendrograms of Jaccard index and histogram of number of OTUs.

327

328 **Fig. S2.** Transmission electron microscopy analysis of Saint Maurice water samples.

329 The pies describe viral morphotype compositions of each sample (at least 100 particles were  
330 considered).

331

332 **Fig. S3.** Electron micrograph of concentrated (10,000x) water sample from the Marne River  
333 (May 14<sup>th</sup>).

334 Examples of Myovirus and Siphovirus are shown by a white or black arrow, respectively. A  
335 membrane vesicle and an untailed virus are shown by a red or green arrow, respectively.  
336 Filaments could correspond to free DNA.

337

338 **Table S1: List of primers used in this study**

339

Primer name	Sequences (5'-3')	Amplicon size (bp)	Target	References
<b>F1427-GC</b>	(GC) <sub>20</sub> TCT GTG ATG CCC TTA GA	229	18S ribosomal DNA	[2]
<b>R1616</b>	GCG GTG TGT ACA AAG GG			
<b>F357-GC</b>	(GC) <sub>20</sub> CCT AGC GGA GGC AGG AG	201	16S ribosomal DNA	[3]
<b>R518</b>	ATT ACC GCG GCT GCT GG			
<b>MZPA1BF</b>	GAT ATT TGI GGI GGT CAG CCI ATG A	550	Major capsid protein of Myoviruses	[4]
<b>MZPA1BR</b>	CGC GGT TGA TTT CCA GCA TGA TTT C			
<b>Pol F</b>	CCA AAY CTY GCM CAR GT	650-750	DNA polymerase of Podoviruses	[5]
<b>Pol Rb</b>	CTC GTC RTG DAT RAA SGC			
<b>Mcp F</b>	GGY GGY CAR CGY AAT	347-518	Major capsid protein of PhycoDNA viruses	[6]
<b>Mcp R</b>	TGI ARY TGY TCR AYI AGG TA			
<b>lbdintF</b>	TGA TAC TGT GCC GGA TGA AA	750	Integrase of Siphoviruses	[7]
<b>lbdintR</b>	TTA GGC AGA GAC AGG CGA AT			

340

341

Figure 1

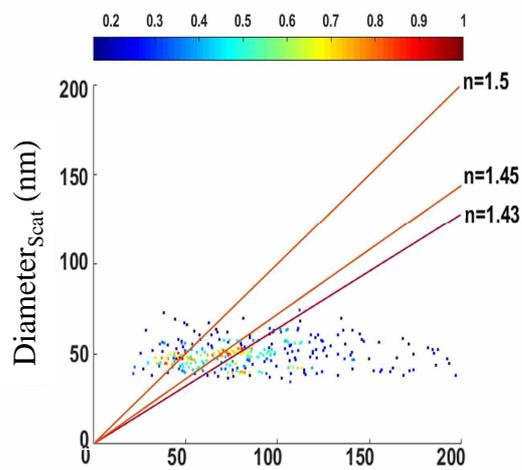
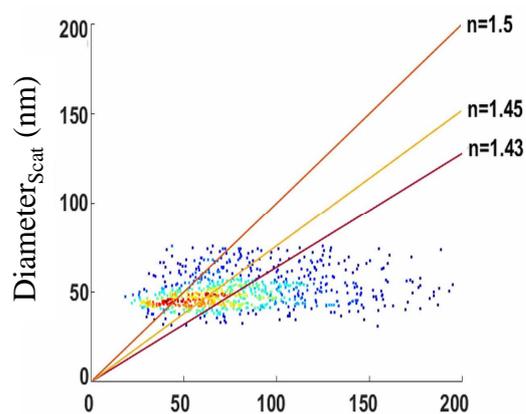
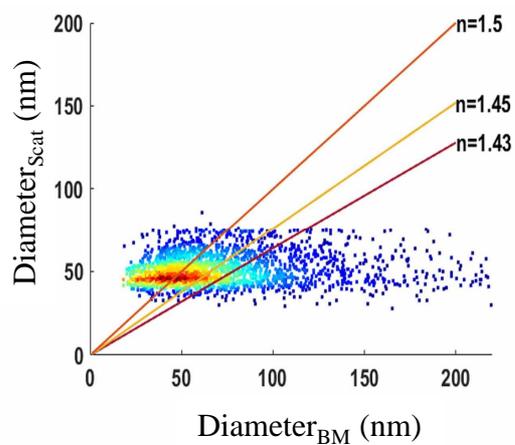
April 1<sup>st</sup>April 22<sup>th</sup>May 14<sup>th</sup>

Figure 2

