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Contribution of hydrological data to the understanding of the spatio-temporal dynamics of F-specific RNA bacteriophages in river water during rainfall-runoff events

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ABSTRACT

Heavy rainfall events were previously reported to bring large amounts of microorganisms in surface water, including viruses. However, little information is available on the origin and transport of viral particles in water during such rain events. In this study, an integrative approach combining microbiological and hydrological measurements was investigated to appreciate the dynamics and origins of F-specific RNA bacteriophage fluxes during two distinct rainfall-runoff events. A high frequency sampling (automatic sampler) was set up to monitor the F-specific RNA bacteriophages fluxes at a fine temporal scale during the whole course of the rainfall-runoff events. A total of 276 rainfall-runoff samples were collected and analysed using both infectivity and RT-qPCR assays. The results highlight an increase of 2.5 $\log_{10}$ and 1.8 $\log_{10}$ of infectious F-specific RNA bacteriophage fluxes in parallel of an increase of the water flow levels for both events. Faecal pollution was characterised as being mainly from anthropic origin with a significant flux of phage particles belonging to the genogroup II. At the temporal scale, two successive distinct waves of phage pollution were established and identified through the hydrological measurements. The first arrival of phages in the water column was likely to be linked to the resuspension of riverbed sediments that was responsible for a high input of genogroup II. Surface runoff contributed further to the second input of phages, and more particularly of genogroup I. In addition, an important contribution of infectious phage particles has been highlighted. These findings imply the existence of a close relationship between the risk for human health and the viral contamination of flood water.

1. Introduction

Surface waters can be contaminated by many pathogens (bacteria, viruses and protozoa) responsible for gastrointestinal diseases (Bosch, 2007; Ramírez-Castillo et al., 2015). The presence of enteric pathogens in surface water is due to faecal contamination, which can come from various sources. The discharge of inadequately treated wastewater is the most common source of pathogens in aquatic environments (Dungeni et al., 2010; Teklehaimanot et al., 2015). Others inputs of faecal pollution appear, particularly during heavy rainfall events. The direct discharge of overflow of wastewater treatment plants in surface water (Rodríguez et al., 2012a), failure of septic tanks in rural areas (Panasiuk et al., 2015), washing out of faecal deposits from domestic or wild animals, breeding activities and agricultural use of manure (Crowther et al., 2002; Doran and Linn, 1979) are possible significant sources of faecal contamination during rainfall-runoff events. Throughout these particular hydrological events, resuspension of riverbed sediment is also suspected to release microorganisms in the water column (Mwanamoki et al., 2014).

With potential climate change and the increase of extreme rainfall events, water quality and its impact on human health are today important issues (Carlton et al., 2014; Hofstra, 2011; Hunter, 2003). Implication of specific microbial pathogens in waterborne disease outbreaks was demonstrated in a comprehensive review compiling 93 studies. The analysis of this data showed that bacteria

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were the most commonly found causative pathogens (89.1%) in comparison with viruses (25.7%), or protozoa (21.6%) (Cann et al., 2012). In the same way, the correlation between hydrological parameters and outbreak occurrences were demonstrated in some other works (Curriero et al., 2001; Nichols et al., 2009). For instance, a relationship between occurrence of gastroenteritis outbreak associated with norovirus (1018 viral gastroenteritis for the period 2002–2007) and rainfall was confirmed through statistical procedures in an Australian study (Bruggink and Marshall, 2010).

Numerous data in the literature highlighted the increase of faecal bacteria concentration in surface water during rainfall events and explored its probable origins (McDonald and Kay, 1981; Sidhu et al., 2012; Staley et al., 2012; Tiefenthaler et al., 2011). The flushing of old bacteria stores in river sediment at the onset of the flood followed by the arrival of the overflow of combined sewer systems in river water were two probable origins (Chu et al., 2011). In order to determine the origins of this higher bacteria concentration in water, the relationship between bacteria and total suspended solids (TSS) was often investigated (Chen and Chang, 2014; McCarthy et al., 2012). The measurement of the TSS concentration in water is commonly used as a proxy to assess the water quality and wet-weather pollution (Rossi et al., 2005). The spatial distribution of catchment sediment sources was also explored using a hysteresis approach to study the relationship between concentration of suspended solids (measured by TSS or turbidity) and water discharge (Williams, 1989). This information tends to give insights into the origins and transport of sediment. A positive correlation between faecal indicator bacteria and suspended material could result from the resuspension of streambed sediment or erosion of soils transported to the stream (Tiefenthaler et al., 2011). The interaction between sediment and water column compartments was recently taken into account to develop a new modelling approach to predict bacterial concentrations in rivers (Ghimire and Deng, 2013).

In comparison with data available on faecal bacteria, few studies investigated the impact of rainfall events on the viral load in surface water. Even though it has been largely documented that viral particles have a different behaviour or survival compared to bacteria (Keswick et al., 1984; Nasser et al., 1993). A higher genome contamination of enteric pathogen viruses, such as human adenovirus, norovirus or enterovirus, were observed during floods using molecular tools (Corsi et al., 2014; Ngaosuwankul et al., 2013; Phanuwan et al., 2006). Nevertheless, no information on their infectivity was available, precluding the assessment of a potential risk of enteric viral infections. Indeed viral genome has a higher persistence compared to infectivity (Gassilloud et al., 2003; Ogorzaly et al., 2010). Hata et al. (2014) drew attention to the reliability of molecular detection steps that can be affected by the complexity of water components during rainfall events. Appropriate indicators of pathogenic viruses, such as F-specific RNA bacteriophages (FRNAPH), detected and quantified by both molecular and infectious tools, were significantly more concentrated during storm events than in baseflow conditions (Cole et al., 2003). FRNAPH are ubiquitous in aquatic environments worldwide, with average concentrations ranging from 1 to 5 $\log_{10}$ plaque-forming units (PFU)/ml in raw sewage (Contreras-Coll et al., 2002; Lucena et al., 2003; Ogorzaly and Gantzer, 2006) and 1 to 3 $\log_{10}$ PFU/ml in river water (Haramoto et al., 2009; Lucena et al., 2003; Ogorzaly et al., 2009). FRNAPH belong to the *Leviviridae* family and are divided into two genus *Levivirus* and *Allolevivirus*. Each genus is divided into two genogroups (genogroups I and II for *Levivirus* and genogroups III and IV for *Allolevivirus*). According to numerous studies performed so far, FRNAPH of genogroups II and III are generally linked with a human associated-contamination, whereas those of genogroups I and IV are mainly derived from animal associated-contamination (Haramoto et al., 2009; Lee et al., 2011, 2009). They may be included as candidates for tracking the origin of faecal pollution. However, this distribution of phage genogroups is not absolute with detection of genogroup I in urban wastewater (Haramoto et al., 2015; Hartard et al., 2015). Furthermore, infectious units from genogroups I and II are described as more resistant in environment than ones from genogroups III and IV, which can potentially biased the original genogroup distribution (Muniesa et al., 2009). These phages and most of the human enteric viruses share similar physical characteristics such as the size, nature of genome and the shape. They are thus proposed as models to predict the presence of pathogenic viruses in surface water (Havelaar et al., 1993; Jofre, 2007). In addition, their ease of detection and their higher presence in surface water compared to enteric viruses allow the performance of a fine-scale analysis of viral pollution in the environment.

The work reported here aims to provide original information on occurrence and transport of FRNAPH during a rainfall-runoff event by exploring the relationship between phage dynamics and hydrological parameters. Specifically, the main objectives are (i) to parameterise the hydrographs in order to analyse the level and the dynamics of phage fluxes during rainfall-runoff events (rising and falling limbs), (ii) to investigate the possible origins of phage pollution wave arrivals in an aquatic environment and (iii) to give new data for risk assessment by detection of infectious phage particles during rainfall-runoff events.

For this study, surface water was sampled from the Alzette River, located in the south of Luxembourg, downstream and upstream from a wastewater treatment plant. The effect of rainfall on water quality was estimated by infectious FRNAPH enumeration and molecular biology tools.

