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Combination of Crossflow Ultrafiltration, Monolithic Affinity Filtration, and Quantitative Reverse Transcriptase PCR for Rapid Concentration and Quantification of Model Viruses in Water

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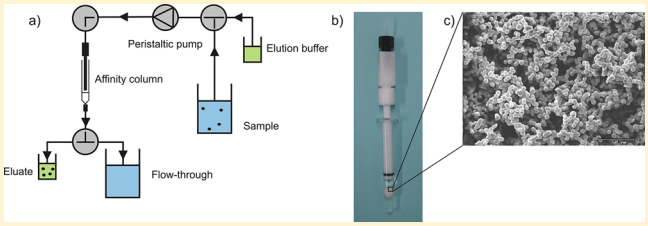
Supporting Information

ABSTRACT: We present a rapid and effective adsorption−elution method based on monolithic affinity filtration (MAF) for the concentration and purification of waterborne viruses. The MAF column consists of a hydrolyzed macroporous epoxy-based polymer. High recoveries were achieved by columns for the bacterial virus (bacteriophage) MS2 110 (±19)%, as model organism, as well as for human adenoviruses 42.4 (±3.4)% and murine noroviruses 42.6 (±1.9)%. This new concentration and purification method was combined with crossflow ultrafiltration (CUF). Because of the adsorption of the examined viruses to the macroporous surface of the MAF column at pH 3, concentrated matrix components by CUF can be removed. Bacteriophages MS2 were spiked in tap water and concentrated with the new CUF-MAF concentration method by a volumetric factor of 10^4 within 33 min. Furthermore, the detection limit for quantification of bacteriophage MS2 by quantitative reverse transcriptase PCR (qRT-PCR) could be improved from 79.47 to 0.0056 GU mL^{-1} by a factor of 1.4×10^4. In a first study, we have shown that this method could also be applied for river water containing naturally MS2 and MS2-like phages.



1. INTRODUCTION

Waterborne viral diseases are a health risk to humans and animals.[1] The risk of infection by consuming drinking water contaminated with viruses is 10−10000-fold higher than that for contamination with pathogenic bacteria at similar level of exposure.[2] Moreover, the long-term persistence in water and the moderate resistance to disinfection methods are further characteristics of waterborne viruses.[3] Therefore, it is emphasized in literature, that bacterial indicator occurrence does not correlate with viral occurrence.[4] The quantification of waterborne viruses at low concentrations demands rapid and efficient concentration methods which are compatible to cell cultivation assays or bioanalytical detection methods (e.g., qPCR) dealing with sample volumes in the milli- or microliter range.[5] These methods are important for studying the virus content in drinking water or raw water,[6,7] for monitoring purposes, and for risk assessment.[8]

The volume of water samples, required to be concentrated for virus detection, depends on several parameters. The recovery rate, concentration factor and the source of the environmental water sample define the volume of a representative sample. In groundwater and drinking water only a low amount of pathogenic viruses will be expected due to filtration effects of soil and sediments or because of water treatment. For these samples, volumes of more than 100 L have to be processed.[9] In contrast, high numbers of pathogenic viruses will be found in raw wastewater samples. Due to the fact, that water treatment methods fail to remove viruses completely, contamination of surface water is expected by discharge of treated sewage water.[10] Therefore, the processing of 10-L samples of surface or marine water will most likely be sufficient to gain detectable concentration levels of viruses.[11]

A volumetric concentration factor of at least 10^4 is hardly achievable in one step. Thus, a combination of two concentration steps is necessary. The goal of the primary step is to rapidly concentrate the viruses in the water samples to a minimized volume and to elute a broad range of viruses into a much smaller volume. Adsorption-elution methods are a commonly used technique as a primary concentration step. In general, viruses can adsorb to a solid matrix by electrostatic attraction or hydrophobic interaction at a defined pH value and

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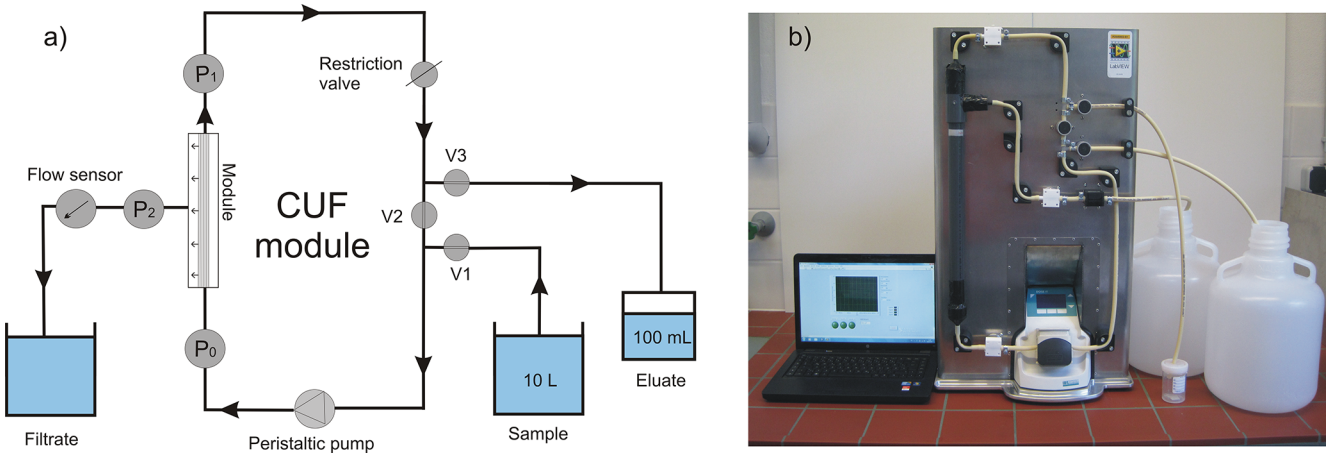

