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Interactions of infectious F-specific RNA bacteriophages with suspended matter and sediment: Towards an understanding of FRNAPH distribution in a river water system

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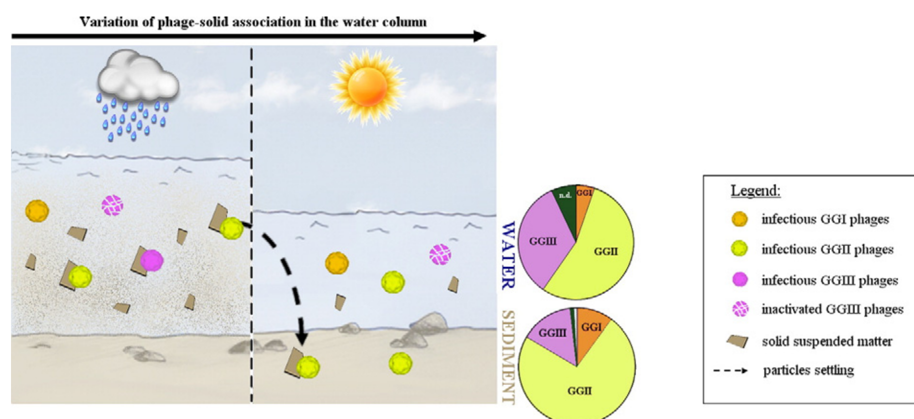
HIGHLIGHTS

- Hydro-climatological conditions influence phage-solid association in water column.
- FRNAPH from genogroups II, III and IV able to sorb to the riverbed sediment
- Fast attachment kinetics to sediment for all strains
- Genogroup distribution differs between water and sediment compartments.

GRAPHICAL ABSTRACT



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ABSTRACT

The association of viruses with settling particles is certainly a major process controlling the spread of viral pollution in surface water and sediment. To better understand the viral distribution in a river system, the behavior of F-specific RNA bacteriophages (FRNAPHs) was investigated in relationship with the suspended solids and sediment. The partitioning of phage particles (free or associated with solids) in surface water and the attachment capabilities of eight distinct strains of phages to sediment were studied in lab experiments. *In situ* observations were also performed with the genotyping of 166 individual plaques of FRNAPHs isolated from surface water and sediment. The results reported here demonstrate a variation of the status of infectious phages as a function of the hydro-climatological conditions. Phage-solid association seems to mainly occur during the peak of rainfall-runoff events but also to a certain extent during the recession phase compared to low flow conditions. The transfer of phages from the water column to sediment may occur at this time. Furthermore, the ability of FRNAPHs to interact with sediment was established for six strains out of eight, belonging to genogroups II, III and IV. A similar dynamic was observed for strains within a same genogroup despite different intensity of attachment and inactivation rates for strains of genogroups III and IV. The latter results match the *in situ* observations in the water and

Abbreviations: FRNAPHs, F-specific RNA bacteriophages; GGs, genogroups; PFU, plaque forming unit; WWTP, wastewater treatment plant; gc, genome copies; PBS, phosphate buffer saline.

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sediment compartments of the studied area. Infectious FRNAPH genogroup II was more abundant in sediment than in surface water. Its capability to sorb to sediment and its higher persistence in the environment compared to genogroups III and IV were the two main explanations. Together, lab and *in situ* experiments produce an overall vision of the mechanisms governing FRNAPH distribution among the water column and riverbed sediment.
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1. Introduction

The quality of water resources is mainly influenced by anthropogenic activities, which can entail the occurrence of human pathogenic viruses in surface water, such as enteric viruses transmitted by the fecal-oral route (Hot et al., 2003; Lodder and de Roda Husman, 2005; Pusch et al., 2005; Wyn-Jones et al., 2011). The spread of viral particles in water resources through fecal pollution is notably demonstrated by a high frequency of virus detection not only in environmental waters, but also in the sediment compartment. To date, presence of viruses has been observed in marine (Danovaro et al., 2001; De Flora et al., 1975; Jakubowska-Deredas et al., 2012) and freshwater (Hargreaves et al., 2013; Leroy et al., 2008; Maranger and Bird, 1996; Skraber et al., 2009) sediments. This compartment is commonly considered as a reservoir of viruses (Bosch et al., 2003). In addition, sediment has been reported to play a major role in the survival rate of infectious viruses. A higher persistence of viruses in sediment than in the water column has been described, as a consequence of a protective effect against inactivating agents (Smith et al., 1978) or a low accessibility of destructive UV rays to trapped viruses (Rao et al., 1984).

Several studies have addressed viral partitioning in surface water to predict sedimentation of viruses (Characklis et al., 2005; Gerba et al., 1978; Hejkal et al., 1981; Metcalf et al., 1984; Payment et al., 1988). Regardless of the matrix analyzed (surface water or treated wastewater), it is suggested that the majority of viral particles are free or associated with particles smaller than 0.3 μm. So, viruses tend to preferentially remain suspended in the water column, which still raises many questions about the transfer of viral particles from contaminated water to sediment. The prediction of virus-solid association in the aquatic environment is difficult due to number of variables, such as intrinsic properties of viruses, physicochemical environmental parameters and surface properties of the support. This was highlighted in the study of Moore et al. (1975) which presented an optimal condition for viral attachment in presence of divalent cations, but also the difference of behavior depending on the viruses tested. Another focus was set on the viral capability to interact with solid particles which influences the distribution of viruses in aquatic systems (Bellou et al., 2015; Bitton, 1975; Gerba et al., 1978; Syngouna and Chrysikopoulos, 2013). These interesting and complex data are essential to predict the fate and transport of viral pollution in aquatic ecosystems.