2. Materials and methods

2.1. Study area

The study was conducted on the Alzette River, one of the main rivers in Luxembourg. The Alzette River runs from Thil (France) for 73 km before joining into the Sauer River at Ettelbrück (Luxembourg). The watershed of the Alzette River covers 1174 km^2 of which 286 km^2 drain into the study area (Fig. 1). The land use in this sub-catchment includes 21.1% of urban areas, 48.2% of agricultural areas and 30.7% of forest and semi-natural areas (data from program EU-CORINE Land Cover, 2006).

The sampling area was close to the city of Hesperange, with about 14,000 inhabitants and located 6 km upstream from Luxembourg City. Three wastewater treatment plants (WWTP) were located upstream from the sampling area with respective capacities of 24,500, 90,000, and 95,000 inhabitant-equivalents. In addition, a fourth WWTP was present directly in the sampling area (Hesperange WWTP). Sampling sites were thus considered under anthropic influence.

The WWTP of Hesperange was renovated in 2006 and converted into a nitrifying activated sludge plant with moving bed biofilm reactor technology. Its maximum capacity is 26,000 inhabitant-equivalents. An average of $2.3 \pm 1.0 \times 10^4$ (mean ± standard deviation) most probable number/100 mL ($n = 6$) of *Escherichia coli* and $4.4 \pm 4.0 \times 10^3$ PFU/100 mL ($n = 6$) of infectious FRNAPH were estimated in the Hesperange WWTP effluents. An efficiency of 91.3% was determined for FRNAPH. Three out of four genogroups were observed in effluents ($n = 4$): genogroup I ($3.0 \pm 2.3 \times 10^5$ genome copies (gc)/100 mL), genogroup II ($3.1 \pm 1.7 \times 10^5$ gc/100 mL) and genogroup III ($2.2 \pm 0.9 \times 10^4$ gc/100 mL).

2.2. Stream water sampling and processing

Stream water samples were collected upstream and downstream from the Hesperange WWTP using automatic samplers

Fig. 1. Main drainage network of Luxembourg. The Alzette River watershed (in green), including the studied sub-catchment (striped area). Sampling locations were upstream and downstream the Hesperange WWTP.

(ISCO). One-litre samples were collected every hour during the course of the studied periods. Samples were transported back to the laboratory in a refrigerated compartment every 24 h for analysis.

Samplings were carried out during two contrasting hydrologic conditions: during low flow (96 samples) and during rainfall-runoff events (276 samples). Two automatic sampling campaigns were performed over 24 h when the water was at its lowest level. For the first campaign, the water discharge was of 2.33 m^3/sec (10th–11th February 2015) and for the second, the water discharge was of 0.63 m^3/sec (8th–9th July 2015). In the same way, two rainfall events were analysed: the first occurred in summer and lasted for 44 h (29th–31st July 2014) and the second occurred in autumn and lasted 94 h (3rd–7th November 2014). A rainfall-runoff event was defined as a significant increase of water discharge in the stream compared to low flow (minimum eight times higher than the low flow). The length of the event was calculated from the increased flow departure until return of the normal low flow.

2.3. Meteorological, hydrological and physico-chemical data

Daily rainfall data of the closest meteorological station (6 km from the studied site) were provided by the "Administration des Services Techniques de l'Agriculture" (ASTA, http://www.agrimeteo.lu). The water discharge (m^3/sec) of the Alzette River was estimated using the rating curve and the water level data provided by the "Administration de la Gestion de l'Eau". Turbidity

of each water sample was measured in nephelometric units (NTU) with a portable turbidimeter (HACH 2100Q).

2.4. Detection of infectious F-specific RNA bacteriophages

The concentration of infectious FRNAPH was determined using *Salmonella enteric typhimurium* strain WG49 and the double-agar-layer technique in accordance with the ISO standard 10705-1:2001. Nalidixic acid was added to media to limit the growth of abundant bacterial flora. Negative and positive controls were included in the determination of infectious FRNAPH. Plates were incubated overnight at 37 °C before counting PFU. FRNAPH were enumerated in 5 × 1 mL of surface water for each sample and their concentrations were expressed in PFU per 100 mL.

2.5. Genogroup detection of F-specific RNA bacteriophages

Prior to the FRNAPH genogroup determination, a clarification of surface water was performed with a centrifugation at 3000 g for 10 min at 4 °C (Centrifuge 5810R, Eppendorf). Then, an ultracentrifugation of 98 mL of supernatant at 235,000 g for 1 h 30 min at 4 °C (L-90K Ultracentrifuge, Beckman Coulter OptimaTM) was performed to concentrate phage particles (Skraber et al., 2011). Each pellet was resuspended in 1 mL of the NucliSens Lysis buffer (BioMerieux, France) and stored at −80 °C until the next steps of sample treatment.

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. Genogroup detection and quantification were performed by the quantitative real-time RT-PCR (RT-qPCR) method developed by Ogorzaly and Gantzer (2006) on ViiA™ 7 Real-Time PCR System (Applied Biosystems, France). Two modifications were brought to this method with the use of TaqMan® Environmental MasterMix 2.0 (Applied Biosystems®) in a final volume of 25 μL.

RNA genomic concentration of each genogroup (gc/100 mL) was determined thanks to standard curves. The target genomic sequences of MS2 (NC001417), GA (NC001426), MXI (AF059242) and SP (X07489), belonging to genogroups I, II, III and IV respectively, were inserted into four plasmids (pEX-A, Eurofins Genomics). Plasmids were transcripted in RNA (RiboProbe® *in vitro* Transcription Systems, Promega) in order to take into account reverse transcription and real-time PCR in the obtaining of standard curves. A detection limit of 10 genome copies per RT-qPCR reaction was determined for the four genogroups.

Negative extraction control and negative and positive RT-qPCR controls were incorporated for each FRNAPH genogroup detection. RT-qPCR inhibition was evaluated on 202 samples of 372 total samples by spiking a known amount of GA plasmids into extracted samples. The quantity of GA plasmid measured in water sample was then compared to the quantity initially introduced.

2.6. Calculation of fluxes of viruses

Each concentration of FRNAPH (PFU or gc/100 mL) detected in the Alzette River was converted into flux (PFU or gc/sec) by taking into account the flow of the river (m^3/sec). FRNAPH fluxes were calculated using the following equation: $F = C \times Q \times 10{,}000$, where F is the flux expressed in PFU or gc/sec; C is the FRNAPH concentration expressed in PFU or gc/100 mL and Q corresponds to water discharge in the stream expressed in m^3/sec.

2.7. Hydrologic variables

The stream flow response of the studied sub-watershed during rainfall events was characterised by the hydrograph. Runoff coefficient (in %) related event runoff to total rainfall, both expressed as a height over catchment area (mm). The rising and falling limbs corresponded respectively to the increase and decrease of water discharge during the event. The discharge peak was the highest point in the hydrograph and it separated the rising and falling phases. On the contrary, the low flow of the river represented the stream discharge during dry conditions. In this study, this terminology was applied to the dynamics of FRNAPH in response of a rainfall-runoff event. The rising and falling phases were defined as the increase and decrease of FRNAPH fluxes during the event. The major peak of FRNAPH, separating the rising and falling limbs, was defined as the first higher infectious FRNAPH flux during the rainfall event.

2.8. Loess analysis

A smoothing algorithm was performed on viral data of rainfall-runoff events in order to reduce the skewness of their distribution. LOESS (Local RegrESSion) algorithm was performed by the SigmaPlot 12.5 software. A smoothing parameter corresponding to a 0.15 sampling proportion was used. Local regressions, resulting from the LOESS algorithm, were used to perform ratio of genogroup II genome on infectious FRNAPH and ratio of genogroup II on genogroup I. Calculations of ratio was performed to give an idea of the relative distribution of infectious FRNAPH and each genogroup in the river water during rainfall-runoff events.