Figure 1. Schematic diagram (a) and image of the CUF system (b).

ionic strength conditions. Other compounds of the matrix are discarded so that purified viruses can be eluted by change of pH. There are two different types of filters: on the one hand electropositively charged filters which concentrate viruses at pH 7[12,13] and on the other hand electronegatively charged ones which concentrate viruses at lower pH.[14,15] Di- and trivalent cations are able to enhance the adsorption efficiency by altering the surface charge of the filter. Glass wool is another inexpensive alternative to microporous filters. Viruses adsorb to oiled sodocalcic glass wool at neutral pH. The surface presents a positive charge and hydrophobic binding sites.[5] Enteric viruses have been concentrated from up to 1500-L samples with a flow rate of 4 L min^{-1}.[16] However, the efficiency of recovery was severely affected by the pH of the water, type of virus and water matrix.

Another method to concentrate viruses from water samples of large volume is ultrafiltration, a size-dependent separation process. All particles larger than the pore size of the membrane are entrapped. The advantage of this method is the applicability without any preconditioning of the sample. Furthermore, a broad range of viruses, but also pathogenic bacteria and protozoa can be concentrated in parallel.[17] High recovery rates can be achieved with crossflow ultrafiltration using sodium polyphosphate precoated hollow fiber dialysis filter made of polysulfone.[18,19] Also automated concentration systems dealing with an ultrafiltration membrane for use in field are described in literature.[20]

Secondary concentration methods need to be combinable with primary ones by further reduction of the final sample volume to enable direct quantification of viruses. Possible examples are size-dependent concentration methods like centrifugal ultrafiltration[21] or ultracentrifugation.[22] On the other hand, the secondary methods additionally serve as purification steps to separate unwanted matrix compounds. Therefore, the most common methods described for this purpose are immunofiltration,[23] immunomagnetic separation,[24] precipitation, and organic flocculation.[25]

A further well suited possibility is a technique called monolithic affinity filtration (MAF).[26] Macroporous epoxy-based monolithic columns were produced in glass columns to achieve a covalent binding to the glass surface.[27] We have observed that viruses can adsorb to the hydrolyzed polymer surface from acidified water samples with high efficiency. The captured viruses were eluted by 1 mL of beef extract buffer at pH 9.5 with high recoveries. This MAF procedure combines concentration with purification and can easily be connected with any other primary concentration method. By means of crossflow ultrafiltration (CUF) in combination with monolithic affinity filtration (MAF), we present a fast and efficient concentration method for viruses which are directly quantifiable by qRT-PCR. For principle study of the two-step concentration process, we have chosen the bacteriophage MS2 as model virus due to the small size of 26 nm.[28] With MS2 we could study the size dependend entrapment efficiency of viruses on the ultrafiltration membrane. Furthermore, they naturally occur in environmental water samples.[29,30] In addition, adenoviruses[31] and murine noroviruses[32] were used as model viruses for human and animal pathogens to study the MAF method.

In this work, the following issues are investigated: (i) characterization of the concentration system by means of spiking experiments, (ii) evaluation of the detection limit for qRT-PCR with and without enrichment, and (iii) analysis of the matrix effects of different river water samples regarding the performance of the rapid combined concentration technique.

