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Assessment of poly-L-lysine dendrigrafts for virus concentration in water: use of MS2 bacteriophage as proof of concept

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Aims: Virus detection has often been difficult due to a low concentration in water. In this study, we developed a new procedure based on concentration of virus particles on an innovative support: poly-L-lysine dendrigrafts (DGL), coupled with directed nucleic acid extraction and real-time PCR quantification.

Methods and Results: This method was evaluated using the bacteriophage MS2 as a model virus. This virus exhibited the size and structural properties of human pathogenic enteric viruses and has often been used to assess new supports of concentration. Moreover, this bacteriophage is also a faecal contamination indicator. In this study, many water filtration conditions were tested (volume of water, concentration, etc.), and more than 80% of bacteriophage were recovered after filtration on polymer, in most conditions. We demonstrated that the method was linear (slope = 0.99 004 and Y intercept when x = 0.02 028), valid (as manipulators, tested concentrations, volumes of sample and batch of polymer did not have any influence on concentration) and sensitive (allowing to concentrate up to 16 600-fold 1 l of sample and to detect and quantify down to 750 GC 11 and 7500 GC 11, respectively).

Conclusions: To conclude, this support exhibits high interest to retain viruses and to allow to detect low concentration of virus in water.

Significance and Impact of the Study: This study gives valuable advance in the methods of concentration and diagnosis of virus in water.

Keywords: concentration, diagnosis, poly-L-lysine dendrigrafts, virus, water safety.

Introduction

Water contamination by viruses is a major cause of concern in public health because it is recognized as an important source of waterborne diseases. More than 120 different types of viruses have been identified to be excreted in extremely high numbers by infected persons, and consequently, they can reach various environmental waters such as, for example, surface water (Lee *et al.* 2005; Lodder and de Roda Husman 2005) and drinking water. Several of these viruses such as adenovirus, enterovirus (Noble *et al.* 2003; Fong and Lipp 2005), norovirus (ter

Waarbeek *et al.* 2010) and hepatitis A virus (Deng *et al.* 1994) have been associated with gastroenteritis, diarrhoea and other gastric diseases. Ramani and Kang (2009) estimated enteric viruses as agents responsible for over 40% of childhood diarrhoea in developing countries. According to the EPA (2006a,b), viruses would be the etiological agents involved in the majority of unidentified outbreaks.

Even at low concentrations, viruses represent a hazard due to their virulence capacities. Consequently, one of the main challenges to overcome in a risk management perspective is the development of methods for detecting viruses present at a low concentration.

Virus detection requires an efficient concentration step from a large volume of water to a much smaller volume. Several concentration methods have been already developed including the adsorption of viruses on cartridges, membranes or glass wool by electrostatic interactions followed by their elution in a small volume (Rose *et al.* 1984; Ma *et al.* 1994; Enriquez and Gerba 1995; Katayama *et al.* 2002; Lambertini *et al.* 2008) or the ultrafiltration technique (Hill *et al.* 2009; Polaczyk *et al.* 2008).

Among these methods, adsorptions on cartridges or membranes constitute the most used and best described methods. However, these techniques are expensive for a routine utilization. On the contrary, glass wool filtration is less costly, but recovery efficiencies are highly variable. The ultrafiltration technique is a promising alternative because it allows a higher recovery of virus at low cost and requires less time than adsorption–elution methods.

Furthermore, after concentration in the support, all these methods necessitate additional steps of nucleic acid extraction, concentration and purification before virus quantification. Nevertheless, these last ones can be responsible for a loss of viral material (Noble and Fuhrman 2001; Katayama *et al.* 2002; Haramoto *et al.* 2004).

In this study, we developed a virus detection method based on concentration of viruses on an innovative material, followed by the direct extraction of the nucleic acid and finally quantification by PCR. This new method requires neither elution nor purification steps and uses an electropositive polymer: poly-L-lysine dendrigrafts (DGL), developed by Colcom. This material is strictly made up of L-lysine and exhibits a high density of amino surface groups (NH_3^+ in the water), about $5 \cdot 10^{15}$ amino groups for filters used in this study.