3. Results

3.1. Situation of the monitored area

The studied area was analysed twice during a 24-h period when the water level of the river was at its lowest (Fig 2). During these periods, the river had an average flow of 0.7 m^3/sec and 2.7 m^3/sec depending on the season. Turbidity was stable whatever the season with values around 10.8 ± 2.1 NTU. Ranges of variation of 1.5 $\log_{10}$ of infectious FRNAPH fluxes (from 6.0 to 7.5 $\log_{10}$ PFU/sec) and 0.7 $\log_{10}$ (from 7.3 to 8.0 $\log_{10}$ PFU/sec) were observed upstream of the WWTP during the low flow period of summer and winter, respectively. The same results were observed for infectious FRNAPH concentrations, with variations of 1.5 $\log_{10}$ ($7.8 \pm 9.9 \times 10^2$ PFU/100 mL) and 0.7 $\log_{10}$ ($1.6 \pm 0.6 \times 10^3$ PFU/100 mL). These daily fluctuations can come from non-regular outputs of all WWTP upstream from the studied area. It is of note that the analysis of water samples collected upstream and downstream from the WWTP showed that the contribution of the Hesperange WWTP mainly took place in summer when the flow of the river was at its lowest. Indeed, in contrast to the winter period during which infectious FRNAPH fluxes were similar upstream ($4.4 \pm 1.8 \times 10^7$ PFU/sec) and downstream ($4.8 \pm 2.4 \times 10^7$ PFU/sec) from the WWTP (Wilcoxon signed rank test, $p = 0.808$), fluxes of infectious FRNAPH were higher downstream ($1.2 \pm 0.9 \times 10^7$ PFU/sec) than upstream from the WWTP ($5.4 \pm 7.0 \times 10^6$ PFU/sec) during summer low flow period (Wilcoxon signed rank test, $p < 0.001$). Fluxes of FRNAPH genogroups were also determined. Genogroup II was most abundant and detected during both low flow periods (Table 1). Similarly to infectious FRNAPH fluxes, a daily variability of FRNAPH II fluxes occurred (1.1 $\log_{10}$ and 0.9 $\log_{10}$ during the low flow period of summer and winter respectively). Other genogroups have not been systematically detected (some points under the detection limit). Genogroup I was detected more than genogroup III while genogroup IV was not found in the river. RT-qPCR inhibition was evaluated for 91 of the 96 low flow samples collected. No sample was inhibited at more than 50%.

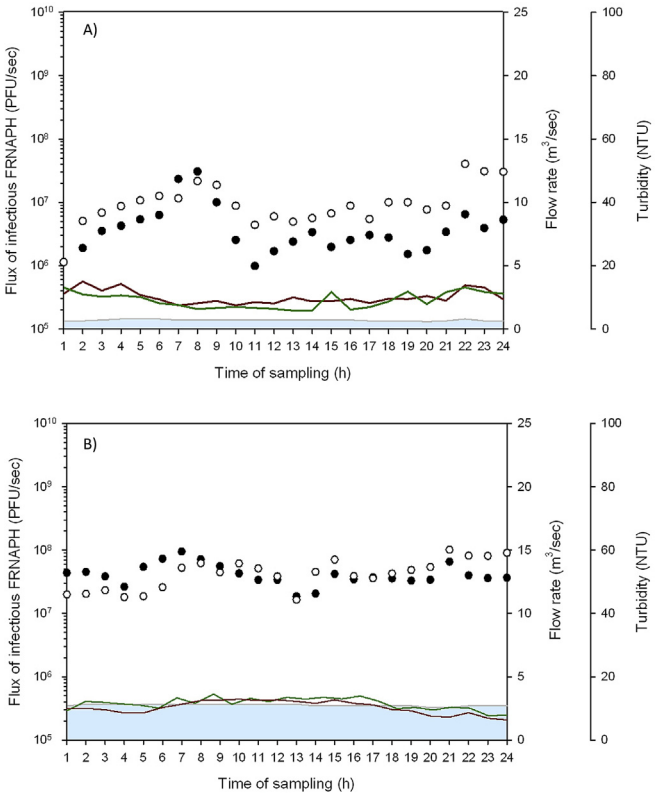

Fig. 2. Hydrological parameters and infectious FRNAPH fluxes during summer (A) and winter (B) low flow. Flow rate in blue area; turbidity upstream and downstream WWTP in red and green lines respectively; ● infectious FRNAPH upstream WWTP; ○ infectious FRNAPH downstream WWTP. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Rainfall-runoff events characterisation

The main parameters for hydrograph description of rainfall-runoff events are reported in Table 2. The autumn event showed a much higher water discharge at the peak of the hydrograph (22.5 m^3/sec) than that of the summer event (7.1 m^3/sec). In the same way, the flow rate prior to the autumn event (2.6 m^3/sec) was much higher than the flow rate prior to the summer one (0.5 m^3/sec). In addition to flow rate, the autumn event lasted twice as long as the summer event with a time of hydrograph duration of 82 h *versus* 39 h. Despite all these differences, the duration of rising and falling limbs lasted the same proportion of time. During the summer event, the runoff coefficient was lower (7%) than during the autumn event (34%). This means that the proportion of total rainfall that becomes runoff during the event was higher in autumn than in summer, most probably due to wetter antecedent conditions and subsequent higher soil saturation. All of these differences between the two studied rainfall events can be explained by the water catchment storage conditions, which differ depending on weather conditions associated with seasons. Observed precipitation patterns were also different in both seasons despite that cumulative rainfall was similar (28.5 and 32.9 mm for the summer and autumn events, respectively). In summer, discontinuous precipitation appeared with one important brief rainfall pulse cumulating in 20 mm in one hour. In autumn, considerable and continuous precipitation occurred during more than 10 h with a maximum of 4 mm per hour (Fig. 3A and C).

For each sampling data, turbidity profiles were similar at the upstream and downstream Hesperange WWTP sites. An increase occurred during the rising limb (from 12.6 to 366 NTU and from 34.4 to 306 NTU, for the summer and autumn events, respectively). Most variations appeared for the summer event with three distinct turbidity peaks. Local rainfall pulses and intense thunderstorms in summer might explain the two distinct water discharge peaks and the repercussion on turbidity.

3.3. FRNAPH detection during rainfall-runoff events

3.3.1. WWTP contribution

The contribution of the Hesperange WWTP in terms of discharge volume was very small compared to stream water discharge coming from upstream (Table 2). Concerning the input of infectious FRNAPH in the river (Fig. 3B and D), the WWTP

Table 1
Detection rate, concentrations and fluxes of FRNAPH genogroups in water samples.

		Detection rate (%)	FRNAPH concentration Min (PFU or gc[a]/100 mL)	FRNAPH concentration Max (PFU or gc[a]/100 mL)	FRNAPH flux Min (PFU or gc[a]/sec)	FRNAPH flux Max (PFU or gc[a]/sec)	Log_{10} difference in flux
Summer low flow (n = 24)	Infectious FRNAPH	100	1.4×10^2	4.4×10^3	9.8×10^5	3.1×10^7	1.5
	Genogroup I	79.1	9.9×10^2	3.7×10^3	6.5×10^6	2.2×10^7	0.5
	Genogroup II	100	2.0×10^4	2.4×10^5	1.4×10^8	1.7×10^9	1.1
	Genogroup III	33.3	6.3×10^2	2.7×10^3	3.7×10^6	2.1×10^7	0.7
Winter low flow (n = 24)	Infectious FRNAPH	100	6.6×10^2	3.4×10^3	1.9×10^7	9.5×10^7	0.7
	Genogroup I	29.1	7.6×10^2	8.1×10^3	2.1×10^7	2.1×10^8	1
	Genogroup II	100	3.8×10^3	2.6×10^4	9.9×10^7	7.5×10^8	0.9
	Genogroup III	4.1	6.1×10^3	6.1×10^3	1.6×10^8	1.6×10^8	–
Summer event (n = 44)	Infectious FRNAPH	100	6.0×10^1	2.5×10^3	5.7×10^5	1.7×10^8	2.5
	Genogroup I	72.7	6.7×10^2	6.0×10^3	1.1×10^7	1.8×10^8	1.2
	Genogroup II	100	4.9×10^3	7.1×10^4	5.9×10^8	6.6×10^9	1.0
	Genogroup III	68.2	5.8×10^2	1.4×10^4	7.9×10^6	1.7×10^8	1.3
Autumn event (n = 51)	Infectious FRNAPH	100	6.7×10^2	7.2×10^3	1.7×10^7	1.2×10^8	1.8
	Genogroup I	54.9	4.0×10^2	4.9×10^3	1.9×10^7	7.5×10^8	1.2
	Genogroup II	96.1	6.3×10^2	4.7×10^4	1.2×10^8	4.5×10^9	1.6
	Genogroup III	39.2	3.2×10^2	6.4×10^3	5.1×10^8	3.1×10^9	0.7

[a] Genome copies.