2. MATERIALS AND METHODS

Description of the CUF System. The setup of the CUF system was based on the crossflow microfiltration system, published elsewhere,[33] with several modifications. An image and a schematic drawing of the CUF system are shown in Figure 1. A peristaltic pump (Pumpdrive 5206, Heidolph, Kelheim, Germany) circulated the water sample through the CUF module with a maximum flow rate of 3.65 L min^{-1}, using Marprene tubing with an inner diameter of 6.4 mm (Watson Marlow, Rommerskirchen, Germany). The CUF module d20 K (Inge AG, Greifenberg, Germany) contained 24 Multibore fibers with a length of 0.5 m and a pore size of 20 nm. Each fiber consisted of seven internal capillaries with an inner diameter of 0.9 mm featuring a total membrane area of 0.2 m^2. The permeability of the module was 990 L h^{-1} m^{-2} bar^{-1} (see Supporting Information Figure S1). Valves, pressure gauges, and the flow meter were maintained from the setup of the crossflow microfiltration unit.[33] CUF module, peristaltic pump, pressure gauge P_o and P_1, and solenoid valve V2 were arranged in a crossflow concentration loop. The solenoid valve V1 was used for the filtration process and V3 for eluting the concentrated bacteriophages. All electronic components were controlled by a computer and operated via the software LabView (National Instruments, Austin, Texas, USA). A simple

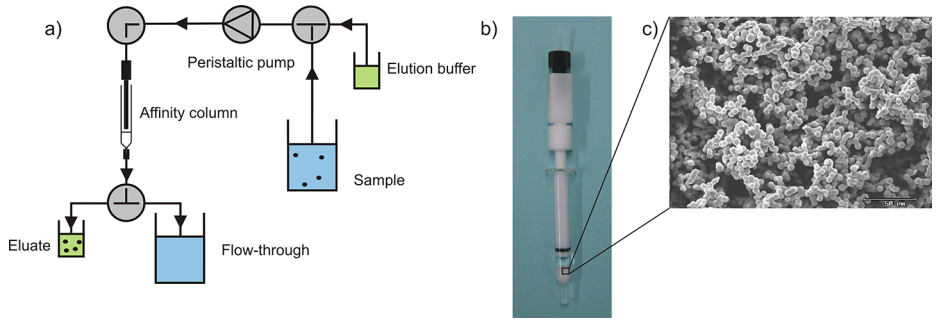

Figure 2. Schematic diagram of the MAF system (a), image of the MAF column (b), and scanning electron micrograph of epoxy-based monoliths with 500-fold magnification (c).

hose clamp was used to restrict the transmembrane pressure (TMP) to determine the permeability of the CUF module.

Description of the CUF Process. The CUF concentration process consisted of four steps (conditioning, filtration, flushing, and elution) and was described elsewhere in detail.[33] The first step was the conditioning of the CUF system. Therefore, all tubings were put in one beaker with ultrapure water (Millipore, Billerica, Massachusetts, U.S.A.). V1 and V3 were opened whereas V2 was closed. The pump was running until the remaining air was completely removed. Then, the inlet tubing was put into the bottle containing the sample. The outlet tubing was placed into an empty bottle to collect the filtrate, and the tubing for eluting the concentrated analytes was fixed in a sterile 100-mL beaker. Filtration was performed at a recirculation flow rate of 3.65 L min^{-1} by closing only V3. A TMP of 0.2 bar was achieved without restriction. A permeate rate of 504 (±21) mL min^{-1} was obtained. The peristaltic pump was stopped when only a small volume of approximately 50 mL was left in the sample bottle to ensure, that no air was sucked into the system. Afterward, the system was flushed with a recirculation rate of 3.65 L min^{-1} for 1 min in the forward flushing mode by closing V1 and afterward 1 min in the opposite direction. Finally, the concentration loop was eluted with 100 mL water at a flow rate of 3.65 L min^{-1}. The remaining sample volume was weighted for the correct calculation of the concentrated water sample volume. In total, a sample volume of 10 L was concentrated in 22 min.

Monolithic and Glass Wool Column Preparation. Monolithic columns were prepared in surface activated glass columns with an inner diameter of 4.5 mm and were produced by self-polymerization of polyglycerol-3-glycidyl ether in organic solvents (m-butyl methyl ether and dioxane) at room temperature within 1 h, as described elsewhere in detail.[27] The monolith had macropores with a size of 22 (±9) μm and no mesopores. The length of the monolith was 6 mm resulting in a volume of the monolithic support of 100 μL. For the activation, the epoxy-containing support was equilibrated first with ultrapure water, and then 200 mL of 0.5 M sulfuric acid were pumped through the column at a flow rate of 0.3 mL min^{-1} under recirculation. The reaction was allowed to proceed for 3 h at 60 °C. The MAF column was washed with 100 mL of ultrapure water and was stored in ultrapure water at 4 °C. Before usage, columns were equilibrated with 100 mL tap water, adjusted to pH 3 with 1 M HCl. Glass wool of the same dry weight as the monolithic polymer (0.017 g) was packed into similar glass columns. Packed glass wool columns were rinsed for 15 min with 200 mL methanol, washed with 200 mL ultrapure water, rinsed for 15 min with 200 mL of 1 M HCl, washed with ultrapure water, rinsed for 15 min with 200 mL of 1 M NaOH, and finally washed with ultrapure water by recirculation with 10 mL min^{-1}.