F-specific RNA bacteriophages (FRNAPHs) are able to replicate in *Escherichia coli*, which are highly present in the animal and human gut. Their concentration in water is thus directly correlated to fecal pollution. The shape and structure of FRNAPHs is similar to the one of enteric viruses, pathogenic for humans (*i.e.* Norovirus, hepatitis A virus). Hence, they help describing the behavior and the fate of pathogenic viruses in the environment or during the process of water treatment. They are generally considered as a valuable tool to evaluate an overall viral hazard in water. FRNAPHs belong to the *Leviviridae* family, which is divided into two genera and four genogroups (GGs): *Levivirus* including GGI and GGII and *Allolevivirus* including GGIII and GGIV. GGI and IV are mainly excreted by animals. GGII and GGIII seem more specific to humans (Hartard et al., 2015; Lee et al., 2011; Lee et al., 2009) especially when primer sequences described by Ogorzaly and Gantzer (2006) are used for the detection by real-time RT-PCR (Ogorzaly and Gantzer, 2006). The reason of such specificity has not been elucidated yet. Nevertheless, some differences between the genogroups have been underlined. GGI and GGII have a higher survival rate in water compared to GGIII and GGIV (Muniesa et al., 2009). More recently, it was established by using a set of reference species (MS2, GA, and Qß for GGI, GGII and GGIII, respectively) that even if the surface charge seems the same for all genogroups, the hydrophobicity characteristics were highly different (Dika et al., 2013; Langlet et al., 2008). Attachment to food (Deboosere et al., 2012) or behavior during wastewater treatment (Boudaud et al., 2012; Haramoto et al., 2015) was demonstrated to be significantly impacted. Sometimes the most hydrophobic phage (GA) displayed the highest attachment (food, sludge) while on other matrixes the less hydrophobic one does (sand, ultrafiltration membranes used for water treatment). Such differences should also influence attachment to suspended matter or sediment in water resources but, to our knowledge, no study has investigated the attachment of different FRNAPH GGs or strains on such a complex environmental matrix. This last point seems however crucial with regard to understanding the behavior of phage particles in aquatic environments.

Thus, the purpose of this study was to define the distribution of the four genogroups of FRNAPHs in a river water system including the suspended matter and the sediment compartments. The attachment capability of laboratory and naturally sediment-related strains of phages to the sediment was also determined, in order to inform on the previously observed distribution patterns. Moreover, this will feed the understanding of the varying comportment of the four genogroups under the different hydro-climatological conditions, especially the part due to sediment mobilization or to the enhanced fecal pollution through wastewater discharge and surface runoff. To the best of our knowledge, the establishment of a relationship between the comparative presence of FRNAPHs in surface water and sediment is still unclear and has not been addressed in the scientific literature yet.

Three distinct approaches were followed. First, the partitioning of FRNAPH particles (free or associated to solids) in surface water was investigated under various hydro-climatological conditions to forecast sedimentation behavior. Second, FRNAPH-solid matter interactions were studied in batch experiments for determining the attachment behavior of viral particles to particulate matter, depending on the surface properties of FRNAPHs. In this purpose, four FRNAPH strains isolated from sediment samples and belonging respectively to the four genogroups were identified and tested using fresh sediment and water samples from the study site. The representativeness of sediment isolates from each genogroup was confirmed with batch experiments performed also with prototypic laboratory strains. Third, genotyping of 166 plaques of infectious FRNAPHs collected from surface water and sediment samples was performed in order to have an insight on genogroup distribution in both compartments. In order to clarify the mechanisms governing FRNAPH distribution in the water column and sediment compartments, the *in situ* observations were finally confronted with the data of the lab experiments.

2. Materials and methods

2.1. Collection and processing of river water and sediment samples

2.1.1. Sampling site

Sediment and river water samples were collected from the Alzette River, one of the main rivers in Luxembourg. The sampling site was close to the city of Hesperange, with about 14,000 inhabitants and

located 6 km upstream from Luxembourg-city. Four wastewater treatment plants (WWTP) were located in the 10 km stretch upstream from the sampling site with respective capacities of 24,500, 90,000, 95,000 and 26,000 inhabitant-equivalents. The nearest WWTP, composed of a primary treatment and an activated sludge plant with nitrifying moving bed biofilm reactor technology, was located 1.4 km upstream from the sampling site. A phage removal efficiency of 91.3% during the sewage treatment process was estimated, resulting in a concentration (mean ± standard deviation) of $4.4 \pm 4.0 \times 10^3$ plaque forming units (PFU)/100 mL ($n = 6$) of infectious FRNAPHs in the WWTP effluents. Three out of the four genogroups were observed in the WWTP effluents ($n = 4$): GGI ($3.0 \pm 2.3 \times 10^5$ genome copies (gc) / 100 mL), GGII ($3.1 \pm 1.7 \times 10^5$ gc/100 mL) and GGIII ($2.2 \pm 0.9 \times 10^4$ gc/100 mL).

2.1.2. Water and sediment sampling

The environmental samples were collected over two different kinds of campaigns. The first one was used to assess the distribution of infectious FRNAPHs in surface water and sediment. A total of 15 surface water and 18 sediment samples were collected during the years 2014–2015. A pump system was developed for vacuuming the surface of the riverbed sediment. It is composed of a sterile 1-L glass bottle connected on one side to a 50-L vacuum container and on the other side to a rigid plastic pipe (diameter = 25 mm). This system is operated by opening a valve allowing sediment to flow through the pipe into the bottle. The second set of water sampling was used to evaluate the partitioning of FRNAPH particles in surface water. Four stream water samples were collected under different hydro-climatologic conditions from low flow to high flow rates during rainfall-runoff events (Table 1). All samples were transported back to the laboratory in a refrigerated compartment. Samples containing sediment were stored for 24 h at 4 °C in order to allow settlement of particles.