Table 2
Hydrological parameters of the summer and autumn rainfall-runoff events.

		Summer event (29th–31st July 2014)	Autumn event (3rd–7th November 2014)
River water	Pre-event flow rate (m^3/sec)	0.5	2.6
	Flow rate at the peak of the hydrograph (m^3/sec)	7.1	22.5
	Event duration (h)	39	82
	Rising limb duration (h)	12 (30.7%)	21 (25.6%)
	Falling limb duration (h)	27 (69.3%)	61 (74.4%)
	Total rainfall[a] (mm)	28.5	32.9
	Antecedent rainfall[b] (mm)	20.8	2.8
	Runoff coefficient (%)	7	34
Effluent WWTP	Effluent flow rate of WWTP (m^3/sec)	0.09	0.11
	Flow rate of the overflow of WWTP in the stream (m^3/sec)	0.03	0.13

[a] On the total duration of the event.
[b] 10 days prior the event.

contributed only to a small extent during the summer rainfall event (Wilcoxon signed rank test, $p < 0.001$) and not significantly during the autumn event (Wilcoxon signed rank test, $p = 1.0$). The flow of the river was so important during the rainfall event that phage input coming from the WWTP effluent was negligible (dilution effect). For the sake of clarity, only upstream data was presented.

3.3.2. Detection of infectious FRNAPH

A total of 276 rainfall water samples were collected and analysed in the Alzette River during both summer and autumn events. All samples analysed were positive for infectious FRNAPH. An increase of 2.5 $\log_{10}$ of infectious FRNAPH flux was measured for the summer event (from 5.7×10^5 to 1.7×10^8 PFU/sec) and 1.8 $\log_{10}$ for the autumn event (from 1.7×10^7 to 1.2×10^9 PFU/sec). Similar results were observed for infectious FRNAPH concentrations, with increases of 1.6 $\log_{10}$ (from 6.0×10^1 to 2.5×10^3 PFU/100 mL) and 1.0 $\log_{10}$ (from 6.7×10^2 to 7.2×10^3 PFU/100 mL) for summer and autumn events, respectively. These increases of infectious FRNAPH were divided into two successive waves of infectious FRNAPH for both rainfall-runoff events. During the summer event, a first increase of infectious FRNAPH flux occurred 8 h after the beginning of the event and a second increase after 16 h of sampling time. These two waves also appeared after 20 and 35 h for the autumn event. Then, for both events, a slow decrease of infectious FRNAPH fluxes occurred during the falling phases, lasting three times longer than the rising phase. Flow rate of the Alzette river got back to its initial flow rate whereas fluxes of infectious FRNAPH always were superior to the pre-event ones (with 0.7 $\log_{10}$ and 0.2 $\log_{10}$ of differences between the beginning and the end of summer and autumn event, respectively).

3.3.3. Characterisation and quantification of FRNAPH genogroups

During rainfall-runoff events, genogroups I, II and III were detected (Table 1). Genogroup II was noted to be the most abundant in all stream water samples. Flux variations of genogroup II genome copies were from 5.9×10^8 to 6.6×10^9gc/sec (1.0 $\log_{10}$) and from 1.2×10^8 to 4.5×10^9gc/sec (1.6 $\log_{10}$) for the summer and autumn events, respectively (Table 1). Flux variations were also observed for genogroup I with 1.2 $\log_{10}$ increase for both events and for genogroup III with 1.3 $\log_{10}$ and 0.7 $\log_{10}$ for the summer and autumn events. Inhibition of RT-qPCR was evaluated for 111 of the 276 rainfall-runoff samples collected. Thirty-one percent of samples were inhibited at more than 50% and only 1.8% at 90%.

For both events, an increase of genogroup II fluxes took place before the water discharge peak whereas a rise of genogroup I occurred at the same time as the peak of hydrograph (Fig. 4). The low detection rate of genogroup III during the autumn event (39.2%) did not allow us to establish dynamics. For the summer event, genogroup III increased at the same time than genogroup II, before the water discharge peak. Decrease of genogroups occurred slowly during the falling phase of the hydrograph until a similar flux of the pre-event.

3.3.4. Input of infectious FRNAPH during rainfall-runoff events

A comparison between genogroup fluxes and infectious FRNAPH can be made to estimate the input of non-infectious *versus* infectious phages during the rainfall-runoff event. Genogroup II, the most abundant of genogroups detected in the water samples, was used to perform the comparison. During the summer event, an increase of 2.5 $\log_{10}$ (Table 1) of infectious FRNAPH flux was observed compared to 1.1 $\log_{10}$ of genogroup II genome. Ratios of genogroup II genome on infectious FRNAPH are presented in Fig. 5. The basal ratio was defined during the pre-event and had a value of about 2 $\log_{10}$ and 1.5 $\log_{10}$ for summer and autumn events, respectively. As concentration of genogroup II was higher than infectious FRNAPH, the ratio of genogroup II to infectious FRNAPH was positive. At the beginning of the rising phase, infectious FRNAPH and genogroup II genome increased in the same proportion, which did not induce a variation of the ratio. A decrease of the ratio (reduction of about 1 $\log_{10}$ compared to the basic value) occurred during the water discharge peak due to an unbalance of fluxes in favour of infectious FRNAPH. An input of infectious phages of genogroup I can be responsible for this difference of ratio. At the end of events, the ratio remained lower than pre-events, particularly for the summer event (0.8 $\log_{10}$ of difference) meaning a higher rate of infectious FRNAPH in the stream compared to the initial situation.

3.4. Analysis of FRNAPH appearance pattern during rainfall-runoff events

3.4.1. Turbidity hysteresis patterns

From a hydrological point of view, a rainfall-runoff event was generally characterised using a hysteresis approach. The relationship between turbidity and water discharge was investigated in order to determine sources and transport of sediment during events. This link was established for both events (Fig. 6A and B). Each hysteresis plot displayed specific patterns. During the summer event, a double clockwise and a single-valued line pattern (Fig. 6A) were the result of the three turbidity peaks observed (Fig. 3A). The double clockwise corresponded to a successive increase and decrease of turbidity as a function of the increase of the flow rate. This chronology was consistent with the first two peaks of turbidity preceding the water discharge peak. The single-valued line pattern was associated with the third peak of turbidity, which was simultaneous with the peak of water discharge. During the autumn event, the turbidity peak also appeared before the peak of the hydrograph inducing a clockwise hysteresis pattern (Fig. 3C).