Description of the MAF Process. The secondary concentration process was based on the adsorption−elution procedure by MAF. MS2 adsorption at different water pH levels, flow rates and binding capacity of the MAF column were evaluated (see Supporting Information). After a primary concentration with CUF, the eluate was acidified with 1 M HCl to pH 3 and mixed intensively by vortexing. Then the sample was pumped through the column at a flow rate of 10 mL min^{-1} using a peristaltic pump from Ismatec (MS-Reglo, Glattbrugg, Switzerland) and a tubing with an inner diameter of 1.85 mm. Afterward, captured MS2 were eluted at a flow rate of 1 mL min^{-1} with 1 mL of glycine-beef extract buffer (pH 9.5) containing 3% beef extract (w/v) and 0.5 M glycine. The MAF process for 100 mL of CUF-concentrated water took 11 min. Adsorption rates were calculated from concentrations of MS2 phages present in the inputs and filtrates whereas recovery efficiency was calculated based on concentrations of MS2 present in the input and the eluate. Concentrations of MS2 were determined by double layer plaque assays (see Supporting Information). An image of the monolithic column and a schematic diagram are shown in Figure 2.

Concentration of Human Adenoviruses and Murine Noroviruses by Monolithic Columns. Human adenoviruses serotype 2 (hAdV2) and murine noroviruses type 1 (MNV-1) were grown in human embryonal kidney cells (HEK 293T, DSMZ ACC635) and mouse leukemic monocyte macrophage cells (RAW 264.7, ATCC TIB-71), respectively, in the biosafety level 2 (BSL-2) facility of the UBA. The protocols for cultivation are described in the literature.[31,32] Virus stocks were prepared by freeze fracture and subsequent centrifugation at 2000 *g* and stored at −80 °C. Concentrations of virus stocks were determined by q(RT)-PCR and calculated as genomic units per mL (GU mL^{-1}). Samples of 300 mL tap water were spiked with 3.2×10^2 GU mL^{-1} for each virus and adjusted to pH 3.0. After concentration, viruses were recovered in 1 mL elution buffer by pure gravity elution. For each virus type at least three replicates were concentrated.

Nucleic Acid Extractions. The RNA of bacteriophage MS2 was extracted using the ViralXpress Nucleic Acid Extraction kit from Millipore (Schwalbach, Germany). In contrast to manufacturer's recommendations, 150 μL instead of 50 μL of the sample were mixed with 200 μL of lysis buffer. For precipitation of nucleic acids, 350 μL of isopropanol were used. The washing step of the pellet was performed with 400 μL of

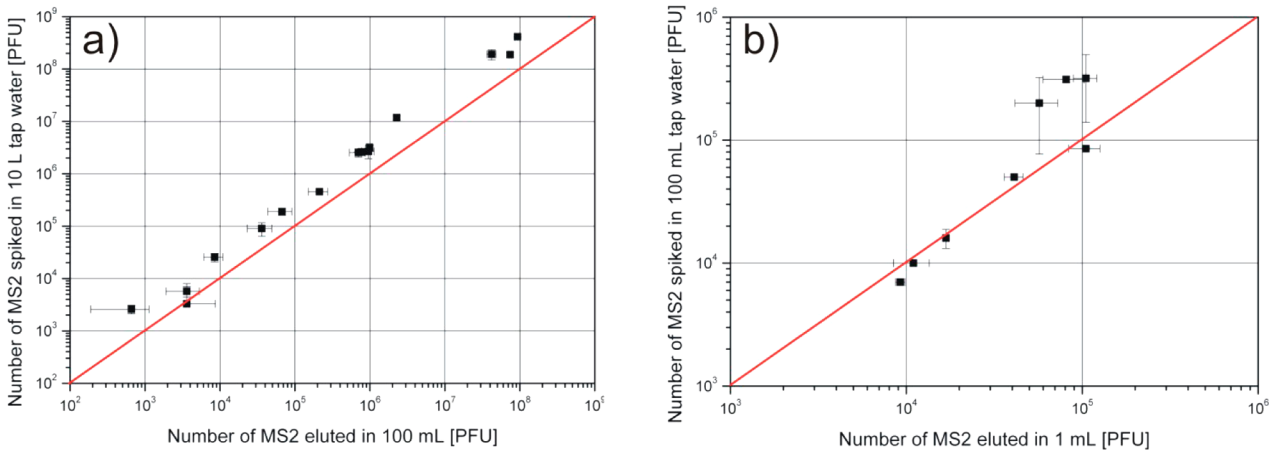

Figure 3. Recovery experiments for concentration of MS2 by the CUF (a) and MAF (b) methods. (Data points are shown with standard deviations. Continuous lines represent fictive recoveries of 100%).

ethanol. Finally, the pellet was resuspended in 20 μL of RNase-free water.