The method was developed on F-specific RNA coliphage MS2 as a model virus. This bacteriophage is a non-enveloped virus with size and structural properties similar to those of many human pathogenic enteric viruses (Grabow 2004). It was often proposed as an indicator of faecal contamination in water even if it is still under debate (Moriñigo *et al.* 1992). Furthermore, MS2 has been widely used as an indicator to quantify virus retention by selected membranes (Langlet *et al.* 2008), and procedures for its quantification have been largely developed (Ogorzaly and Gantzer 2006).

Material and methods

Micro-organisms and media

Escherichia coli K12 Hfr (NCTC 12486) and *Salmonella Typhimurium* WG49 (NCTC 12484) strains were used as host strains for the RNA F-specific bacteriophage MS2 (NCTC12487).

Bacterial and bacteriophage strains were kindly provided by the CRECEP (Paris, France).

Various media were used in this study as illustrated in Table 1.

Phage culture

A 250-ml flask containing 25 ml of LB broth Lennox medium was inoculated with *E. coli* K12 Hfr cells (NCTC 12486) and grown overnight in LB broth Lennox medium. The culture was incubated for 90 min at 37°C under shaking (100 rev min^{-1}). A MS2 bacteriophage (NCTC12487) solution was added to the culture to give a final concentration of 10^7 PFU ml^{-1} . After 5 h of incubation at 37°C (shaking at 100 rev min^{-1}), 2.5 ml of chloroform (Merck, Darmstadt, Germany) was added to the culture; then the culture broth was cooled to 4°C for 18 h. After this incubation, the culture was centrifuged at 3000 g for 20 min and the supernatant was carefully collected. Initial viral stocks of phages were about $10^{12} \text{ PFU ml}^{-1}$. Aliquots of phages were then stored at -80°C in 20% glycerol.

Bacteriophage counting

The number of bacteriophages (plaque forming units, PFU) per volume unit was determined by standard titration (NF EN ISO 10705-1 (2001), using *Salmonella typhimurium* WG49 (NCTC 12484) as host. Five hundred microlitres of a *Salmonella typhimurium* WG49 (NCTC 12484) stock, stored at -80°C , was inoculated in a 250-ml flask containing 50 ml of TYGB medium. When optical density reached 0.250, the culture was placed in wet ice. Serial dilutions of the bacteriophage solution were performed. Alternatively, 1 ml of each dilution was mixed

Table 1 Composition of the different media

Medium name	Composition
LB Broth Lennox	1% tryptone, 0.5% yeast extract, 0.5% NaCl
TYGA	1% tryptone (Sigma Aldrich, Saint Louis, MO, USA), 0.1% yeast extract (ThermoFisher Scientific, Waltham, MA, USA), 0.8% sodium chloride (VWR, Radnor, PA, USA) and 10 ml l^{-1} of calcium chloride–glucose solution containing (3 and 10%, respectively) (Fisher Scientific), 2% agar (VWR)
TYGA top agar	1% tryptone (Sigma-Aldrich), 0.1% yeast extract (Fisher Scientific), 0.8% sodium chloride (VWR), 1% agar (VWR)
TYGB	1% tryptone (Sigma-Aldrich), 0.1% yeast extract (Fisher Scientific), 0.8% sodium chloride (VWR), 1% agar (VWR)

with 1 ml of *Salmonella typhimurium* WG49 culture and 2.5 ml of TYGA top agar medium and poured onto a TYGA plate. Plates were incubated for 18 h at 37°C. For each tested dilution, two series were performed. One of them was treated to RNase A (Sigma-Aldrich) at final concentration of 40 $\mu\text{g ml}^{-1}$ and the other was not.