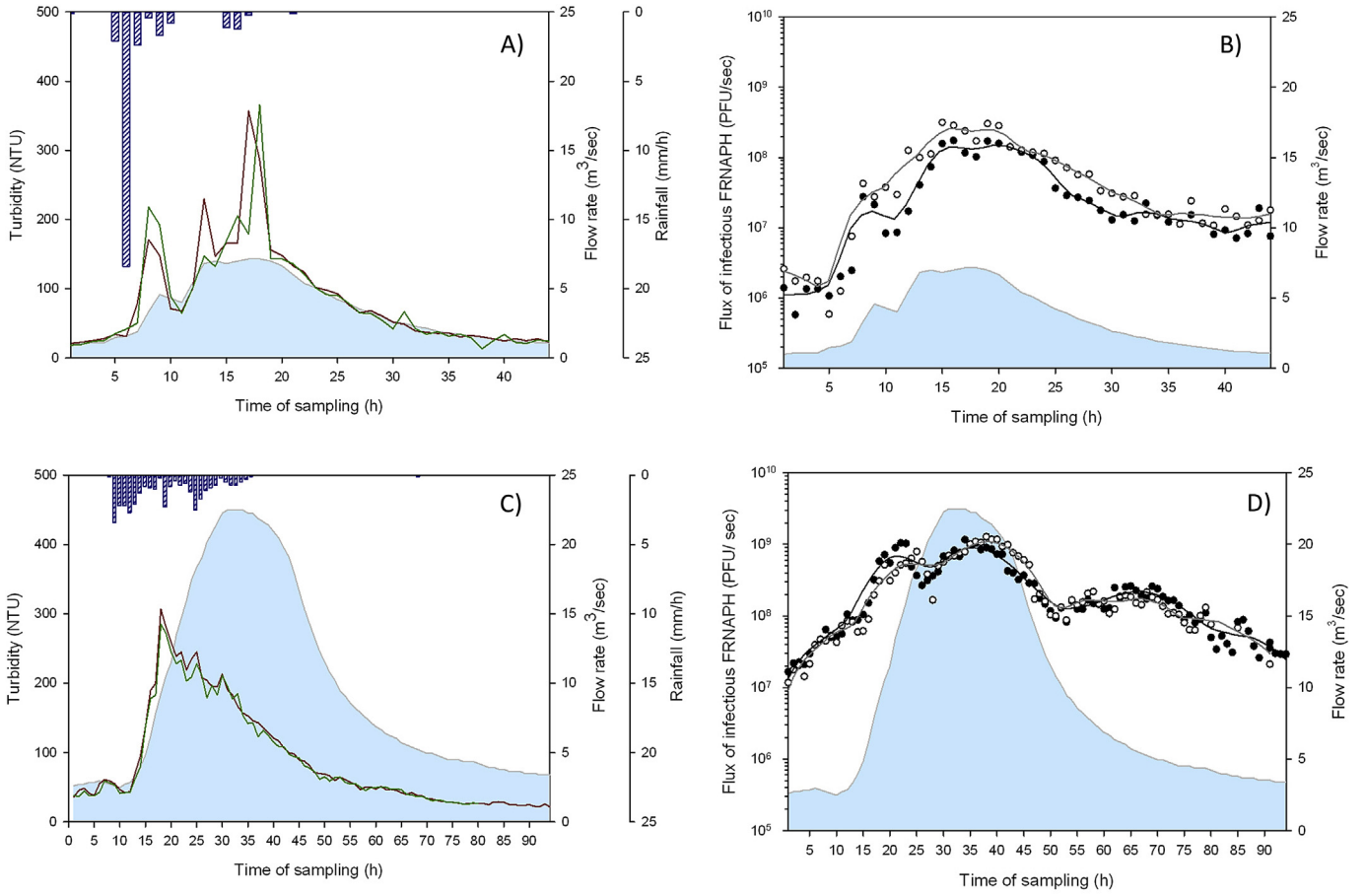

Fig. 3. Hydrological parameters and infectious FRNAPH fluxes during summer (A, B) and autumn (C, D) rainfall-runoff events. Flow rate in blue area; rainfall in bar chart, turbidity upstream and downstream WWTP in red and green lines respectively; ● infectious FRNAPH upstream WWTP; ○ infectious FRNAPH downstream WWTP; smoothed curves of infectious FRNAPH upstream and downstream WWTP in dark and grey lines respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4.2. *Infectious FRNAPH hysteresis patterns*

Since viral particles can be considered as colloid in aquatic environment, we suggested applying the hysteresis approach to F-specific RNA bacteriophages. This analysis allowed us to clarify the sources and transport of phage particles during event and to establish their link with other solids, such as suspended matter or sediment. The infectious FRNAPH concentrations and water discharge relationships were presented in Fig. 6C and D. For both events, a pattern close to a figure of eight appeared which was the result of the two waves of infectious FRNAPH. This profile was first a brief clockwise pattern and then an anti-clockwise pattern. This profile allowed us to describe the chronological arrival of the two distinct waves of infectious FRNAPH during both events (Fig. 3, Section 3.3.2). It is interesting to note the size difference of loops for both figure of eight patterns of the summer and autumn events. In the hysteresis plot of summer event, the first clockwise loop was smaller than the anti-clockwise loop. This observation was reversed for the hysteresis plot of autumn event. Dynamics of infectious FRNAPH as a function of the flow rate progressed in the same way in both events studied though in different response proportion (size of loops).

3.4.3. *Distribution of FRNAPH genogroups*

Assuming an equivalent efficiency of the four RT-qPCR assays, specific to each FRNAPH genogroup, it was possible to compare the distribution of different genogroups during rainfall-runoff events. The faecal pollution was characterised considering the ratio between genogroup II (human-associated contamination) and genogroup I (mostly animal-associated contamination). Both of these genogroups were the most abundant in water samples. The variation of this ratio during rainfall-runoff events is presented in Fig. 7. The basal ratio was defined during the pre-event and had a value of about 1 $\log_{10}$ for the both events. As genogroup II was more abundant than genogroup I, the ratio of genogroup II to genogroup I was almost always positive. An increase of the ratio occurred in parallel to the first peak of turbidity with a ratio gengroup II to genogroup I of 1.6 $\log_{10}$ for both events. These increases were due to input of genogroup II and not a decrease of genogroup I (as described in Section 3.3.3). A second variation of the ratio genogroup II to genogroup I occurred in the second part of hydrographs with a decrease of the ratio inferior to the initial value. For the summer event, the ratio decreased right from the beginning of the hydrograph peak until the end of the falling phase with an average value of 0.8 $\log_{10}$. For the autumn event, the low values of ratio occurred a few hours after the peak of the hydrograph with an inversion of the ratio (-0.1 $\log_{10}$). An important input of genogroup I explained this counterbalance. These observations showed different faecal pollution sources at different time of the rainfall-runoff events.

4. Discussion

The objective of this study was to provide new comprehensive information on the origin, dynamics and transport of viral particles

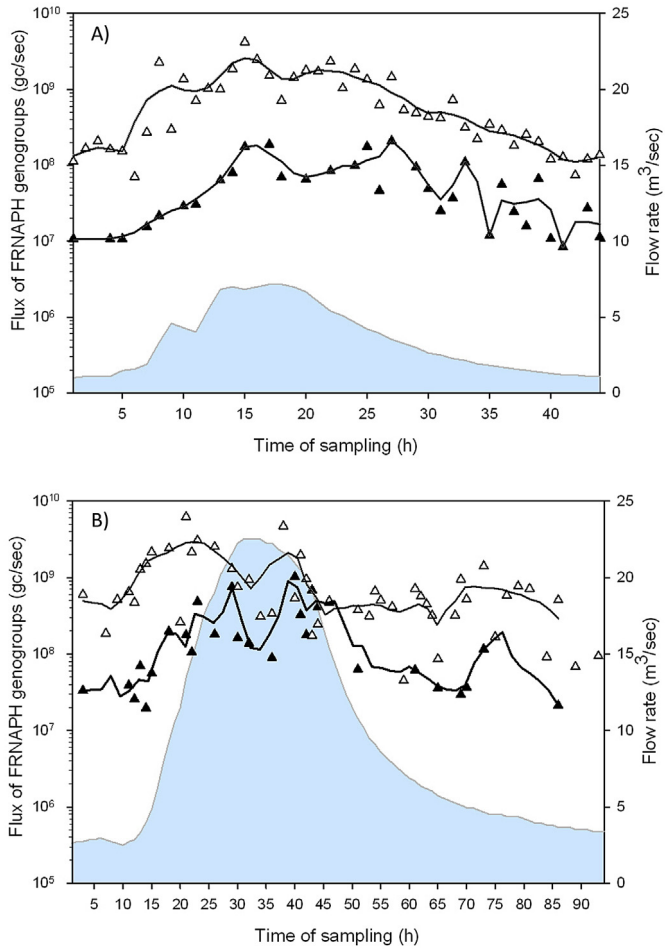

Fig. 4. Fluxes of genogroups I and II upstream the WWTP during summer (A) and autumn (B) rainfall-runoff events. Flow rate in blue area; ▲ genogroup I; △ genogroup II; smoothed curves of genogroup I and II in dark and grey lines respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in surface water during rainfall-runoff events. To this purpose, F-specific RNA phages, selected as faecal and viral pollution indicators, were detected and quantified using both culture and molecular assays. Data was then analysed in an innovative way integrating hydrological, climatological and microbiological parameters.