2.1.3. Elution of phages from sediment

Twenty milliliters of pyrophosphate buffer (0.01 M) at pH 7.0 were added to 10 g of wet sediment (sediment/liquid ratio = 0.5). The mixture was submitted to sonication (37 kHz) on ice for 3 min and interrupted for 30 s every minute for mixing manually the samples (Danovaro et al., 2001). Samples were then shaken at 400 rpm for 30 min at 4 °C (orbital shaker, KS 260 basic IKA®). Supernatant was retrieved twice after successive centrifugations at 10,000*g* for 10 and 5 min. Ten 1-mL fractions of the supernatant were used for the determination of infectious FRNAPH concentration by plaque assay. The recovery of the infectious FRNAPH elution step was assessed on different sediment samples by spiking a known amount of the most representative strain in sediment *i.e.* BZ13 strain DL20 (FJ483839) belonging to GGII. More than 10% of infectious viral particles were found after the elution process with a recovery variability which could be influenced by the sediment matrix ($29.3 \pm 16.0\%$, $n = 6$).

Table 1
Physical parameters and F-specific RNA bacteriophage (FRNAPH) concentration of the four surface water samples collected under different hydro-climatological conditions.

	Sample #1	Sample #2	Sample #3	Sample #4
Sampling date	02/29/2016	10/07/2014	03/02/2015	03/03/2015
Flow rate (m^3/s)	4.0	4.9	19.5	6.0
Hydrological phases	Low flow	Rising phase	Peak of rainfall-runoff event	Recession phase
Total rainfall[a] (mm)	0	59.8	16.1	16.1
Antecedent rainfall[b] (mm)	0	3.8	15.2	20.5
Turbidity (NTU)	11.2	93.6	457	56.7
FRNAPH concentration ($\log_{10}$ PFU/100 mL)	3.4	3.2	3.8	3.2

[a] On the total duration of the event.
[b] 5 days prior the event.

2.2. F-specific RNA bacteriophages

2.2.1. Detection of infectious FRNAPHs

The concentration of infectious FRNAPHs was determined using *Salmonella enteric* serovar Typhimurium strain WG49 as host bacteria and the double-agar-layer technique as described in the ISO standard 10705-1 (2001). Nalidixic acid was added to media for limiting the growth of the abundant bacterial flora. Negative and positive controls (MS2 phage) were included in the determination of infectious FRNAPH concentrations. Plates were incubated overnight at 37 °C before PFU counting.

2.2.2. Plaque isolation from environmental samples

From surface water and sediment samples collected during the years 2014–2015, 10 well-separated phage plaques formed on agar plates were randomly collected by micropipette aspiration and suspended in a 1 mL volume of phosphate-buffered saline (PBS 0.01 M, pH 7.4, Sigma-Aldrich) supplemented with 5% of glycerol (Ogorzaly et al., 2009). After 1 min of agitation at 30 °C (1400 rpm, Thermomixer, Eppendorf), phage isolates (57 isolates from surface water and 109 from sediment) were stored at −80 °C until characterization. Genotyping and identification of the field-collected strains were performed using a high throughput sequencing technology based on whole genome sequencing (unpublished protocol).

2.2.3. Bacteriophage strains and stock production

Once collected and identified, FRNAPH strains belonging to each genogroup (GGI to GGIV) was replicated before use in batch experiments. MS2 strain DL16 (EF108464), BZ13 strain DL20 (FJ483839), Qß strain HL4-9 (FJ483841) and FI strain BR1 (FJ539134) were selected as the most representative sediment isolates of the GGI (predominance of 73% of strain DL16, $n = 14$), GGII (39% of strain DL20, $n = 111$), GGIII (75% of strain HL4-9, $n = 35$) and GGIV (100% of strain BR1, $n = 1$), respectively. In the same way, phage stock suspensions of prototypic laboratory strains, MS2 (GQ153927), GA (X03869), Qß (AB971354) and SP (X07489) belonging to GGI, GGII, GGIII and GGIV, respectively, were carried out.

Production of phage stock suspensions was achieved by growing host bacteria *Escherichia coli* K-12 *Hfr* (CIP 104130) to the exponential growth phase and subsequent inoculation with the respective phages according to the standard procedure ISO 10705-1 (2001), without the chloroform step. After production, the phage suspensions were centrifuged at 3000*g* for 20 min to separate bacteria from bacteriophage particles. The supernatant was decontaminated by successive filtrations through sterile membrane filters (first 0.8 μm then 0.22 μm, Millipore). The initial concentrations of phage stocks were estimated at about 10^{11} PFU/mL. All phage stocks were stored at 4 °C until use.

2.2.4. Bacteriophage genogroup quantification using a two-step RT-qPCR

First, RNA extraction was done by the addition of the NucliSENS® Lysis buffer (BioMerieux, France) (sample/lysis buffer ratio = 0.5). The RNA extraction was performed using the NucliSENS® Magnetic Extraction kit (BioMerieux, France). The elution step was carried out in 100 μL of elution buffer and extracted nucleic acids were stored at −80 °C until detection by RT-qPCR.

Then, genogroup quantification was performed by the RT-qPCR method developed by Ogorzaly and Gantzer (2006) on a ViiA™ 7 Real-Time PCR System (Applied Biosystems, France). Two modifications were brought to this method with the use of the TaqMan® Environmental MasterMix 2.0 (Applied Biosystems®) in a final volume of 25 μL. Construction of standard curves with target genomic sequences of each genogroup was developed to determine RNA genomic concentrations (Fauvel et al., 2016). A detection limit of 10 genome copies per

RT-qPCR reaction was determined for the four genogroups. A negative extraction control as well as negative and positive RT-qPCR controls were incorporated into each analysis.