Synthesis of filters grafted with dendrigrafts of polylysine

Grafting of poly-L-lysine DGL onto polypropylene surfaces was obtained by plasma activation of the PP surfaces. Briefly, 5 g of native DGL-G3 was modified in the presence of 0.700 g sodium bicarbonate by adding 0.21 g of succinic anhydride in 100 ml of H₂O/THF (1 : 1) to get from 5 to 10% carboxylic groups onto the DGL surface. Two hours later, THF was removed under reduced pressure and then modified DGL was purified by steric exclusion on sephadex G25 resin (XK50 column) in ammonium carbonate buffer (0.1 mol l⁻¹) at 20 ml min⁻¹. After removing ammonium carbonate in excess under reduced pressure, the modified DGL was freeze-dried. Just before grafting, the modified DGL was dissolved in water at 10 g l⁻¹ and then degassed for 1 h. Prior to plasma treatment, polypropylene filters (SICAM Géotextiles, Albine, France, 8.4 mg, 7 mm diameter, fibre diameter 25 μm) were washed in an ultrasonic bath successively with water and ethanol for 90 s and finally dried in an oven at 60°C. The plasma reactor was composed of two cylindrical parallel aluminium electrodes (diameter: 13 cm, distance between electrodes: 6 cm) placed in a cylindrical vessel coupled with a RF generator (13.56 MHz) and fitted with an efficient vacuum pump (18 m³ h⁻¹ flow rate, initial pressure inside the device lower than 4 Pa). The PP samples were set on the bottom electrode in a glass container. The pressure of the reactor was adjusted to 4 Pa. Then, argon was introduced into the chamber and was monitored with a regulated Pirani gauge to reach the desired pressure. Activation was performed with a power of 10 W with 40 Pa argon plasma for about 90 s. Then, plasma-activated filters were covered up with 10 g l⁻¹ of the modified DGL solution for 2 h at 25°C to complete grafting yield. After a quick washing in water, the filters were immersed in a bath of 0.25 mol l⁻¹ carbonate–bicarbonate buffer pH = 11.25 with 50% of MeOH and sonicated for 1 h at room temperature. After an important washing with ultrapure water, filters were freeze-dried and kept under vacuum at –20°C.

Filtration on DGL

The DGL polymer was placed in the bottom of a serological pipette. A 50-ml syringe was used as a reservoir. Virus solutions were percolated 10-fold through the DGL (Fig. 1).

Virus retention on DGL was assessed by the difference between virus initial concentration and virus quantification on percolate by PCR.

Viral RNA extraction

Viral RNA was extracted using QIAamp Viral RNA Mini kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. The extraction was performed on 140 μl (viral suspensions in water were treated by diethylpyrocarbonate (DEPC) (Sigma-Aldrich)). RNA was eluted with 60 μl of AVE buffer. The extracts were immediately stored at –80°C.

Real-Time PCR

The following optimized PCR conditions were used for the real-time PCR (qPCR). A 168-pb fragment was amplified in triplicate, using KAPA SYBR Fast One-Step qRT-PCR kit (Clinisciences, Nanterre, France). Reaction



Figure 1 Description of system used to concentrate viruses. (a) System of filtration (b) dendrigrafts.

mixtures contained 10 μl of KAPA SYBR FAST qPCR master mix (Clinisciences), 0.4 μl KAPA RT mix (Clinisciences), 100 nmol l^{-1} each primer, 2 μl of purified RNA and water to make up to the final volume of 20 μl .

Two oligonucleotides were used in this study. The forward primer was 5'-TCGATGGTCCATACCTTAGATGC-3' corresponding to the region 1255–1277 and the reverse primer ACCCCGTTAGCGAAGTTGCT 1423–1404 of the MS25' bacteriophage genome (NC_001417-3') (Ogorzaly and Gantzer 2006).

The cDNA synthesis was performed at 42°C for 5 min followed by 95°C for 5 min and 40 cycles of 3 s denaturation at 95°C, 20 s annealing at 59.7°C and 1 s extension and data acquisition at 72 °C. This last step was followed by a melting curve analysis, which was performed by increasing the temperature from 50 to 90°C, with a raise in temperature by 0.2°C every 2 s with a plate read step in order to acquire the fluorescent signal.