Nucleic acids from the vertebrate viruses hAdV2 and MNV-1 were extracted using the NucliSens Magnetic Extraction kit (Biomerieux, France) according to the manufacturer's manual. Independent of the sample volume processed, 50 μL of bead suspension were used. Nucleic acids were eluted in 100 μL elution buffer and 10 μL volumes were subjected to qRT-PCR.

cDNA Synthesis for Phage PCR. For this step the DyNAmo cDNA Synthesis kit from Thermo Fisher Scientifics (Schwerte, Germany) was used. The reaction was performed according to the manufacturer's recommendations by adding 4 μL of extracted template RNA.

PCR Amplification of Phage MS2. In the PCR reaction, a 314 bp long fragment was amplified coding for the replicase gene of bacteriophage MS2. The used oligonucleotides were synthesized by Eurofins MWG (Ebersberg, Germany), as reported elsewhere.[34] The forward primer was 5′-Digoxigenin-CTGGGCAATAGTCAAA-3′ (position 2717−2732 according to GenBank Accession Number V00642) and the reverse primer was 5′-Biotin-CGTGGATCTGACATAC-3′ (position 3031−3016 according to GenBank Accession Number V00642). The PCR reaction was performed with the LightCycler 480 system from Roche Diagnostics (Mannheim, Germany). The reaction solution was composed of 10 μL of LightCycler 480 SYBR Green I Master reagent from Roche Diagnostics, according to the manufacturer's recommendations, 1.6 μL of cDNA template and primers were used 0.4 μM each. The reaction volume was adjusted by using PCR water to achieve a final volume of 20 μL. The following program containing 55 cycles consisted of an initial denaturation step at 95 °C for 15 min and a three-step cycling at 94 °C for 15 s, 50 °C for 30 s, and 72 °C for 30 s. For quantification of MS2 a RNA standard (Roche Diagnostics) was used. The concentration was given to 0.8 μg μL^{-1}, which corresponds to 4×10^{14} GU mL^{-1}.

PCR Amplification of Vertebrate Viruses. The presence of human adenoviruses was quantified by TaqMan qPCR on an ABI Prism 7300 sequence detection system (Applied Biosystems, Germany), basically as described in literature,[35] with minor modifications. Murine norovirus (MNV-1) RNA was amplified using a single-step qRT-PCR published elsewhere.[36] The detection limits for both viruses were 0.03 GU mL^{-1} of the original 300-mL samples.

Water Sample. Experimental contamination was performed by seeding 10-L prefiltered (25 μm, Apic Filter GmbH, Weil der Stadt, Germany) tap water samples with the bacteriophage MS2. For these experiments, a tap water sample was spiked with MS2 to a final concentration between 0.53 and 1120 GU mL^{-1}. The water sample was prepared in autoclavable Nalgene bottles (VWR International GmbH, Ismaning, Germany) and shaken by hand for several minutes. Ten liters of the surface water (kept overnight in a cool box and tested within 24 h after sampling) were taken from an urban, as well as an alpine, river. The combined CUF-MAF concentration method and qRT-PCR were performed on the following day.

Recovery Experiments. Each experiment was performed at least twice. The recovery was calculated by dividing the total number of bacteriophages in the eluates by the total numbers of spiked bacteriophages. For characterizing the individual concentration systems, quantification of MS2 was done by plaque assay. Results of the combined process were achieved by using qRT-PCR as quantification method.

3. RESULTS AND DISCUSSION

Characterization of the CUF System. In a first concentration step, the sample was entrapped in a concentration loop of a CUF system. Fifteen 10-L tap water samples were spiked with MS2 to get final concentrations between 0.2 and 2×10^4 PFU mL^{-1} (Figure 3a). We determined stable recovery rates of 31 (±8)% (n = 11, m = 3) over 5 orders of magnitude for spiked concentrations between 9 and 2×10^4 PFU mL^{-1}. For lower concentrations only a few plaques could be measured in the eluate. Counting these plaques, a recovery of 58 (±38)% (n = 4) was calculated. The high standard deviations resulted from increasing variation of plaques numbers with decreasing concentrations. The reproducible recovery rates have shown that the concentration process can be repeated at least 15 times without negative effects on the CUF module. Bacteriophages MS2 were recovered with a mean efficiency of 31%, which is in the range of other membrane techniques.[18] Higher recovery rates would be achieved by precoating the membranes. The CUF system was able to concentrate bacteriophages within 22 min with a volumetric concentration factor of 100. The concentration rate was 4.5 min^{-1} and was calculated by dividing the volumetric concentration factor by the time. A CUF system equipped with the dialysis membrane F80A had a smaller concentration

