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Comparison of Coliforms and Coliphages as Tools for Assessment of Viral Contamination in River Water

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The aim of the study was to evaluate the presence of pathogenic viruses in the Moselle River and to compare the usefulness of thermotolerant coliforms and somatic coliphages as tools for river water quality assessment in terms of viral contamination. Thermotolerant coliforms and somatic coliphages were enumerated by standardized methods in 170 samples of river water drawn from five sampling sites along the Moselle River (eastern France). BGM cell culture and integrated cell culture-reverse transcription-PCR DNA enzyme immunoassay were used to determine the presence of pathogenic viral genome (*Enterovirus* and *Norovirus* genogroup II [GGII]) and infectious *Enterovirus* spp. in 90 1-liter samples. No infectious *Enterovirus* spp. were isolated, but *Enterovirus* and *Norovirus* GGII genomes were detected in 38% of the samples. *Norovirus* GGII genome was mostly detected in winter, whereas *Enterovirus* genome was mostly detected in summer and fall. Somatic coliphages appeared to be less sensitive to higher river water temperature than thermotolerant coliforms. Furthermore, the number of river water samples positive for pathogenic viral genome increased with increasing concentration of somatic coliphages, whereas coliform concentration was unrelated to viral genome contamination. Consequently somatic coliphages, which are less sensitive to environmental factors than thermotolerant coliforms in river water, would provide a promising tool for assessment of river water quality in terms of fecal and viral pollution.

Many factors influence viral pollution of surface water. Factors involved include the distance from wastewater discharge traveled by the virus (30), the epidemic period and thus the initial viral concentration (16), the survival (6) and transport (42) properties of the virus, the fact that the viruses are free or attached to suspended solids (31), and the environmental conditions (temperature, flow rate, salinity, and pH, etc.) (35). As a consequence, the crucial task of assessing viral contamination of river water, a major source of drinking water, is most difficult. Currently, viral contamination can be estimated either by specifically detecting pathogenic viruses or by evaluating the level of fecal contamination using indicators. Specific detection of pathogenic viruses is not adapted to routine analysis. Cell culture, which is the reference technique for the detection of environmental viruses, is time-consuming and does not allow the detection of all viral serotypes (e.g., *Norovirus*). Molecular biology techniques overcome some of the disadvantages of cell culture, but protocols are not standardized and the high genetic variability of some viruses (e.g., *Norovirus*) can be a source of underestimation. Inversely, molecular biology techniques can yield an overestimation since no information is obtained concerning viral infectivity (7). Furthermore, no matter which technique is used, it is not realistic to search for all virus serotypes that might be present in water. For this reason, some authors have suggested *Enterovirus* spp. could be used as indicators of the global viral pollution (19, 26). Using this approach however, other viruses such as hepatitis viruses (hepatitis E or A virus) or gastroenteritis viruses (*Norovirus* and

Rotavirus, etc.) are not taken into consideration despite their high prevalence (18, 23). Epidemic peaks also influence the density of pathogenic viruses in surface waters. For example, enteroviruses are mainly present in summer or fall (16, 20, 36) whereas noroviruses are principally detected in winter (27). This difference in seasonal distribution of viruses may seriously discredit the use of a single pathogenic virus as an indicator of global contamination. Several authors have shown that *Enterovirus* spp. are not representative of all viruses (4, 6, 28, 29, 38).

An alternative to specific search for pathogenic viruses is to use indicators of fecal contamination which are supposed to be representative of all pathogenic microorganisms of enteric origin and, among them, viruses. Bacterial indicators, and more particularly coliforms and streptococci, have been used for this purpose for a hundred years, but in some cases they are less resistant than viruses and consequently underestimate viral pollution (10, 34). The search has focused on other indicators better correlated with viral contamination. Some studies have shown that bacteriophages, viral particles similar in size and structure to pathogenic viruses, could be good indicators of viral contamination. The bacteriophages most frequently studied in this context are somatic coliphages (3, 11, 13), F-specific phages (9, 13, 39), and *Bacteroides fragilis* phages (13, 17, 24, 37). It is noteworthy that unlike methods specifically designed to detect pathogenic viruses, methods used to detect phages and bacteria are inexpensive, rapid, and easy to perform and thus applicable in both industrialized and developing countries. The diversity of indicators already in use or still in the research phase highlights the fact that no universal indicator has been identified. Consequently, it may be useful to determine which indicator(s) is best suited to practical conditions of application (e.g., type of water to analyze). In earlier work on a 30-km stretch of the Moselle River, we compared the behav-