2.3. Partitioning behavior of FRNAPHs in surface water

Surface water samples were collected on four occasions under different water flow rates and turbidities. Each sample represented a particular hydro-climatological condition (Table 1). Daily rainfall data were provided by the "Administration des Services Techniques de l'Agriculture" (ASTA, http://www.agrimeteo.lu) whereas the water flow rate of the Alzette River was provided by the "Administration de la Gestion de l'Eau". The low flow condition corresponded to a 5-days period where the water flow rate was stable. The rising and recession phases were respectively the increase and decrease of the water flow rate during an event while the peak of rainfall-runoff event was the highest point of the water discharge. These various conditions were used to study the degree to which bacteriophages in the water column are associated with settling particles. Turbidity was measured in nephelometric units (NTU) with a portable turbidimeter (HACH 2100Q). The fraction of phages associated with settling particles in water was estimated through the use of a centrifugation technique. Three successive centrifugations were performed. A first centrifugation at 3000*g* (Centrifuge 5810R, Eppendorf) for 10 min at 4 °C was carried out on raw surface water; a second was done at 10,000*g* (Centrifuge 5810R, Eppendorf) for 10 min at 4 °C on the supernatant resulting from the 3000*g* centrifugation. An ultracentrifugation of 235,000*g* for 90 min at 4 °C (L-90K Ultracentrifuge, Beckman Coulter OptimaTM) was finally performed on the supernatant from the 10,000*g* centrifugation. At each step, the entire pellet was resuspended in PBS (0.01 M, pH 7.4, Sigma-Aldrich), and part of the supernatant was stored at 4 °C in view of infectivity assays. Results were expressed in PFU per 100 mL.

2.4. Attachment of bacteriophages to solid matter in batch experiments

Batch experiments were performed in two distinct media *i.e.* phosphate buffered saline (PBS 0.01 M, pH 7.4, Sigma-Aldrich) and raw river water, using laboratory and/or environmental phage strains. Experiments in river water were performed for studying attachment and inactivation behavior whereas experiments in PBS were carried out for comparing behavior of distinct phage strains belonging to the same genogroup. Phage suspensions were added to sterile glass bottles of 100 mL containing 5 g of sediment collected during low flow condition and 10 mL of liquid media (solution/solid ratio = 2.0) to obtain a final concentration of 2 x 10^4 PFU/mL. This concentration was chosen high enough to neglect phages naturally occurred in surface water and sediment but not too high to avoid overloading the system. At the beginning of the experiment ($t = 0$), the theoretical concentration of each infectious phage suspension was evaluated by quantification of the phage stocks. The experiment starting point was set immediately after the contact of phages with sediment (corresponding to $t = 1$ min). Solid matter was constituted of fresh sediment and was a complex environmental matrix. The four phage stocks were used independently. Sediment-phages suspension was shaken at 250 rpm at 4 °C (orbital shaker, KS 260 basic IKA®) for different time intervals (1, 30, 60, 120 and 180 min). Mixed suspensions were centrifuged at 10,000*g* for 10 min for removing solid particles. The supernatant was then analyzed to determine remaining FRNAPH concentrations using infectivity and/or molecular biology assays. In parallel, a control batch receiving phage suspensions without sediment was submitted to the same procedure in order to monitor bacteriophage inactivation and sorption to the container surface.

RT-qPCR inhibition was evaluated on negative controls by spiking a known amount of transcribed MS2 plasmids into extracted samples. No inhibition was observed for tested controls except for two samples which presented an inhibition of 2.5% and 69.7%.

3. Results

3.1. Partitioning of F-specific RNA bacteriophages in surface water

Surface water was collected under different hydro-climatological conditions including low flow period as well as rainfall-runoff events at the selected sampling point in the Alzette River. Environmental conditions and water quality parameters of each sample are presented in Table 1. Besides the rising and the recession phases, two extreme situations were sampled: the prolonged low flow period and the peak of a heavy rainfall-runoff event. Between both situations, turbidity and flow rate increased about 40 and 5 times, respectively, and phage concentration increased by 0.4 $\log_{10}$.

Each of the four samples was analyzed by successive centrifugations at varying rotational speeds in order to determine the partitioning of infectious FRNAPHs in the water column. Fig. 1 represents the percentage of viral and suspended particles removed from the water phase (supernatant) after each centrifugation step. Despite water samples with different initial turbidities, the loss of suspended particles was equivalent. The major particle removal (from 86.7% to 96.9%) occurred at the first centrifugation step (3000*g*). In contrast, the dynamics of phage particle settlement varied depending on the hydro-climatologic conditions. Huge differences were especially noticed for the two extreme conditions (low flow period *vs* peak of rainfall). After the first centrifugation at 3000*g*, 54.9% of phages were removed from the supernatant for the peak of rainfall compared to 15.5% for the low flow condition. This difference was even higher after a centrifugation at 10,000*g* (approx. 80% *vs* about 20%), while the settlement of suspended solids reaching 97.7 ± 2.5% for both conditions. Concerning the two other conditions, the results obtained for the rising phase were similar to those of the low flow condition, whereas withdrawal of phages from the supernatant was observed for the water level recession phase. Concerning the third step, *i.e.* ultracentrifugation, no difference was observed with an equivalent loss of phages in the supernatant. The concentration of infectious FRNAPHs determined in the pellets supported all these results (data not shown). This difference of sedimentation behavior in relation to hydro-climatological conditions suggests that the status of infectious FRNAPHs (free or associated with settling particles) varies in the water column.

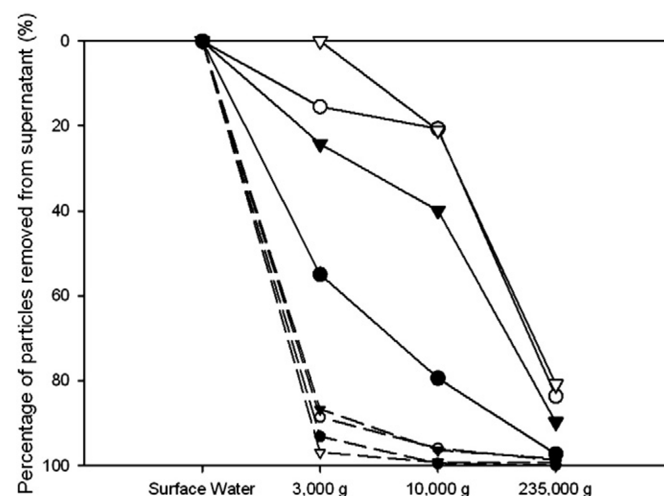

Fig. 1. Percentage of infectious F-specific RNA bacteriophages (solid line) and suspended particles (dashed line) removed from surface water after successive centrifugation steps (from surface water at 3000*g*, supernatant from 3000*g* at 10,000*g* and supernatant from 10,000*g* at 235,000*g*) according to the four distinct hydro-climatological conditions (○ low flow, ▽ rising phase, ● peak of rainfall-runoff event, ▼ recession phase).