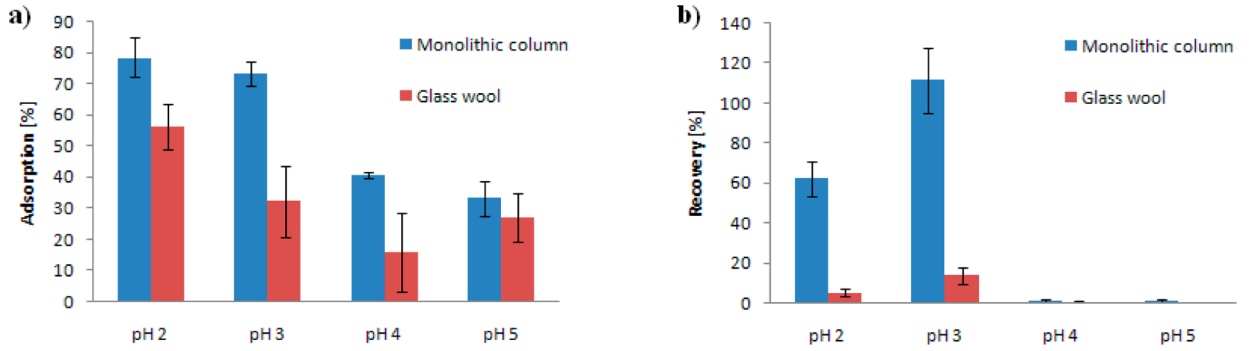

Figure 4. Comparison of MS2 adsorption rates at different pH (a) and their corresponding recoveries (b).

rate of 3 min^{-1}.[18] Reasons are the higher permeability of 990 L h^{-1} m^{-2} bar^{-1} of the used Multibore membrane and the small elution volume. The automated CUF system containing the Multibore membrane has a high potential for routine virus concentration. Using larger areas of the Multibore fibers, the permeate flow rate would increase and the concentration process would be faster. However, the elution volume would increase as well. Therefore, a higher amount of shorter fibers would be favorable. Moreover, the design of the CUF unit has to be adjusted to the MAF process to get the optimal concentration process with high concentration rates.

Characterization of the MAF System. The secondary concentration and purification step was an adsorption−elution method based on macroporous monolithic affinity filtration columns. The epoxy-based polymer support was activated by sulfuric acid. Functional groups at the surface were converted into hydroxyl groups. These functional groups are similar to that on activated glass wool surfaces.

To determine whether the surface of the monolithic column is comparable to the negatively charged glass wool at low pH and as well is able to capture positively charged viruses,[37] the dependency of the adsorption of MS2 on the pH value was examined (Figure 4a). Therefore, water samples were acidified to different pH values (pH 2, 3, 4 and 5). The highest adsorption, 78.5 (±10.7)% for monolithic columns and 56.2 (±7.2)% for glass wool columns, respectively, was determined at pH 2. At pH 3 the adsorption to monolithic columns (73.3 (±6.3)%) was much more effective than to glass wool columns (32.2 (±11.5)%). The adsorption efficiency decreased at pH 4 and 5. This can be explained by the isoelectric point of 3.9 for MS2,[38] indicating the contribution of electrostatic interaction to the adsorption process. This new method was compared to glass wool filtration. Glass wool filtration is a cost-effective method and able to concentrate multiple types of viruses. Compared to epoxy-based polymeric monolithic columns, lower amounts of MS2 were adsorbed on glass wool columns at each applied pH value.

For the elution step, the retained MS2 phages on the columns were recovered by glycine-beef extract buffer of pH 9.5, which is a high-ionic-strength protein solution.[39] However, 62.1% recovery of MS2 in tap water at pH 2 was much lower than at pH 3 (111.8 ± 16.3%, $n = 4$, $m = 3$) (Figure 4 b). This is most likely due to inactivation of the bacteriophage at pH 2. To examine inactivation effects at pH 3, samples prepared with the same concentration, but diluted separately by SM buffer and tap water of pH 3, were kept at room temperature for 1 h. Concentrations of 1.7 (±0.09) × 10^3 and 1.6 (±0.1) × 10^3 PFU mL^{-1} ($n = 4$) were determined by plaque assays. No inactivation effect was observed at pH 3 and MS2 was captured and recovered with high efficiency. A pH of 3 was chosen for acidification of water samples subjected to the MAF process. Recoveries occasionally higher than 100% may be explained by disaggregation of MS2 aggregates during the filtration procedure, although vigorous vortexing was involved to disperse the MS2 stocks during serial dilution procedure. Because of the varying aggregation behavior at different pH values,[40] samples were adjusted to neutral pH before testing in the plaque assay. Furthermore, using the same method for glass wool column experiments, only 2.8 (±1.9)% of seeded MS2 could be recovered, which is comparable to glass wool recoveries reported for MS2 in literature (range 0.3 to 1.8%).[5] The fact, that with the MAF procedure nearly 100% of seeded MS2 was recovered, shows the promising high potential of the macroporous epoxy-based polymer material.