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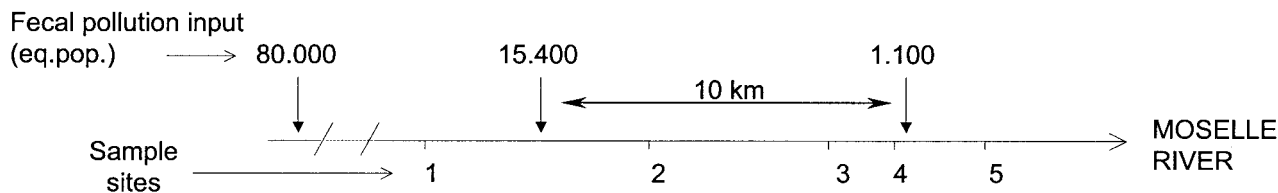
FIG. 1. The five sampling sites along the Moselle River.

ior of three bacterial indicators of fecal pollution (thermotolerant coliforms, enterococci, and spores of sulfite-reducing anaerobes) with that of three bacteriophages (somatic coliphages, F-specific phages and *B. fragilis* phages) (35). The results showed that spores of sulfite-reducing anaerobes, F-specific phages and *B. fragilis* phages can underestimate fecal pollution in comparison with thermotolerant coliforms. Enterococus concentrations are highly correlated to thermotolerant coliform concentrations, while somatic coliphages give original information tracking fecal pollution longer and farther. This difference in the behavior of coliforms and coliphages has been confirmed in a recent study of 392 water samples coming from 10 different rivers located in four countries (Argentina, Colombia, Spain, and France) (25). Thus, in river water, somatic coliphages could be valuable indicators of fecal pollution, taking into account viral contamination. In this context, the aim of this study was to define which indicator of fecal pollution, thermotolerant coliforms or somatic coliphages, is the most appropriate for assessing viral contamination in river water. To achieve this objective, we worked on the same sample sites along the Moselle River as in our previous study (35) looking for fecal indicators (thermotolerant coliforms and somatic coliphages) and pathogenic viruses (*Enterovirus* and *Norovirus* genogroup II [GGII]). Standardized methods were used to enumerate fecal indicators. *Enterovirus* spp. were identified by cell culture, integrated cell culture (ICC)-reverse transcription (RT)-PCR, and RT-PCR, while *Norovirus* GGII spp., which are not cultivable, were identified by RT-PCR alone.

MATERIALS AND METHODS

River water samples. All of the river water samples were taken from the Moselle River in eastern France. This river is about 70 m wide and is open to navigation. Its flow rate can exceed 130 m^3/s in winter, decreasing to less than 20 m^3/s in summer. Sampling sites (Fig. 1) were selected on the basis of levels of fecal contamination. Sites 1 to 5 correspond to the following towns, respectively: Méréville, Maron, Villey le Sec, Pierre la Treiche, and Chaudenay (see also reference 35). It can be noticed that there is a stretch of water between sites 2 and 4 where there is no source of human or animal fecal contamination over a distance of more than 10 km. Thirty-four river water sampling campaigns were undertaken between February 2000 and May 2002 at each of the five sampling sites ($n = 170$). The physicochemical characteristics of the water are given in Table 1. Samples were taken from the middle of the river, at a depth of 1 m. The

samples were maintained at 4°C. They were transported to the laboratory and analyzed within 2 h.