3.2. Influence of viral genogroup on the attachment of FRNAPH to sediment in river water

Fig. 2 presents results of batch experiment where environmental FRNAPH strains were put in contact in river water with or without sediment. Infectious FRNAPHs and total phage particles were quantified during the whole experiment through both an infectivity assay (PFU) and molecular tool quantification (genome copies). Inactivation (loss of PFU only) and/or attachment (loss of both PFU and genome copies) were the main causes of variation of the FRNAPH concentration. The inactivation level was estimated as the loss of PFU in absence of sediment whereas the attachment rate was calculated by comparing total phage numbers (genome copies) in the absence and presence of sediment at a same time point.

Strain DL16 (GGI) underwent neither inactivation nor attachment in reason of a stable concentration of infectious and total particles during the whole stirring period (180 min) in absence or presence of sediment. The observations were contrasting for the others tested strains from GGs II, III and IV.

In absence of sediment, the persistence of strain DL20 (GGII) was demonstrated with a constant concentration of infectious particles and genome copies during the whole experiment. In presence of sediment, the behavior of this strain was modified with a rapid reduction of 1.3 $\log_{10}$ of total particles after 30 min and 1.4 $\log_{10}$ of infectious phages at the end of the experiment. The stability of strain DL20 in absence of sediment supports the hypothesis that, in presence of solids, the reduction of total phage particle concentration was due to attachment to sediment (attachment rate of 1.3 $\log_{10}$ at 30 min). Moreover, reduction kinetics in presence of sediment seems to be different between the total and infectious particles (Spearman correlation test, $r = 0.714$, p-value $= 0.136$). A fast reduction was observed for total particles compared to a slower reduction over the entire time of the experiment for the infectious DL20 particles.

For GGIII, a decrease of 0.9 and 1.8 $\log_{10}$ was measured at the end of the experiment for total and infectious strain HL4-9 particles, respectively, in absence of sediment. Attachment of phages to the glass container inner surfaces, degradation of viral RNA and inactivation of infectious particles could explain these reductions. In presence of sediment, an instantaneous reduction of 2.1 $\log_{10}$ and 1.6 $\log_{10}$ occurred for total and infectious phages and continued to reach a maximal reduction of 2.8 $\log_{10}$ for both. The higher reduction of total phage particle concentration in presence of sediment than in absence of sediment at a same time (2.7 $\log_{10}$ compared to 0.8 $\log_{10}$ at 30 min, respectively) confirmed the attachment of strain HL4-9 to sediment (attachment rate of 1.9 $\log_{10}$ at 30 min). Furthermore, reduction kinetics in presence of sediment again appears to be faster for total particles with a maximum reduction after 30 min whereas reduction continued up to 120 min for infectious phages (Spearman correlation test, $r = 0.429$, p-value $= 0.419$).

For the GGIV strain, a decrease of total and infectious particles was shown in the absence and in presence of sediment. As for the GGIII strain, viral RNA degradation and/or attachment to the container were