In low flow conditions, infectious FRNAPH were detected in the Alzette River with concentrations ranging from 2.1 to 3.2 $\log_{10}$ PFU/100 mL, which was comparable to the results of previous studies (Lucena et al., 2003; Ogorzaly et al., 2009; Skraber et al., 2009a; Stewart-Pullaro et al., 2006). It corresponded to the initial faecal pollution of the river and correlatively suggested possible viral pollution. In rainfall-runoff conditions, the FRNAPH concentration reached 3.4 and 3.9 $\log_{10}$ PFU/100 mL, with an average increase of 1.6 and 1.0 $\log_{10}$ for the summer and autumn events, respectively. Faecal pollution and consequently viral pollution were therefore significantly increased. Viral loads were significantly higher during storm event conditions than under low flow conditions as has previously been reported (Hata et al., 2014). Different trends of coliphage concentrations during the storm event as a function of the sampling schemes were also reported (Cole et al., 2003). In order to determine the input of faecal pollution during a rainfall-runoff event, relevant water samples covering the different phases of the hydrograph are essential. Thus, the high frequency sampling (automatic samplers) seems to be the most appropriate sampling method to monitor the dynamics of viral fluxes to a fine temporal scale during a rainfall-runoff event. Furthermore, FRNAPH concentrations were converted to fluxes. For both events, the flow rate of surface water increased more than eight times compared to low flow conditions, making a comparison of phage concentrations difficult. The conversion in fluxes avoids this problem and so provides a precise idea of the total number of phages present in river water.

Besides the detection of the infectious FRNAPH increase, two distinct waves of phage pollution were also highlighted all along both the rainfall-runoff events. Integration of hydrological parameters in addition to microbial data allowed interpretation of these two chronological arrivals. Phage behaviour observed during both events showed a similar hysteresis pattern in two steps, named figure of eight (Williams, 1989). The first part is a clockwise loop and the second one is an anti-clockwise loop, reflecting two different origins of infectious FRNAPH separate in time. In order to determine these distinct origins, a parallel with turbidity *versus* water discharge hysteresis patterns can be made. Indeed, the basic relationship between turbidity (or total suspended solid) and flow rate is frequently used in the literature to identify the spatial distribution of sediment origins (Brasington and Richards, 2000; Karimaee Tabarestani and Zarrati, 2014; Smith and Dragovich, 2009). Five classes of hysteresis are classically described: single-valued line, clockwise loop, anti-clockwise loop, single line plus a loop and figure of eight, each motif supplying an indication on the origin of suspended solid delivery through the catchment during rainfall-runoff events (Williams, 1989). In this study, the clockwise loop firstly observed is due to a rapid increase of FRNAPH flux preceding the water discharge peak. This type of loop is attributed to a flushing-out of sediment available in the riverbed before the flow peak (Lawler et al., 2006; Smith and Dragovich, 2009). The assumption of sediment resuspension occurring at the beginning of the rainfall-runoff events was confirmed by the turbidity *versus* water discharge hysteresis, which also displayed a clockwise schema. Moreover, the presence of viral particles in river sediments was already reported, and some authors considered it as a probable reservoir of virus (Staggemeier et al., 2015; Wu et al., 2009). Then, the anti-clockwise loop observed for FRNAPH was the result of a second increase of viral flux slightly shifted in time compared to the water discharge peak. Since viral particles tend to travel at a velocity closer to the mean flow velocity, the infectious FRNAPH flux tends to lag behind the flood wave. Thus, the lag time increased with the distance of source contaminants downstream (Williams, 1989). The input of wastewater excess from the WWTP (Lawler et al., 2006) can be one of the explanations of this interval. The second reported cause of the anti-clockwise loop is the surface runoff, which depends of cumulative effects of the seasonal precipitations and the degree of soil saturation in water. These chronological origins progressed in the same way for both events studied but with different proportions. For the summer rainfall-runoff event, the hysteresis pattern of discharge water against FRNAPH concentration presented a clockwise loop smaller than the anti-clockwise loop. This observation was reversed for the autumn event hysteresis pattern. The infectious FRNAPH behaviour and turbidity variations in surface water were a combined result of the intensity of the rainfall and the status of the watershed. During summer, water catchment storage was low mainly due to high temperatures and evapo-transpiration. This resulted in low pre-event discharge (0.5 m^3/sec) and low runoff coefficient (7%). Observations of antecedent rainfall prior the rainfall-runoff event also allowed us to determine the amount of available sediment at the bottom of the riverbed. Ten days prior to the summer rainfall-runoff event, 20 mm of precipitation occurred flushing out the

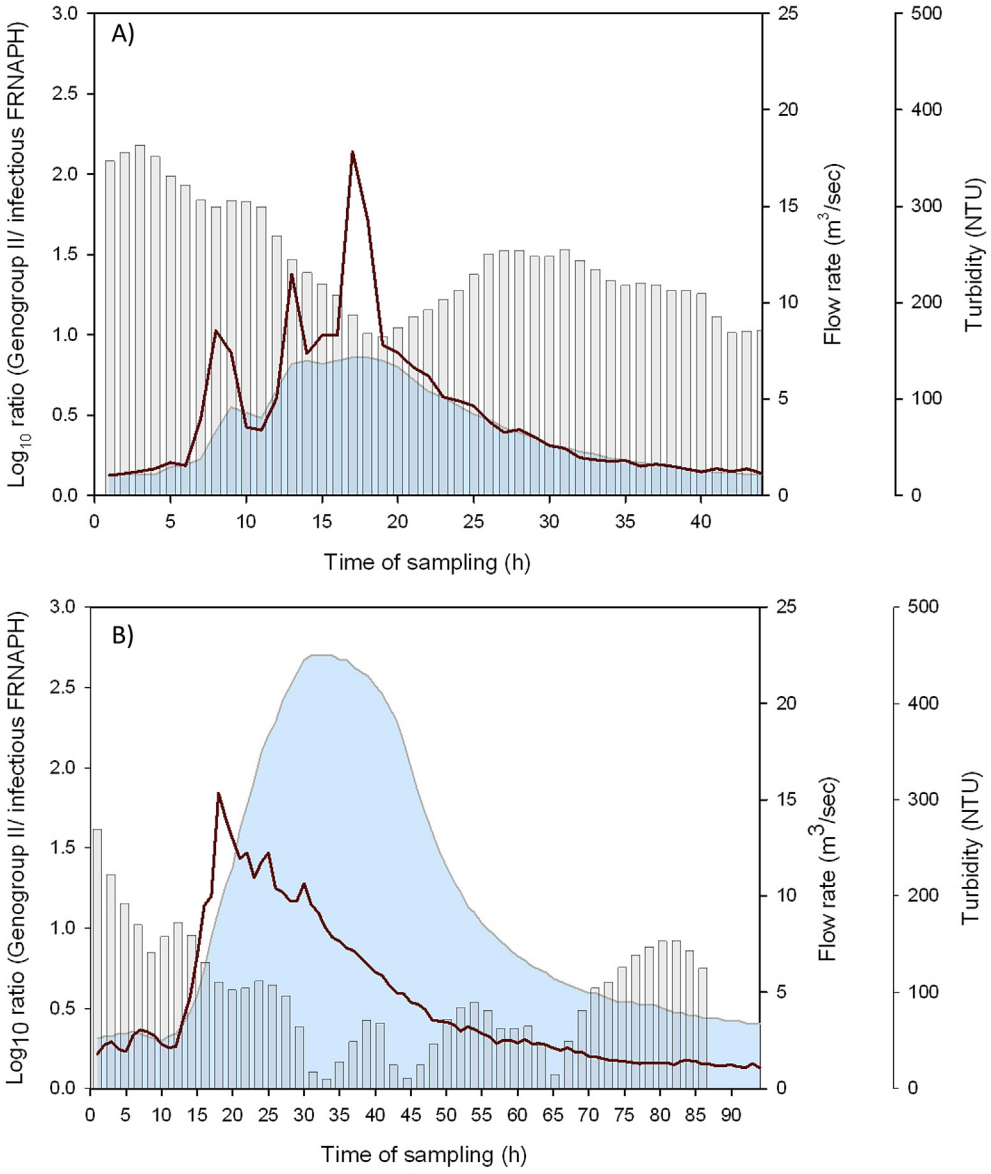

Fig. 5. Variation of $\log_{10}$-transformed ratio genogroup II genome on infectious FRNAPH fluxes (bar charts) during summer (A) and autumn (B) rainfall-runoff events. Red line represents turbidity for each event. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