Pathogenic viruses. Ninety samples out of 170 were analyzed for pathogenic viruses. The concentration of pathogenic viruses was measured in 1 liter of river water by filtration using a protocol adapted from Gilgen et al. (8). Briefly, water was filtered through a positively charged membrane with 0.45-μm pores (CUNO NM04701 045SP). An 8-μm-porosity cellulose ester prefilter (Millipore SCWP04700) was used to limit clogging. The filter and prefilter were then eluted by immersion in 4.6 ml of sterile glycine buffer (0.4%) meat extract (1%) at pH 9.5. After stirring for 10 min at 100 rpm, the eluate was centrifuged at 1,500 × *g* for 10 min and the volume of the supernatant was measured with precision. The pH was adjusted to 7.2. The eluate was then divided into two equal volumes in cryotubes (2 ml/tube) and held at −80°C.

Detection of infectious *Enterovirus* by cell culture. Each 2 ml of eluate was treated with 10% antibiotic and antimycotic solution (catalog no. A-5955; Sigma). Qualitative detection of infectious *Enterovirus* was performed by adding treated samples on monolayer BGM cells. After 2 h of contact, cells were covered with MEM solution (catalog no. M-5650; Sigma) containing 2% fetal calf serum. After 6 days of incubation at 37°C under 5% CO$_2$ atmosphere, the flasks were examined under microscope for cytopathogenic effect, and 140 μl of triple freeze-thaw cell culture were analyzed for *Enterovirus* genome by ICC-RT-PCR.

RT-PCR detection of pathogenic viral genome. (i) Ultrafiltration. Each 2 ml of eluate was introduced into an ultrafiltration tube (catalog no. UFV4BHK00; Millipore) and centrifuged 5 min at 3,100 × *g*. One hundred forty microliters of sample was collected for viral RNA extraction.

(ii) Viral RNA extraction. Viral genome was extracted from 140 μl of ultrafiltrated sample with the Qiamp viral RNA kit (Qiagen, 52904) according to the manufacturer's instructions. Hence, 60 μl of final extracted RNA solution were obtained and conserved at −80°C.

(iii) Primers, probes, and standard RNA. Primers and probes are presented in Table 2. To avoid false-negative results, 20 μl of a standard RNA solution was added during the viral RNA extraction step to each sample. This standard was a 281-nucleotide RNA fragment which was amplified using the same primers as for *Enterovirus* and detected using a specific probe (Std).

Standard RNA solution was added to each sample at the limit of detection concentration.

(iv) cDNA synthesis. Five microliters of extracted sample was mixed with either 1 μl of 10 μM *Enterovirus* primer (Ent2) (final concentration of 0.5 μM in 20 μl of mixture) or 1 μl of 66 μM *Norovirus* GGII primer (NVP110) (final concentration of 3.3 μM in 20 μl of mixture) and heated at 95°C for 3 min. To this 6-μl mixture was added 14 μl of a mixture containing 4 μl of 5× RT buffer (250 mM Tris-HCl [pH = 8.4]), 50 mM MgCl$_2$, 350 mM KCl, 15 mM dithiothreitol, 2.5 mM spermidine (catalog no. M515A; Promega), 1 μl of RNase inhibitor (40 U · μl^{-1}; catalog no. N211A; Promega), 2 μl of 10 mM each deoxynucleoside triphosphate (catalog no. N8080260; Perkin-Elmer), 1 μl of reverse transcriptase (8 U · μl^{-1}; catalog no. M510A; Promega), 4.6 μl of DNase- and RNase-free water (catalog no. W4502; Sigma), and 1.4 μl of T4gene32 (1 μg · μl^{-1}; catalog no. 972991; Roche). RT was performed at 42°C

TABLE 1. Physicochemical characteristics of Moselle River water samples ($n = 170$)

Parameter	Flow rate (m^3/s)	Temp (°C)	pH	Turbidity (NTU)	Conductivity (μS/cm)	Coliforms log CFU/100 ml	Coliphages log PFU/100 ml
Mean	51	13.6	7.9	6.1	280	2.53	3.06
SD	35	6.0	0.3	4.3	95	0.78	0.51
Minimum	8	1.5	7.1	1.1	40	0.88	1.30
Maximum	133	24.0	9.2	30.1	464	3.75	4.14