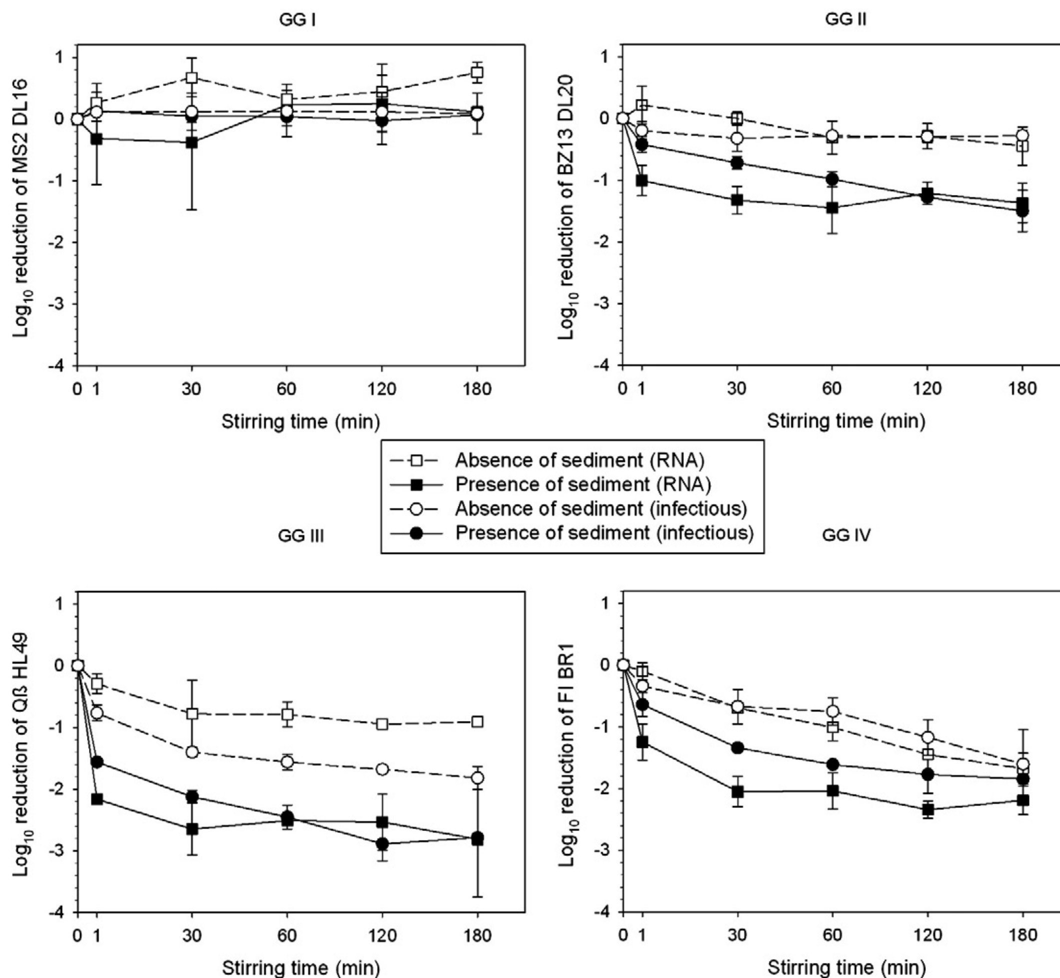

Fig. 2. Log_{10} reduction of infectious particles (circle symbol) and genome (square symbol) of F-specific RNA bacteriophage strains in surface water upon stirring in absence (white symbol) or presence (black symbol) of sediment. The starting concentration of 2.0 x 10^4 PFU/mL was the same whatever the infectious strain genogroup whereas this one varied for total phage particles (4.3 x 10^4 gc/mL for MS2 DL16, 2.4 x 10^6 gc/mL for BZ13 DL20, 4.6 x 10^4 gc/mL for Qß HL4-9 and 6.8 x 10^5 for FI BR1). Vertical bars indicate standard deviation from triplicate experiments.

challenged in absence of solid whereas attachment to solid matter occurred in presence of sediment (reduction of 2.0 log_{10} of total particles after 30 min in presence of sediment compared to 0.7 log_{10} in absence of sediment: 1.3 log_{10} attachment rate at 30 min). Nevertheless, the difference of reduction between total and infectious BR1 particles seems to be less obvious (Spearman correlation test, $r = 0.886$, p-value $= 0.033$).

To conclude, a distinct behavior of the four FRNAPH strains was demonstrated with no attachment effect for the strain belonging to GGI, whereas attachment and/or inactivation were for the other ones. A difference of attachment kinetics was also suspected between total and infectious particles for the GGII and GGIII strains with reduction kinetics slower for infectious phages than total viral particles.

3.3. Influence of FRNAPH viral strain further to contact to sediment

In order to complete the investigation on the attachment of FRNAPH to sediment, the dynamic of two strains belonging to the same genogroup was compared in PBS in presence and absence of sediment, using only infectivity assay (Fig. S1).

No reduction occurred for both strains belonging to GGI and GGII, displaying therefore a similar behavior, whereas a decrease of infectious FRNAPH concentration was measured for both strains of GGIII and GGIV. For GGIII, higher reduction rates were consistently observed for the environmental strain compared to the lab strain (Wilcoxon signed rank test, $p < 0.001$), with a final reduction rate differences at 180 min of 0.5 log_{10} (without sediment) and 0.9 log_{10} (with sediment). For GGIV, a difference of reduction between the environmental strain and the lab strain occurred exclusively in absence of sediment (Wilcoxon signed rank test, $p = 0.007$), with a final reduction rate differences at 180 min of 1.8 log_{10}. In presence of sediment, both strains of GGIV present a similar behavior (Wilcoxon signed rank test, $p = 0.135$). Then, despite different intensity of reduction in absence or presence of sediment for strains of GGIII and GGIV, a similar dynamic was observed for strains within a same genogroup. It was however not possible to clearly distinct the inactivation to attachment dynamics since only infectivity was performed during the PBS experiment.

A variation in the BZ13 DL20 (environmental GGII strain) strain behavior was observed between experiments performed in PBS and in river water, reflecting a media effect on the strain dynamic. In presence of sediment, no reduction of infectious strain BZ13 DL20 was observed in PBS (Fig. S1) whereas 1.4 log_{10} of reduction was measured in surface water (Fig. 2). To confirm the media-dependent behavior for GG II strains, the fate of the GA strain was investigated in river water with and without sediment (Fig. S2). Once again, BZ13 and GA strains exhibited similar persistence and reduction profiles in a given media.

3.4. F-specific RNA bacteriophage diversity and distribution in surface water and sediment

Infectious FRNAPHs were quantified from river water and sediment samples collected every month. The mean concentration was estimated to be 2.8 ± 0.7 log_{10} PFU/100 mL of surface water ($n = 19$) and 1.4 ± 0.7 log_{10} PFU/g of dry sediment ($n = 15$). One hundred sixty-six individual plaques of infectious FRNAPHs were characterized from surface water and sediment samples. Fig. 3 shows the distribution of the four genogroups in both compartments. The distribution of infectious FRNAPH genogroups in water was different to that observed in sediment, especially for GGII (Pearson's chi-squared test, p-value $= 0.013$) and GGIII (Pearson's chi-squared test, p-value $= 0.005$).

4. Discussion

The objective of this study was to provide new elements in the understanding of viral particle distribution in a dynamic river system. To this purpose, detection and distribution of infectious FRNAPHs were assessed in surface water and in sediments of the Alzette River. In parallel, two distinct laboratory experiments were carried out to clarify the solid-viral particle interactions and to forecast transfer of phages from the water column to sediment.