Standard curves

Hundred-fold dilutions of F-specific RNA phage stocks ($10\text{--}10^{12}$ PFU ml^{-1}) were performed using DEPC water in order to draw standard curves. The Ct values of each dilution amplified in triplicate by qRT-PCR were plotted as a function of the logarithm of the starting amount of phages expressed in genome unit. Correspondence between plaque forming unit and genome copy (GC) was determined using amount of RNA measured in 10^{12} PFU ml^{-1} and molecular weight of viral genome. One PFU μl^{-1} corresponds to 5 GC μl^{-1} (GC). This link was in agreement with O'Connell *et al.* (2006).

The slope of the standard curve was used for determining the PCR efficiency (E) according to $E = 10^{(1/s)} - 1$ (Kubista *et al.* 2006). Thus, a standard curve with a slope of -3.33 corresponds to a reaction with 100% efficiency.

Recovery calculation and statistical analysis

Percentage recovery was calculated as the ratio between the virus concentration after water filtration and the theoretical virus concentration seeded into the water. A quantifiable dilution of viruses was used as PCR standard. Statistical analysis was performed with R Development Core Team software.

Results

Standard curve, efficiency and sensitivity of qRT-PCR assays

qRT-PCR assays were run in triplicate with RNA extracted from 100-fold serial dilution of phage MS2 in

DEPC water ($10^{12}\text{--}10^1$ PFU ml^{-1} corresponding to $5\cdot 10^{12}\text{--}10^1$ GC ml^{-1}). A negative control, corresponding to the RNA elution buffer AVE, was added for each assay. No amplification was observed in negative control, and no Ct value could be determined. Ct values of each amplified dilution were calculated and plotted as a function of the logarithm of the initial phage concentration expressed in GC ml^{-1} . A standard curve was established for concentrations between $5\cdot 10$ and $5\cdot 10^{12}$ GC ml^{-1} , with a slope of -3.28 (Fig. 2). The calculated efficiency (see Material and Methods) was 101.8%, and the squared correlation coefficient was >0.998 .

For each qRT-PCR, melting curves were established. Only one peak (melting temperature of the amplicon) was obtained in *melting curve* analysis, and it occurs at 83°C. No amplification was observed in negative control. These data confirm the specific amplification of the PCR product. Moreover, 5 μl of each concentration was loading on 2% agarose gel. Coloration was performed using SYBR Gold (Lifes technologies, Saint Aubin, France). Bands obtained are between 100 and 200 pb, in agreement with expected fragment length (168 pb) (Supporting Information).

The detection limit corresponds to the smallest number of genome units that provides a PCR-positive result at the 90% threshold. 10 separate dilutions prepared at $5\cdot 10^3$ and $5\cdot 10^2$ GC ml^{-1} were analysed. The value of the obtained detection limit was $5\cdot 10^3$ GC ml^{-1} corresponding to 1 GC/PCR well, with 90% of positive samples.

The limit of quantification (Lq) corresponds to the first level of the calibration range.

According to the standard ISO_TS_12869, ten samples at the target LqPCR ($5\cdot 10^4$ GC ml^{-1}), which were obtained after dilution of solution stock of bacteriophage followed by RNA extraction, were quantified (Table 2).

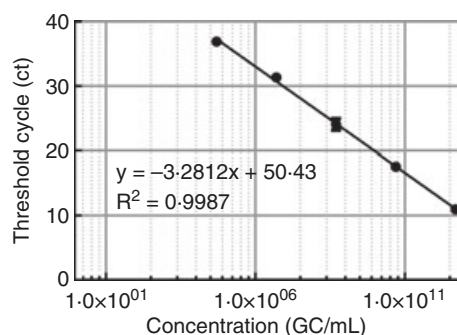


Figure 2 Standard curves for MS2 bacteriophage quantification by qRT-PCR. Means and standard deviation of Ct (triplicate).