TABLE 2. RT and PCR primers used in this study

Virus	Primer	Sequence[a]	Position	Reference
Enterovirus	Ent2	5′-ATTGTCACCATAAGCAGCCA-3′	578–597	29
	Ent1	5′-CGGTACCTTTGTACGCCTGT-3′	64–83	29
Norovirus GGII	NVP110	5′-ACDATYTCATCATCACCATA-3′	4865–4884	22
	SR46	5′-TGGAATTCCATCGCCCACTGG-3′	4754–4773	1

[a] Single letter code: D = G, A, or T; Y = C or T.

for 60 min. Finally, RNA-DNA hybrids were denatured, and reverse transcriptase was inactivated by heating to 95°C for 5 min. The resulting cDNA was then amplified by PCR.

(v) PCR amplification. PCR assay was performed with 5 μl of cDNA and 45 μl of the mixture containing 0.5 μl of each 10 μM *Enterovirus* primer (Ent1 and Ent2) or 0.5 μl of each *Norovirus* GGII primer (SR46 and NVP110, each at a concentration of 66 μM), 0.3 μl of Hotstart DNA polymerase (5 U · μl^{-1}; catalog no. 1007837; Qiagen), 23 μl of nuclease-free water (Sigma W4502), 11 μl of a 20% glycerol solution (catalog no. 101186 M; BDH), 5 μl of 10× PCR Mix (catalog no. 1005479; Qiagen), and 5 μl of each deoxynucleoside triphosphate (each at a concentration of 10 mM; catalog no. N808-0260; Perkin-Elmer). Amplification was performed using GeneAmp 2700 (Applied Biosystems). The amplification included (i) 15 min at 95°C to release the activity of the Hotstart DNA polymerase; (ii) 45 cycles of 30 s at 94°C, 30 s at 50°C, and 30 s at 72°C; and (iii) 10 min at 72°C. Amplified DNA was kept at 4°C until the DNA enzyme immunoassay (DEIA) detection which was performed the same day. RT and PCR mixture with 5 μl of phosphate-buffered saline buffer instead of sample were used as negative control.

(vi) DEIA detection. Amplified DNA samples were analyzed for the presence of viral genome (*Enterovirus* and *Norovirus* GGII) using the DEIA kit (DiaSorin PS0001) according to the manufacturer's instructions. The different probes are described in Table 3 and were used at a concentration of 100 pg/μl.

Indicators. Thermotolerant coliforms were quantified on m-FC medium after membrane filtration according to standard methods (15). Somatic coliphages were quantified using the bacterial host strain *Escherichia coli* (WG5) according to standard methods (14).

RESULTS

Influence of temperature on coliform and coliphage concentrations in river water. Among the 170 river water samples analyzed between February 2000 and May 2002, all were positive for both thermotolerant coliforms and somatic coliphages. The concentration-water temperature relationships are shown in Fig. 2 (river water temperatures varied from 1.5 to 24.0°C). Concentrations of thermotolerant coliforms were relatively homogeneous below 7°C (mean value: 3.29 ± 0.26 log CFU/100 ml; n = 25) but decreased and became highly heterogeneous at higher temperature (2.40 ± 0.77 log CFU/100 ml; n = 145). For somatic coliphages, the same phenomenon was observed, but at a higher temperature: below 15.7°C, concentrations were relatively homogeneous (3.29 ± 0.28 log PFU/100 ml; n = 101), whereas above 15.7°C, concentrations decreased and became heterogeneous (2.73 ± 0.59 log PFU/100 ml; n = 69). Those variations above 7°C for thermotolerant coliforms and above 15.7°C for somatic coliphages were mainly due to a