The environmental spread of infectious FRNAPH particles was confirmed by their presence in surface water and in sediment compartments of the study area. Detection of infectious FRNAPHs in both compartments was comparable to the results of previous studies (Lucena et al., 2003; Ogorzaly et al., 2009; Skraber et al., 2009). In the present work, genogroup determination was carried out by whole genome sequencing directly from individual infectious phage plaques. Among the 166 plaques isolated, GGII isolates were proportionally the most abundant in both surface water and sediment compartments. FRNAPHs are often proposed as microbial source tracking tracers to distinguish human (GGII and GGIII) from non-human (GGI and GGIV) fecal contamination sources (Lee et al., 2011; Lee et al., 2009). Abundance of GGII in both compartments confirms the anthropogenic character of the fecal pollution sources found in the Alzette River, with a WWTP at <2 km upstream from the sampling site. This is also confirmed by direct analysis of water samples by RT-qPCR (Fauvel et al., 2016). The repartition of GGs was differed in both, water and sediment compartments, especially for GGII and GGIII. Infectious FRNAPHs belonging to GGII were more present in sediment than in surface water, which was the opposite for GGIII phages. This difference of distribution might be elucidated based on the results obtained from the laboratory batch experiments discussed hereafter.

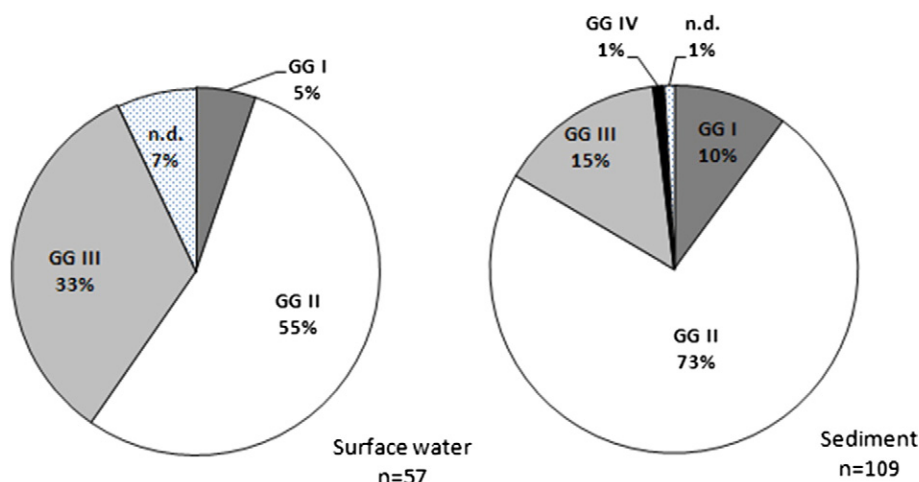

Fig. 3. Distribution of the four genogroups (GGs) of F-specific RNA bacteriophages in surface water and sediment samples. n.d., not detected (viral particle not assigned to a FRNAPH or failure of genome sequencing).

The interactions of FRNAPHs with their direct environment are mainly controlled by their surface properties. In order to detect any variation of behavior of different viral particles, four strains of FRNAPHs, directly isolated from sediment, were used in batch experiments. Results obtained suggest a difference of behavior for the four strains tested in terms of inactivation and attachment under experimental conditions (surface water at 4 °C and shaking at 250 rpm). On the one hand, inactivation of bacteriophage strain DL16 (GGI) and strain DL20 (GGII) were much less important than strain HL4-9 (GGIII) and strain BR1 (GGIV) in absence of sediment. The greater survival of GGI and GGII compared to GGIII and GGIV was previously reported (Muniesa et al., 2009; Schaper et al., 2002). A variability in the survival of strains within a same genogroup still remains in debate with some contradictory data reported (Brion et al., 2002; Long and Sobsey, 2004). Results of batch experiments performed in PBS with prototypic laboratory and environmental phages present a similar persistence for strains belonging to GGI and GGII, whereas higher inactivation rates were suspected for the GGIII and GGIV environmental strains. Even if results obtained by infectivity assay did not allow us to clearly conclude in favor of phage inactivation, the reduction rates observed were always stronger for environmental strains of GGIII and GGIV than for laboratory strains. All these results confirm the difficulty to establish original proportions of infectious FRNAPH genogroups in environment by reason of their survival difference, hindering accurate conclusions in a microbial source tracking framework.

On the other hand, the variation of the attachment behavior of infectious FRNAPHs to a surface was also documented (Armanious et al., 2015; Dika et al., 2013; Hébrant et al., 2014). In our batch experiments performed in river water, sorption to complex environmental solid matrix was detectable for strains belonging to GGII, GGIII and GGIV (BZ13 DL20 and GA, Qß HL4-9 and FI BR1, respectively). Thus, despite the attachment properties of infectious HL4-9 and BR1 strains (GGIII and GGIV, respectively) highlighted, their instability and low environmental persistence can explain their sparse presence in sediment. Furthermore, the predominance of GGII in sediment can be explained by (i) the inhomogeneous distribution of the four genogroups in surface water in favor of GGII, (ii) the persistence of infectious DL20 strain (GGII) observed in the batch experiment, and (iii) its capability to adsorb to solid matter.

Results of batch experiments also inform on the attachment kinetics of strains able to sorb to sediment. For all of them (DL20 (GGII), HL4-9 (GGIII) and BR1 (GGIV) strains), attachment occurred mainly in the early stages of the stirring time with a rate of 1.3 $\log_{10}$, 1.9 $\log_{10}$ and 1.3 $\log_{10}$ from the first 30 min, respectively. The major part of reduction was observed in the first minute of experiment. This fast attachment of viral particles to a surface was already underlined elsewhere (Gantzer et al., 1994). Furthermore, some discrepancies between the three strains tested were observed with a variation in the attachment rates. This difference of attachment rate pointed out surface properties of FRNAPHs as one of the factors responsible for distinct behaviors in their attachment to solid matter. According to our results, the variability in attachment capacities seems to be mainly influenced by the viral genogroup than by the strains. Hébrant et al. (2014) reported also a difference of attachment rate for two FRNAPH strains belonging to GGII and GGIII. In contrast to our results, the authors present an attachment rate of bacteriophage Qβ (GGIII) less effective than bacteriophage GA (GGII) on biofilms under hydrostatic conditions and a same attachment rate under hydrodynamic conditions. These results highlighted that, besides the surface properties of viral particles, sorbent matrix plays an important role in the phage attachment dynamic. Moreover, the difference of attachment profile observed in our study for GGII strains in surface water and in PBS demonstrated also the influence of surrounding conditions in phage-solid interactions.