bottom of the river. Therefore, little sediment was available during the summer event. In autumn, the catchment progressively water saturated due to the frequency of seasonal precipitation and the increase of water storage. Consequently, pre-event discharge values (2.6 m^3/sec) and runoff coefficients (34%) were higher. However, unlike during the summer event, a lot of sediment was hypothetically accessible at the bottom of the riverbed due to no antecedent rainfall prior to the event. Thus, FRNAPH fluxes observed during the rainfall-runoff events resulted from two successive waves of FRNAPH pollution coming from two chronological origins: first the resuspension of riverbed sediment and later the surface runoff or the WWTP effluents. Furthermore, the intensity of each pollution wave can vary depending on the status of the water catchment storage acting on the intensity of the surface runoff, and antecedent rainfall, acting on sediment resuspension.

In order to establish the origin of the faecal pollution encountered in the Alzette River in low flow and rainfall-runoff conditions, FRNAPH genogroups were quantified using a molecular approach. The application of RT-qPCR can be hampered by the presence of molecular inhibitors such as humic and fulvic acids, organic compounds, bacterial debris or nucleases (Gibson et al., 2012; Rodríguez et al., 2012b), especially in the case of complex water samples gathered during rainfall-runoff events. Virus recovery efficiency and nucleic acid extraction can also be affected (Hata et al., 2014). Only 1.0% of tested samples (n = 202 of 392 total samples) had an inhibition rate superior or equal to 90%. Molecular data reported here was under estimated to a certain extent, but that did not prevent the data treatment. Moreover, molecular data can be considered as more appropriate than infectivity data for source identification, since the FRNAPH genogroups exhibit difference environmental persistence characteristics. Infectious units from genogroups I and II are universally described as more persistent than genogroups III and IV. These distinct survival properties of the FRNAPH genogroups can hindered the establishment of their original proportion in water, thus giving misleading information when they are used for microbial source tracking (Long and Sobsey,

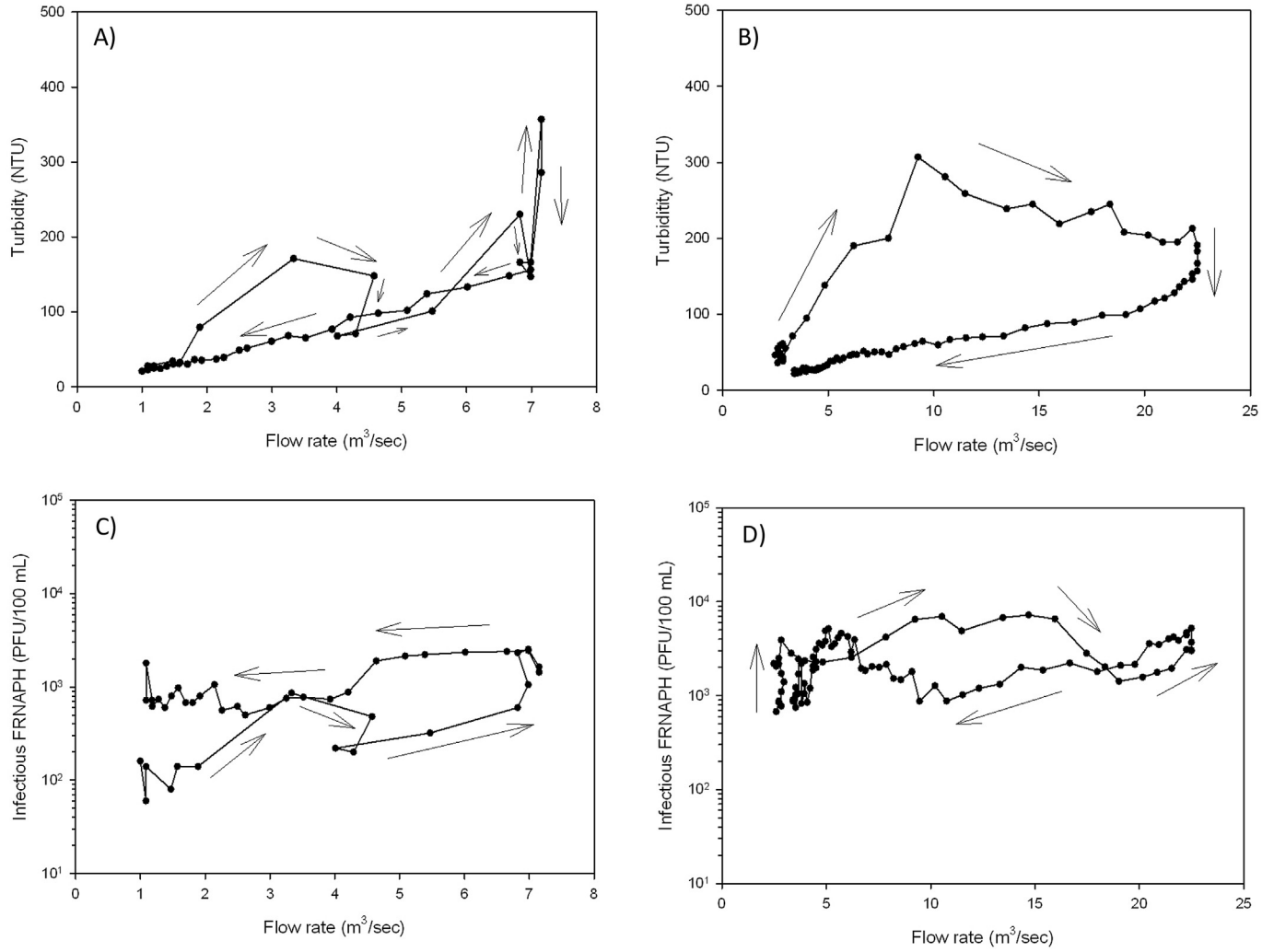

Fig. 6. Hysteresis plots of water discharge against turbidity (A, B) and infectious FRNAPH concentration (C, D) during summer (A, C) and autumn events (B, D), respectively.

2004; Muniesa et al., 2009; Ravva and Sarreal, 2016; Yang and Griffiths, 2013). Considering the molecular data, the viral genome persists longer than the infectious status of the corresponding virus (Bertrand et al., 2012; Gassilloud et al., 2003), and no significant difference of RNA persistence has been showed for the four genogroups (Kirs and Smith, 2007; Ogorzaly et al., 2010). The use of the RT-qPCR results for the source identification allows the prevention of this limitation. In addition, the origin of human or animal faecal pollution was determined here with the ratio of genogroup II to genogroup I, both considered as the most persistent among the four genogoups.