significant decrease between sites 2 and 5 (Student's test, $P <$ 0.05) (data not shown). Indeed, for each sampling date, the highest value of concentrations for both coliforms and coliphages always corresponded to the most polluted sample site (site 2) located 3 km upstream from an urban wastewater discharge. Thermotolerant coliform concentrations were stable for temperatures under 7°C and started decreasing between samples sites 2 and 5 for higher temperatures with a 0.66-log reduction for temperatures between 7 and 15.7°C and a >1.31-log reduction for temperatures over 15.7°C. Regarding somatic coliphages, concentrations were stable for temperature less than 15.7°C and started decreasing when temperature rose above that value with a 0.62-log reduction between samples sites 2 and 5. Results show clearly that thermotolerant coliforms were more sensitive than coliphages to the temperature effect. The two indicators thus described different patterns of fecal pollution.

Presence of pathogenic viruses in river water. Ninety samples out of 170 were analyzed for pathogenic viruses between December 2000 and May 2002. We looked for *Enterovirus* and *Norovirus* (GGII) contamination. *Enterovirus* spp. were identified using cell culture, ICC-RT-PCR, and RT-PCR while *Norovirus* GGII spp., which are not cultivable, were identified with only RT-PCR. Cell culture and ICC-RT-PCR failed to isolate infectious *Enterovirus* spp. from any of the 90 river water samples. Nevertheless, using RT-PCR-DEIA which is a more sensitive method, 34 samples out of 90 (38%) were positive for *Enterovirus* (13%) and/or *Norovirus* GGII (27%) genome. These results are detailed as a function of water temperature range in Table 4. *Norovirus* GGII genome was largely detected when temperature was below 7°C with 93% of the samples being positive while the proportion of positive samples fell to 7% for temperature over 15.7°C. This observation can be explained by the winter prevalence of *Norovirus*. Conversely, *Enterovirus* genome was detected less often for temperature under 7°C whereas 16% of the above 15.7°C samples were positive. This observation can also be explained by the summer-autumn prevalence of *Enterovirus*. Our results show that the viral contamination of river water was mainly represented by *Norovirus* GGII in winter and mainly by *Enterovirus* in summer. When considering the sampling sites as well as the

TABLE 3. Probes for viral genome detection (DEIA)

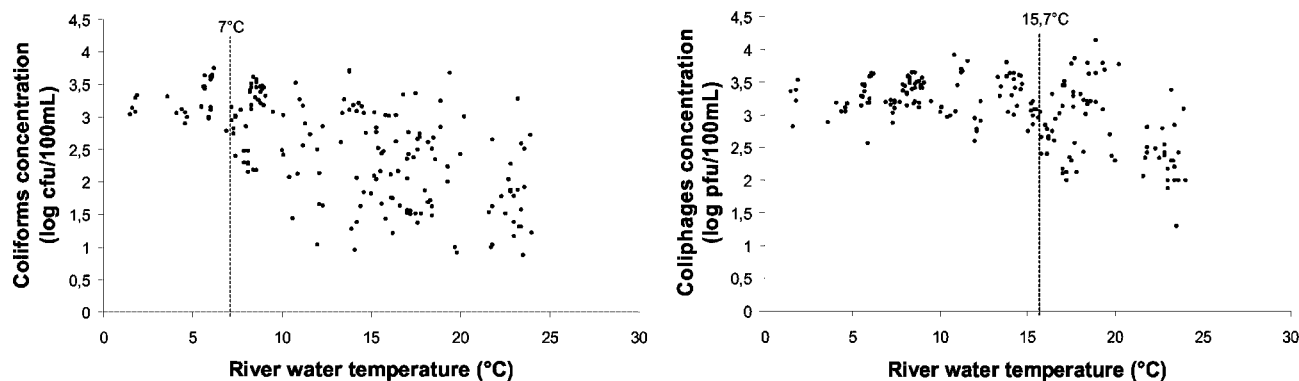| Virus	Probe	Sequence	Position	Reference
Enterovirus	Ent	5′-TCCTCCGGCCCCTGAATGCG(biotin)-3′	445–464	43
Norovirus GGII	SR47	5′-ATGTCAGGGGACAGGTTTGT(biotin)-3′	1804–4823	1
	SR61	5′-ATGTCGGGGCCTAGTCCTGT(biotin)-3′	1804–4823	1
	SR67	5′-ACATCTGGTGAGAGACCTGA(biotin)-3′	1804–4823	1
Standard	Std	5′-GACGGCCAGGTCCGGATG(biotin)-3′		