In parallel, different kinetic of reduction was observed in presence of sediment between infectious and total phage particles from a same strain. This was particularly the case for GGII where the reduction kinetics of infectious strain DL20 was much lower than total phage particles. Assuming that this reduction was not due to inactivation of infectious DL20 particles upon contact with the sediment, this difference of reduction, for a same sorbent, under the same conditions, revealed a possible variation of surface properties between infectious and non-infectious phages leading to a different kinetic attachment. The major role of the outer capsid surface (amino-acids) in virus-solid interactions was demonstrated by the use of virus-like particles (Armanious et al., 2015), but no study has already explored surface properties of non-infectious FRNAPH in attachment profiles. Recently, Brié et al. highlighted different surface properties of non-infectious and infectious MS2 phages (GGI) further to exposition to high temperatures (Brié et al., 2016).

The main hypothesis about presence of viruses in sediment was a viral particle settling controlled by their association with solid particles (Rao et al., 1986). Previous studies indicate that viral particles appeared to be mainly free or associated with particles <0.25 μm in diameter in the surface water (Payment et al., 1988). Results obtained from our centrifugation experiments suggest that the status of viral particles (free or associated with settling particles) varied according to hydro-climatological conditions. In periods of low flow and at the rising phase of a rainfall-runoff event, results indicated that viral particles in surface water are free or associated with small particles remaining in suspension in the water column. Under these conditions, the transfer of viral particles to sediment appears as improbable. Nevertheless, at the peak of a rainfall-runoff event but also to a certain extent during the recession limb, infectious FRNAPHs appear to be associated with or included in solid particles with >50% and 24% of infectious FRNAPH lost in supernatant after the first centrifugation, respectively. From these results, association of FRNAPHs with settling particles during last stages of rainfall-runoff events could facilitate the transfer of viral particles from the water column to sediment. The concept of variations in partitioning under the influence of hydro-climatological conditions was broached in other studies (Characklis et al., 2005). Krometis et al. (2007) demonstrated that settling concentrations of microorganisms, such as enterococci or *Clostridium perfringens*, were generally highest in the period briefly after start of the storm. An exception occurred for total coliphages (merging of FRNAPHs and somatic coliphages), which exhibited a lower and constant rate of association regardless of hydro-climatological events. This last result is not directly comparable to the results in the present study because of the difference of phages studied. Nevertheless, it appears that most microorganisms seem to be more favorably associated with solid particles during a rainfall-runoff event than during low flow conditions.

The spatio-temporal dynamics of FRNAPHs during rainfall-runoff events were previously investigated in the studied area (Fauvel et al., 2016). It was demonstrated that the resuspension of sediment was one of the possible origins of viral pollution during rainfall-runoff events. A high input of infectious GGII bacteriophages was linked to the release of phages from the riverbed. In the present study, the presence and abundance of GGII in sediment was confirmed, and the persistence and attachment capacity of a field-collected GGII strain was also demonstrated. From all these results, sediment of the studied area constitutes a reservoir of GGII, which can be released into the water column through the action of rainfall.

In order to explain the partitioning variation of infectious FRNAPHs in collected samples, the origin of suspended particles in the water column was determined by the use of a hydrological approach. If the origin is different, we can suppose that the nature of the suspended particles would also differ, favoring or avoiding infectious FRNAPH attachment. Resuspension of sediment and/or surface runoff are both main possible origins of suspended particles in the water column. The analysis of antecedent rainfall five days prior to the rainfall-runoff event allowed us to make this distinction. For the sample collected during the rising phase of the rainfall-runoff event, the suspended particles could come from available sediment in the bottom of the riverbed. Its accumulation was possible due to low precipitation (only 3.8 mm) prior to the event. On the contrary, the surface runoff is the preferential origin of suspended

particles present for the samples collected at the peak and at the last phase of a rainfall-runoff event. The frequency of antecedent precipitation (15.2 mm and 20.5 mm, respectively) induced a saturation of the catchment in water. The distinct origins of suspended solids in the analyzed samples (sediment resuspension or surface runoff) represent one possible explanation of the variation of partitioning of infectious FRNAPHs.

This study was conducted in a river mainly polluted by human fecal pollution (abundance of GGII). It will be interesting to study the dynamics of FRNAPHs in a river with less anthropogenic pollution in order to confirm the behavior of genogroups in the water cycle and the environment. For example, the absence of GGI in sediment and the input of GGII from resuspended sediment during rainfall-runoff events could be tested in a river where GGI is the major genogroup.

5. Conclusions

Interactions of F-specific RNA bacteriophages with solid particles were demonstrated in both freshwater and sediment compartments. In contrast to data previously published, viral particles are not systematically free or associated with small solid particles in surface water. The results reported in this work underline a variation of the free or bound status of infectious FRNAPHs depending on the hydro-climatological conditions. The distinct origins of suspended solid particles (sediment or surface runoff) and consequently the nature of solid particles in the water column can be one explanation of the partitioning variation of infectious phages. The transfer of viral particles from the water column to sediment could be facilitated after a rainfall-runoff event, while the water flow rate is returning to normal conditions. Association of infectious FRNAPHs with sediment was also demonstrated as well as the influence of viral surface properties and surrounding conditions on their attachment to sediment. In addition, a difference in the attachment kinetics to sediment between infectious and total viral particles was highlighted. These laboratory results match the *in situ* observations in water and sediment compartments, and bring new elements towards the understanding of the distribution of FRNAPHs in a river system under the influence of varying hydrological fluxes.

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