The faecal pollution observed in the Alzette River can be described as of human origin, as evidenced by the broad occurrence (100% of positive samples) and the high concentration (more than 4.3 $\log_{10}$ genome copies/100 mL) of genogroup II in low flow conditions. The presence of the three wastewater treatment plants upstream from the studied area can be a cause of this anthropic pollution. Furthermore, the comparison of the infectious FRNAPH flux upstream and downstream from the Hesperange WWTP in summer and in winter showed a small impact of the WWTP only during lowest flow rate of the river (in summer). Animal sources of faecal pollution were also possible in the Alzette River as highlighted by the presence of genogroup I in water samples (around 2.2×10^7 gc/sec), but its link to human pollution is not excluded (Hartard et al., 2015; Harwood et al., 2013). FRNAPH genogroups were also detected during both events studied with a presence and increase of genogroups I, II and III. For instance, genogroup II presented an increase of 1.0 $\log_{10}$ (5.9×10^8 to 6.6×10^9 gc/sec) and 1.6 $\log_{10}$ (1.2×10^8 to 4.5×10^9 gc/sec) for summer and autumn events, respectively. Given that genogroups I and II were mostly detected during both studied events rather than genogroup III, only the ratio of genogroup II to genogroup I was investigated. Considering that genogroup II is highly specific to human pollution and that genogroup I may be excreted in high quantities by animals this ratio may inform about both animal and human inputs. This approach was similar to that used by Ogorzaly et al. (2009). A significant increase of the ratio genogroup II to genogroup I occurred in parallel to the first peaks of turbidity, regardless of the event examined. This variation mainly resulted in a significant input of genogroup II from resuspended sediment. The sediment may contain more genogroup II because the baseline of the faecal pollution was mainly human. Indeed, hysteresis analyses demonstrated the contribution of sediment resuspension phenomenon in the first wave of FRNAPH contamination. Moreover, this assumption was supported by the major occurrence of genogroup II in the sediment of the riverbed of the Alzette River (data not shown). In a second phase, the ratio genogroup II to genogroup I decreased for both events with a more pronounced drop during the rainfall-

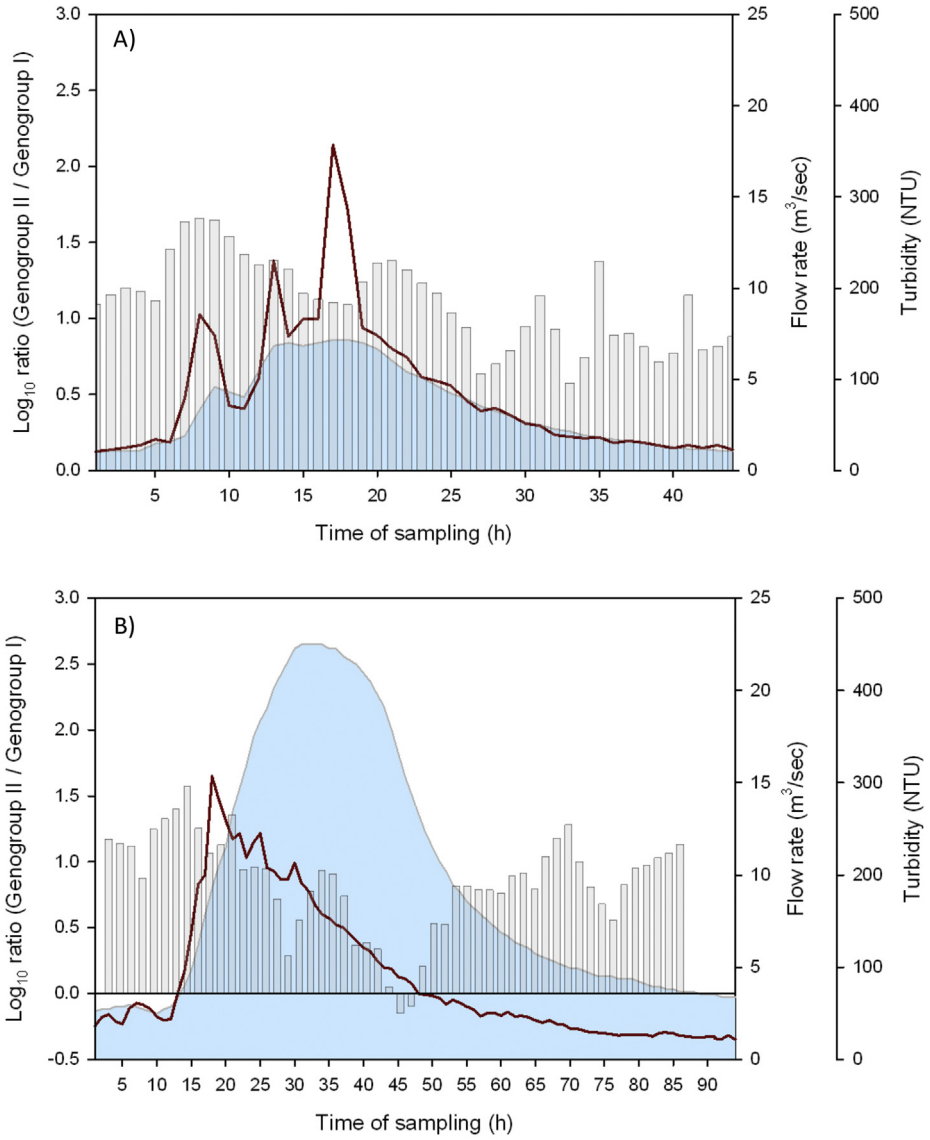

Fig. 7. Variation of $\log_{10}$-transformed ratio genogroup II on genogroup I fluxes (bar charts) during summer (A) and autumn (B) rainfall-runoff events. Red line represents turbidity for each event. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

runoff autumn event. In addition to the genogroup II, an input of genogroup I counterbalanced the ratio genogroup II to genogroup I. Hysteresis pattern interpretations showed that the second input of infectious FRNAPH arrived slightly shifted in time in comparison to the wave of discharge water. Runoff of urban and rural areas or excess water of WWTP effluents were possible origins coming from upstream of the measuring station. Genogroup I, which were mainly derived from animal faeces, came mainly from surface runoff as explained by the runoff coefficient of summer 7% and autumn events 34%. During the summer event, genogroup I was also meaningful during the falling phase, where the ratio genogroup II to genogroup I was lowest. Surface runoff can take more time during the summer season due to the drought of the soil absorbing rainwater.

In some other studies, the risk concept was broached with molecular results of enteric virus pathogens presenting a higher occurrence in flood water (Ngaosuwankul et al., 2013; Sidhu et al., 2012). However, given that a viral genome has a higher persistence compared to infectivity, an exaggerated conclusion can be made. Compared to these studies, monitoring of infectious FRNAPH, in parallel with genogroups, informs on the real amount and proportion of infectious phages during a rainfall-runoff event. The genogroup II to infectious FRNAPH ratio may thus give additional information linked with the age of the pollution. At the beginning of the events, the ratio was stable due to an equivalent increase of infectious FRNAPH and genogroup II genome. The input of genogroup II coming from resuspended sediment can be considered as infectious. Much data in the literature has shown the persistence of viruses in sediment (Skraber et al., 2009b; Smith et al., 1978). Indeed, it was suggested that the adsorption to solid particles could protect viruses and prolong their infectivity (Labelle et al., 1980). Accessibility of UV rays to sediment is limited by the depth and turbidity of surface water. Then, the genogroup II genome to infectious FRNAPH ratio decreased for both events during the peak of discharge water. The arrival of infectious genogroup I from surface runoff can explain this variation of ratio. At the end of the events, the ratio of genogroup II genome to infectious FRNAPH was lower than prior to the event. The rate of infectious phage particles was much more important at the end of the event than at the beginning. It therefore appeared, for the first time, that the infectious FRNAPH

input continued for some time after rainfall-runoff events despite the fact that flow rate of the Alzette river return to its normal level. If similar dynamic patterns occur for pathogenic viruses, this implies that a human health threat is not only present during the rainfall-runoff event but also after. These new data have to be taken into account in the risk assessment studies. In conclusion, these results provide chronological information on infectious FRNAPH that could allow a better estimation of the risk for human health during a rainfall-runoff event.

5. Conclusions

A combination of microbiological and hydrological approaches allowed a better understanding of the origin and dynamics of viral particles in a stream during rainfall-runoff events. These findings clearly highlighted the importance of the hydrologic cycle to virus fate and transport. Specifically, these results revealed that:

- The FRNAPH flux in the water column increase in accordance with hydrograph variations.
- FRNAPH load observed during the rainfall-runoff events result from two successive waves of FRNAPH pollution coming from two distinct origins: first the resuspension of riverbed sediment and later the surface runoff or WWTP effluents.
- Sediment available on the riverbed contributes to a supply of genogroup II in the water column, while genogroup I principally comes from the surface runoff.
- The polluted charge present a higher flux of infectious phage particles during rainfall-runoff events compared to low flow.
- Viral pollution decreases more slowly than the flow of the river with a flux of infectious FRNAPH always higher in post-rainfall runoff event compared to pre-events.

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