FIG. 2. Coliform and coliphage concentrations in river water as a function of water temperature ($n = 170$).

temperature (Table 5), viral contamination expressed as percentage of samples positive for viral genome did not vary with sampling site irrespective of the temperature range.

Indicators and pathogenic viruses. Among the 90 river water samples analyzed for pathogenic viruses, no infectious enteroviruses were detected by either cell culture or ICC-RT-PCR. While the absence of genome can be interpreted as an absence of virus, the presence of genome cannot be construed as a guarantee of the presence of infectious particles. Nevertheless, as we did not detect any infectious enterovirus, we compared the results for thermotolerant coliforms and somatic coliphages to those of *Enterovirus* and *Norovirus* GGII genome and thus classed the concentrations of both indicators into three categories (100 or less, over 100 to 1,000, and more than 1,000 PFU or CFU/100 ml). Results given in Fig. 3 clearly show that coliforms were not linked to the viral contamination represented by the presence of virus genome. Indeed, when the level of coliforms fell below 10^2 CFU/100 ml, 8 samples out of 32 (25%) contained viral genome (6 samples were positive for *Enterovirus* and 2 were positive for *Norovirus* GGII). Conversely, the number of samples positive for viral genome decreased with decreasing concentration of coliphages. Nevertheless, it must be underlined that only 7 samples out of 90 contained 10^2 PFU/100 ml or less, and one of them was positive for *Enterovirus* genome.

DISCUSSION

To determine which indicator of fecal pollution, thermotolerant coliforms or somatic coliphages, is the most appropriate for assessment of viral contamination in river water, we propose to analyze our results in three distinct parts.

First, following up on earlier work (35), we compared indicators of fecal contamination. Our earlier results showed that

thermotolerant coliforms and somatic coliphages were the most representative indicators of the fecal pollution along the river. Here, we examined their respective behavior over a longer period of time and a wider range of temperature. Our present results confirm that between sites 2 and 5 concentrations of thermotolerant coliforms decline faster than those of somatic coliphages. Tracking fecal pollution of the river over a longer distance with somatic coliphages thus corroborated the difference in fecal contamination estimated from bacteria and bacteriophages (25). This self-clearance capacity of the river could be related to water temperature, in agreement with our previous results.

Secondly, cell culture and ICC-RT-PCR detection of pathogenic viruses did not reveal any infectious *Enterovirus* spp. This could be explained either by the distance (in time and space) between the sample sites and the potential sources of viable viruses and/or by the small volume analyzed (equivalent to 500 ml). Nevertheless, considering the molecular biology results, 34 samples out of 90 (38%) were positive for *Norovirus* GGII (27%) and/or *Enterovirus* (13%) genome. These positive results could be explained by the fact that RT-PCR is known to be more sensitive than cell culture (19, 29, 41), possibly because RT-PCR allows detecting genome from both infectious and noninfectious particles, which increases the number of targets for the detection technique. Moreover, our observation is consistent with previous results showing the presence in surface water of *Enterovirus* genome (2, 5, 8, 33) or *Norovirus* genome (12, 32, 40). Looking at the qualitative results, overall

TABLE 4. *Enterovirus* and *Norovirus* GGII genome-positive samples as a function of river water temperature

Temp (°C)	n	No. (%) of samples positive for:	
		Enterovirus	*Norovirus* GGII
<7	15	1 (7)	14 (93)
≥7–≤15.7	30	4 (13)	7 (23)
>15.7	45	7 (16)	3 (7)

TABLE 5. Number of samples positive for pathogenic viral genome in river water as a function of sampling site and river water temperature range