To be valid, the accuracy at the limit of quantification (ELq) must be <0.15.

ELq was calculated as follows:

$$ELq = \sqrt{\text{Standard Deviation}^2 + \text{bias}^2}$$

In our study, ELq is equal to 0.12 and then can be accepted; therefore, the quantification limit at $5 \cdot 10^4$ GC ml⁻¹, that is, 250 GC/PCR well, is validated.

Retention capacity and recovery efficiency of bacteriophage MS2

The absorption of bacteriophage to DGL was first determined by comparing the concentration of virus in the water before and after filtration through the DGL. Ten millilitres of standard water solutions with virus concentration of $5 \cdot 10^6$ and $5 \cdot 10^8$ GC ml⁻¹ was used to determine the absorption maximum capacity of DGL.

Figure 3a shows the average adsorption yield of bacteriophage MS2.

For both concentrations, 80% of viral particles were retained on the DGL support.

In a second step, the ability to recover viruses was assessed. Ten millilitres of standard virus solution at $5 \cdot 10^6$ or $5 \cdot 10^8$ GC ml⁻¹ was filtered through DGL and eluted using a direct virus lysis on the support followed by RNA purification.

The percentage of recovery was calculated from the ratio between the number of particles recovered and the number of particles adsorbed on support (Fig. 3b).

An average recovery of 77% was obtained independently of the concentration used in the experiments.

Table 2 Determination of the limit of quantification

	x_i^*
LQ $5 \cdot 10^4$ GC ml ⁻¹ – test 1	4.629
LQ $5 \cdot 10^4$ GC ml ⁻¹ – test 2	4.646
LQ $5 \cdot 10^4$ GC ml ⁻¹ – test 3	4.767
LQ $5 \cdot 10^4$ GC ml ⁻¹ – test 4	4.666
LQ $5 \cdot 10^4$ GC ml ⁻¹ – test 5	4.766
LQ $5 \cdot 10^4$ GC ml ⁻¹ – test 6	4.705
LQ $5 \cdot 10^4$ GC ml ⁻¹ – test 7	4.489
LQ $5 \cdot 10^4$ GC ml ⁻¹ – test 8	4.489
LQ $5 \cdot 10^4$ GC ml ⁻¹ – test 9	4.699
LQ $5 \cdot 10^4$ GC ml ⁻¹ – test 10	4.836
x_i^*	4.669
Bias	-0.03
Standard deviation	0.11
ELq	0.12

* x_i^* is the log value of GC ml⁻¹, calculated by the inverse calibration, from the Ct values and the calibration curve equation.

Linearity assessment of the virus concentration method

Linearity assessment was required to validate performances of the concentration method. Actually, it was necessary to ensure that the number of particles in the sample and the number of particles recovered after concentration and extraction were linearly correlated.

Ten millilitres of three titrated solutions containing, respectively, 10^4 , 10^6 and 10^8 PFU ml⁻¹ were percolated through the DGL support. The number of retained particles was then quantified by qRT-PCR after RNA extraction. With the use of this method, the concentration factor obtained is 166-fold.

Figure 4 shows the data obtained by analysing the quantity of viral particles recovered after concentration on support and the number of expected particles.

The data exhibited a good linearity with a slope close to 1 (0.99 ± 0.04), and Y intercept of $x = -0.02 \pm 0.28$ with a good correlation coefficient ($R^2 = 0.99$).

Statistical analysis of retention performances of DGL

The ability of DGL filter to retain viruses was evaluated using low concentrations of viruses (below the limit of detection). Two virus water solutions at 50 GC ml⁻¹ and $5 \cdot 10^2$ GC ml⁻¹ in a final volume of 1 l and 100 ml, respectively, were used. The RNA extraction was performed directly on DGL and eluted in $60 \mu\text{l}$. These conditions led to a concentration factor of 1660- and 16 600-fold when the percolated volume was, respectively, 100 ml and 1 l.