Studies in the past suggested that virus removal in columns was inversely related to the flow velocities.[41] For Brownian motion driven particles $\log(C/C_0)$ is proportional to $v^{-2/3}$, whereas C and C_0 are the concentration and the initial concentration of free viruses in the aqueous phase, respectively, and v is the applied flow rate.[42] In our study, MS2-seeded samples were concentrated by monolithic columns at different flow rates varying from 5 to 40 mL min^{-1}. As Supporting Information Figure S3 illustrates, the adsorption of MS2 to the stationary phase follows the expected linear dependency with correlation coefficient of 92%. It indicates the significance of convective-diffusive transport in the fast mass transfer between monolithic column and mobile phases.[43]

The binding capacity of the monolithic column for MS2 bacteriophages was evaluated by using a sample volume of 1 L spiked with MS2 ($c = 316 \pm 16$ PFU mL^{-1}) and was quantified by plaque assay. A saturation of the monolithic affinity column (0.017 g) was observed after passage of 300 mL (see Supporting Information Figure S2). The binding capacity of the MAF column was determined to 5.6 × 10^6 PFU g^{-1} for MS2 in tap water. After optimization of the parameters the MAF was examined with samples of various concentrations (Figure 3b). 300-mL samples spiked with MS2 (final concentration = 23−1100 PFU mL^{-1}) were tested. The maximum concentration to be eluted from the column in 1 mL was 1.0 ± 0.2 × 10^5 PFU mL^{-1}, which is consistent with the binding capacity determined above. For samples containing MS2 concentrations below the capacity limit high recovery rates of 110 ± 19% ($n = 5$) were obtained.

The small volume of the macroporous monolith (100 μL) allowed the elution of MS2 into a small volume of 1 mL, which can be directly analyzed by qRT-PCR or plaque assay. The aim

to establish a rapid concentration method was achieved. The MAF process concentrates MS2 in 11 min with a volumetric concentration factor of 100, which results in a concentration rate of 9.1 min^{-1}. Furthermore, human adenoviruses and murine noroviruses, as surrogate for human noroviruses, were examined. Three experiments were performed using human adenovirus type 2 and four experiments using murine norovirus type 1 (Supporting Information Table S1). Under the optimized conditions described above, average recoveries of 42.4 (±3.4)% and 42.6 (±1.9)% were achieved, respectively. Recoveries of adenoviruses and noroviruses using monolithic columns were lower than those for bacteriophage MS2. Nevertheless, the MAF process was more effective than glass wool filtration published elsewhere.[16]

Characterization of the Two-Step Concentration Process. The two concentration methods were combined with rapid entrapment of MS2 by CUF followed by concentration and purification of the bacteriophages by MAF. In the first step, microorganisms from the 10-L sample were concentrated by using a Multibore hollow fiber module with pore sizes of 20 nm, and 100 mL of concentrate were eluted into a sterile beaker. After acidification to pH 3, bacteriophages were captured on the macroporous hydrolyzed epoxy-based polymer. The combinability of concentration and purification method with fast detection by qRT-PCR was tested. In this regard, the MAF should separate the inhibiting compounds from environmental water which were coeluted after the CUF process. For quantification of the bacteriophage MS2 with qRT-PCR, a dilution series of standard MS2 RNA was measured. The resulting Cp values were plotted against the concentration of standard MS2 RNA. Quantification of bacteriophage MS2 was possible in a range of 4×10^3–4×10^{11} GU mL^{-1} (see Supporting Information Figure S4).

To determine the effectivity of the CUF-MAF/qRT-PCR method, the limits of detection (LOD) of the molecular assay itself and of the combined analysis method were determined. Therefore, as published recently,[44] the resulting fluorescence signal gained at a predetermined PCR cycle was referred to the given concentration. The cycle was selected in a way to include most data points within the linear range of the sigmoidal calibration curve. In Figure 5, the resulting calibration curves with or without preconcentration of the phages are shown. For the curve without preconcentration (analyzed at cycle 40), a series of dilution for MS2, spiked in tap water, was measured by qRT-PCR. The LOD was determined to be 79.5 GU mL^{-1}. For determing the calibration curve for the combined method (analyzed at cycle 42), five 10-L tap water samples were concentrated by CUF and MAF, followed by qRT-PCR detection. A LOD of 0.0056 GU mL^{-1} was calculated. These data show that the sensitivity of the quantification assay was improved by a factor of at least 1.4×10^4.

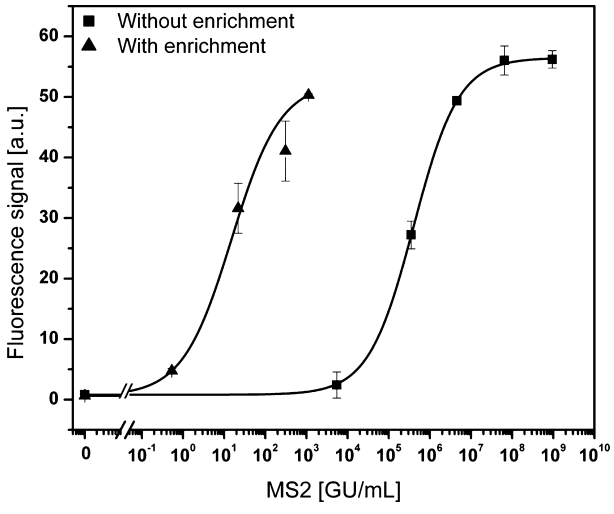

Figure 5. Calibration curves for bacteriophage MS2 in water samples measured with qRT-PCR.