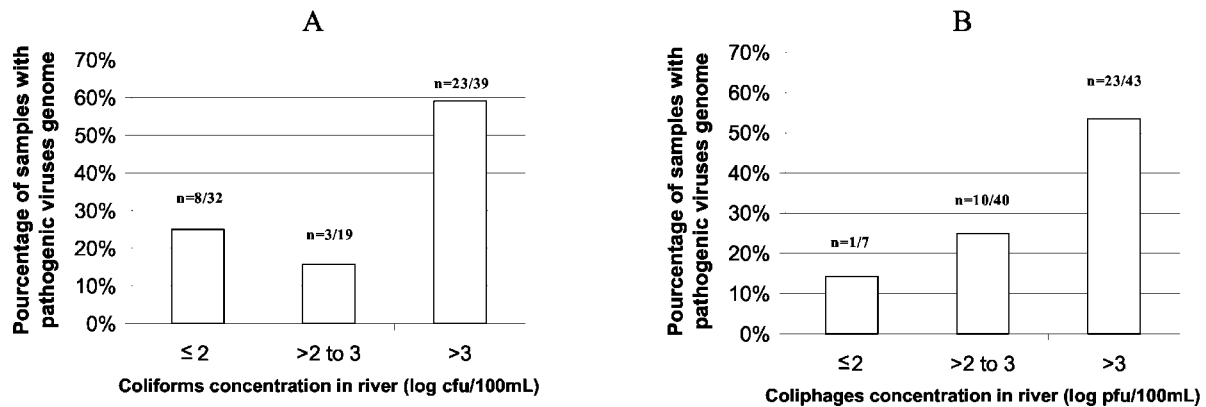| Site no.	n	No. of samples positive in water temp range			Total no. of positive samples ($n = 90$)
		<7°C ($n = 15$)	≥7°C–≤15.7°C ($n = 30$)	>15.7°C ($n = 45$)	
1	18	3	3	2	8
2	18	3	2	2	7
3	18	3	2	2	7
4	18	3	2	2	7
5	18	2	1	2	5
Total	90	14	10	10	34

FIG. 3. Number of samples containing pathogenic viruses genome as a function of coliform (A) or coliphage (B) concentration.

viral pollution of the river appeared to remain constant at the different sampling sites, while individual viral distributions exhibited seasonal patterns. These results are in agreement with previous studies showing that *Enterovirus* spp. are mostly detected in summer (16, 20, 36), whereas noroviruses are mainly isolated in winter (27). As previously noted (16, 21), this observation argues against proposing the use of a single pathogenic virus as an indicator of global viral contamination in river water.

Finally, we compared thermotolerant coliform and somatic coliphage concentrations for each sample positive for *Enterovirus* or *Norovirus* GGII genome. Our results show that the number of virus genome-positive samples decreased with decreasing concentration of coliphages, while no such relation was observed for thermotolerant coliforms. In fact, concentrations of thermotolerant coliforms fell off between sites 2 and 5 when temperature increased (temperature > 7°C), whereas the number of samples positive for pathogenic viral genome did not vary in the same way. Inversely, somatic coliphages appear to be interesting because of the following observations.

(i) A relation seems to exist between the concentrations of these indicators and viral contamination of river water in terms of pathogenic viral genome. This apparent relation is based on qualitative results and would have to be confirmed by quantitative molecular biology. Nevertheless, this is in agreement with another study (2) which concludes that bacteriophages (somatic coliphages and F-specific phages) but not fecal indicators are correlated with different enteric viral genomes in river water downstream from a wastewater discharge.

(ii) The number of samples positive for viral genome, and as a result for infectious particles, was low when the concentrations of somatic coliphages remained under the value of 100 PFU/100 ml. This threshold might be very useful for forecasting virological contamination in river water, but further confirmation would be necessary since this condition was only found for seven samples in our study.

In conclusion, somatic coliphages appear to be a promising tool for river water quality assessment in terms of fecal pollution that takes into account the virological parameter.

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