The experiments were performed five times by three different experimenters and with two DGL batches. A total of 60 experiments were carried out using a randomization to fit the day effect.

Considering each individual filtration condition (manipulator, batch of DGL, concentration), DGL allowed a high retention and recovery of bacteriophage (Fig. 5): the percentage of recovery ranged from 87 to 258%.

An analysis of variance (ANOVA) (Snedecor and Cochran 1980; Armstrong *et al.* 2000, 2002) was then performed to assess the influence of the variables or association of variables (manipulators, batch and concentration) on virus recovery. After verification of the normal distribution of the values (using Q-Q plots and a scatter plot of the standardized residuals against the fitted values), the interquartile range test (test representing the distribution of the data towards the median) was used to identify and exclude outlier values, which are identified when:

- value <1st Quartile-1,5*InterQuartile Range
- and value >3rd Quartile+1,5*InterQuartile Range

Figure 3 Adsorption (a) and recovery (b) efficiency of bacteriophage MS2. The bars in the histogram represent the means of adsorption efficiency calculated from six independent experiments and the means of recovery efficiency calculated from three independent experiments, respectively. Error bars illustrate standard deviations.

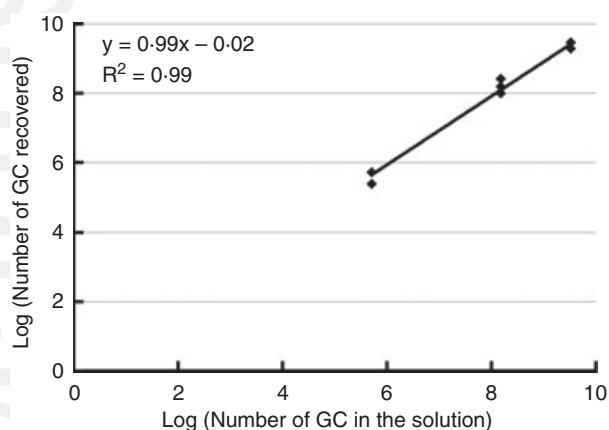
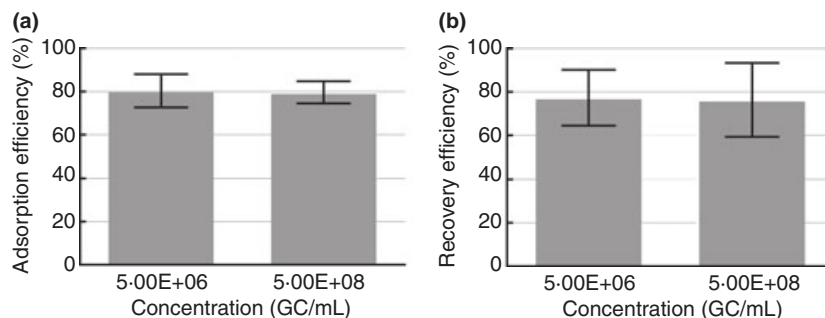


Figure 4 Linearity assessment of the virus concentration method. The curve represents the log of the expected number of virus particles in solution as a function of the number of virus particles recovered after concentration. Each measure was taken in triplicate.

Six outlier values were removed from the analysis.

Then the ANOVA calculation shows that:

- the three variables were found not to be associated with virus recovery: virus concentration ($P = 0.20$), experimenters ($P = 0.99$) and batch of DGL ($P = 0.19$) (Table 2).
- there was no significant interaction between the factors (Table 3).

Thus, these results showed that the DGL support allowed high retention and high recovery efficiency of viruses. The results demonstrated that the filtered volume, the experimenters and the batch of DGL production do not have any influence on the retention ability of the filter.

Conclusion

One of the main challenges to improve the detection of enteric viruses in water was to develop new concentration and extraction methods that allowed to detect low concentrations of viruses in water.