To characterize the combined concentration system, spiking experiments with initial concentrations of MS2 between 0.53 and 1120 GU mL^{-1} were conducted (Supporting Information Table S2). In pure tap water no MS2 could be found. At low concentrations of bacteriophage MS2 (<1 GU mL^{-1}) the recovery was nearly 100% and a concentration factor of 10^4 was achieved. Therefore, the first concentration step by CUF was highly efficient at low concentrations. Lower recovery efficiencies analyzed by plaque assay could be partially caused by agglomeration of bacteriophages during the CUF process. With increasing concentrations of MS2, the recovery decreased from 97.2% to around 10%. The decrease of recovery can be explained by lower recovery from CUF at high concentration levels and the limited capacity of the monolithic columns, which were determined to be 5.9×10^6 PFU g^{-1} for MS2. Correspondingly, when the concentrations of MS2 in 10 L water were lower than 1 GU mL^{-1}, the MAF step was highly effective. The full concentration process lasted 33 min and resulted in a concentration rate of 303 min^{-1}. Upscaling of the CUF units and monolithic columns are ongoing in our group, so that a better recovery at higher numbers of viruses and wider working ranges with high efficiencies can be expected.

Real Water Experiments. Moreover, the CUF-MAF/qRT-PCR method was further tested with environmental water samples to show its potential for analyzing MS2 and MS2-like bacteriophages with more complex matrix effects than tap water. Therefore, two 10-L surface water samples from an urban and an alpine river were analyzed. The urban water sample, taken from a shallow canal, should represent a worst case scenario due to high amounts of algae and particulate organic load, which may easily block the ultrafiltration membrane. Additionally, the macropores of the MAF column could also be blocked by the matrix concentrated by the CUF process. Supporting Information Figure S5 illustrates the requirement of environmental water concerning the two-step concentration process. The high amount of matrix components coeluted in the CUF process could severely inhibit sensitive molecular detection methods, such as the enzyme reactions of the qRT-PCR. Therefore, the separation of MS2 from other matrix components is necessary before performing the qRT-PCR. During the MAF process, most matrix components passed through the columns. Purified eluates of 1 mL were generated and analyzed by qRT-PCR. The concentration of the MS2-like bacteriophages (F^+RNA bacteriophages) in the urban river sample was calculated to be in the range of 3.2 (±0.3) × 10^3 GU mL^{-1}. Furthermore, it can be stated that PCR-inhibiting compounds, like humic acids, were effectively separated by MAF. In comparison to the urban river, the alpine river water displayed a lower turbidity, although the 10-L water samples were collected after a wastewater treatment plant. Here, a corresponding concentration of 8.6 (±2.5) GU mL^{-1} was determined. In our study, after processing of 10-L surface water samples with our concentration and detection

system, the amount of detected MS2 and MS2-like phages was clearly above the detection limits. The two-step concentration and purification method combined with qRT-PCR detection, allows fast quantification of MS2-like bacteriophages in surface water within 3−4 h.

Potential Applications. The combined concentration method tested with phage MS2 can also be applied to other viruses. MAF is inexpensive and easy to apply. With MAF, not only MS2 but also adenoviruses and noroviruses, which occur in environmental waters, were concentrated. The adsorption−elution method concentrates different viruses and separates the matrix of environmental water which is important for rapid bioanalytical detection methods like PCR. With MAF, inhibitors in matrix for subsequent biomolecular detection can be removed but the diversity of microorganisms in the original sample can be maintained up to the final eluate. With CUF, microorganisms larger than the pore size of the Multibore ultrafiltration membrane can be enriched simultaneously. As the CUF and MAF methods can be used in line, an automated combined system could be established for monitoring of environmental water in the field. This rapid and effective combined analytical method could be used to monitor microbial levels in raw water and could be applied for risk assessment of drinking water resources.

■ ASSOCIATED CONTENT

Supporting Information

Permeability of the multibore CUF-membrane (Figure S1), binding capacity measurement of the monolithic affinity column for MS2 in tap water (Figure S2), linear correlation between the removal of MS2 and $v^{-2/3}$ (Figure S3), qRT-PCR calibration curve of bacteriophage MS2 referred to standard MS2 RNA (Figure S4), photography of the 100-mL eluate after the CUF process (a), a MAF column after (b) and before the MAF process (c) (Figure S5), recovery of human adenoviruses and murine noroviruses by MAF (Table S1), and recoveries of the RNA bacteriophage MS2 in samples after CUF-MAF/qRT-PCR (Table S2). This material is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

The authors declare no competing financial interest.

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