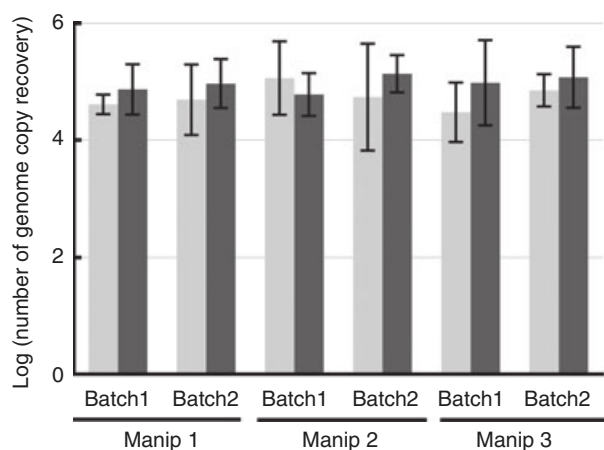


Figure 5 Viral particles recovery. The histogram represents the means of particle recovery for each condition: a/concentration, b/batch and c/ manipulator. Five replicates of each condition were performed, and error bars show standard deviations. Two concentrations were tested 50 GCu mlI (grey) and 500 GC mlI (dark grey).

A number of strategies had been already described. The most commonly used techniques are based on virus adsorption and elution, ultrafiltration and flocculation (Bosch *et al.* 2008). These techniques show various efficiencies of recovery (between 20 and 95%) according to the structure and physico-chemical properties of viruses (Rose *et al.* 1984; Ma *et al.* 1994; Enriquez and Gerba 1995; Katayama *et al.* 2002; Lambertini *et al.* 2008; Girones *et al.* 2010).

In this study, DGL was shown as an affective support for concentrating bacteriophage MS2 in water, whatever the filtered volume. The direct RNA extraction on support permits a concentration directly proportional to the volume of sample up to 16 600-fold, when 1 l of sample is used. This technique allows a quantification of bacteriophage in water from 250 GC l⁻¹ (when using 1 l of sample). The sensitivity of the method may be improved, using high-volume samples or low-elution RNA volumes. Moreover, Ogorzaly and Gantzer (2006) showed that the

Table 3 Variance analysis

Effect	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Manipulator	2	0.05	0.02	0.01	0.99
Concentration	1	6.15	6.15	1.71	0.20
Batch	1	6.49	6.49	1.81	0.19
Manipulator/ concentration	2	0.44	0.22	0.06	0.94
Manipulator/batch	2	1.21	0.61	0.17	0.85
Concentration/batch	1	0.52	0.52	0.14	0.71
Manipulator/ concentration/batch	2	2.29	1.15	0.32	0.73
Residuals	27	96.85	3.59		

Df, degrees of freedom; Sq, square; F value, variance ratio; Pr(>F), probability.

use of probe as detection chemistry led to a gain of ten factors in sensitivity.

The adsorption of bacteriophage by DGL filters was higher to that found for other electropositive filters such as NanoCeram cartridge filters, cellulose ester filters or 1 MDS cartridge filters (Sobsey *et al.* 2004; Hill *et al.* 2009; Ikner *et al.* 2011). Besides, the linearity of concentration method was verified and highlighted that our method was reliable.

In support of this, a statistical analysis confirms the validity of this method; adsorption and retention of viral particles are not impacted by volume sample, virus concentration, experimenters or batch of polymer.

Development of concentration methods allowing the detection of a large variety of viruses from drinking water with high sensitivity is a major challenge. To this purpose, it should be interesting to assess the potentiality of DGL.

Indeed, in their review, Cashdollar and Wymer (2013) highlighted that the most important parameters in virus retention development method were not the matrix or the type of filter used, but the type of virus and more particularly the strain used. To confirm the interest of the new DGL materials for virus concentration, studies are ongoing with other viruses such as hepatitis A and norovirus I. First results are encouraging. Nevertheless, further studies will be necessary to better understand the interactions between the DGL materials and viruses. Moreover, according to Cashdollar and Wymer (2013), it will be necessary to compare performances of DGL materials with other concentration methods and focus the work on the method implementation.

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