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Monolithic adsorption filtration (MAF)-Based Methods for Concentrating Viruses from Water

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ABSTRACT

Waterborne infectious diseases caused by viral infections are a health risk for humans and animals. The direct analysis of viruses in drinking water is difficult, since very low detection limits are needed. Therefore, rapid and efficient concentration methods are needed, which are compatible to cell cultivation assays or bioanalytical detection methods.

Rapid and effective methods were developed based on monolithic adsorption filtration (MAF) for the concentration and purification of waterborne viruses. Almost all seeded bacteriophage MS2, as model organism, could be recovered by MAF in tap water. Good recoveries were also obtained for human adenoviruses and murine noroviruses. MAF was successfully combined with ultrafiltration (UF) to concentrate viruses from large volume water samples. For processing samples in a 10-L scale, a volumetric concentration factor of 10^4 could be achieved within 0.5 h either by combining crossflow ultrafiltration (CF-UF) and MAF(Small) or by MAF(Big) and centrifugal ultrafiltration (CeUF). The detection limit of a nucleic acid amplification test (NATs) RT-qPCR was improved by a factor of the same order of magnitude for MS2. After principle studies in tap water these combined concentration techniques were applied to environmental samples. A three-step concentration process (UF/MAF(Big)/CeUF) was designed to concentrate viruses from water volumes larger than 10 m^3. Tap and ground water samples with a volume of 30 m^3 were reduced to 1 mL in 20 hours by the described three-step concentration method. Combining the concentration methods MAF and UF a wide range of viruses could be simultaneously concentrated. It was shown that next generation sequencing approaches for metagenomics studies could be enabled without cultivation by applying the developed new combined concentration method.

ZUSAMMENFASSUNG

Wasserinfektionskrankheiten, die durch virale Infektionen verursacht werden, stellen ein Gesundheitsrisiko dar. Um die direkte Analyse von Viren in Wasser zu erleichtern, wurden, basierend auf der monolithischen Adsorptionsfiltration (MAF), schnelle und effektive Methoden zur Aufkonzentrierung entwickelt. Mittels einer Kombination von Ultrafiltration und MAF wurde bei 10 L Proben ein volumetrischer Konzentrationsfaktor von 10^4 innerhalb von 0.5 h erhalten. Mit den entwickelten Methoden können verschiedene Viren gleichzeitig aufkonzentriert werden.

Contents

1 Introduction

Waterborne infectious diseases caused by viral infections are a health risk for humans and animals[1]. The total number of waterborne illnesses associated with exposure to pathogens in drinking water is estimated to be 19.5 million/year in the US[2]. The risk of infection by consuming drinking water contaminated with viruses is 10 - 10,000 fold greater than that for contamination with pathogenic bacteria at a similar level of exposure[3]. Furthermore, the infectious dose for most viruses is quite low. For example, exposure to 10 viral particles is enough to cause illness for a child and only 1 infectious unit of rotavirus is enough to cause infection for adult with no antibodies against this virus[4, 5]. Moreover, the long-term persistence in water and the moderate resistance to disinfection methods are further characteristics of waterborne viruses[6, 7]. Viruses in raw wastewater are the source of contamination in drinking water but water treatment facilities often fail to ensure the complete disinfection of viral pathogens[8]. It is emphasized in literature, that bacterial indicator occurrence does not correlate with viral occurrence[9]. Therefore, methods to routinely quantify viruses are highly recommended for raw and drinking water[10]. This is one part of the risk assessment of drinking water, which is suggested by the WHO water safety plan[11]. However, the direct analysis of viruses in drinking water is difficult since very low detection limits are needed. The quantification of waterborne viruses at low concentrations demands rapid and efficient concentration methods which are compatible with cell cultivation assays or bioanalytical detection methods, like PCR or immunoassays, dealing with sample volumes in the milli- or microliter range[12].

The aim of this work was to develop fast and effective methods, i.e. monolithic adsorption filtration (MAF) to concentrate viruses in water. Due to the small size and polar surface of viruses, a new adsorption-elution strategy was established to capture and recover viruses. Under optimized conditions, almost all seeded bacteriophage MS2, as

model virus, could be recovered. To achieve high flow rates and increased binding capacities, monolithic disks of different diameters, from 4.5 mm to 35.5 mm, were prepared. For processing samples in 10-L scale, MAF was combined with UF. A volumetric concentration factor of 10^4 was achieved in 0.5 h. The established methods were also applied in environmental samples. For concentrating viruses from large-volume water samples (> 10 m^3), a three-step concentration process, UF/MAF(Big)/CeUF was designed. 30-m^3 tap and ground water samples were reduced to 1 mL in 20 hours. Various viruses were simultaneously concentrated by these combined concentration methods. The final concentrates were compatible with cultivation methods (i.e. plaque assay) as well as molecular biological methods (i.e. PCR or next generation sequencing).

Fundamentals

2 Fundamentals

2.1 Water virology

Virus transmission via water was firstly proved in 1945[13]. After a large poliovirus-caused outbreak in the community, water from the local creek was fed to mice in the lab. Following this treatment, the mice were poliovirus infected. The viral risk in water was realized until the outbreak of hepatitis E happened in New Delhi, India, between 1955 and 1956, which caused 30,000 infections and 73 deaths[14, 15]. Until now, more than 140 virus types are found in human sewage. The number of viruses in the faeces of patient could be up to 10^{10} to 10^{13} per gram of stool[16]. The concentration of virus of 10^{6} to 10^{8} genomic units per liter could be detected in raw sewage[17-20]. As there is no regulation concerning the limits of viruses in discharge of sewage, wastewater treatment plants do not guarantee that the effluent is free from viruses. Therefore, viruses find their ways into surface water like ground, sea, lake, or river water. These water resources are used for recreation, irrigating or production of drinking water. Human beings expose themselves to enteric viruses when they are directly in contact with contaminated water and consume seafood, fresh vegetable and unsafe drinking water (Fig. 1). According to the report of WHO in 2007, consumption of unsafe water and inadequate sanitation and hygiene caused 88% of the 4 billion annual cases of diarrhoeal disease and led to 1.8 million deaths every year[21]. In conclusion, enteric viruses in water pose a threat to human health.

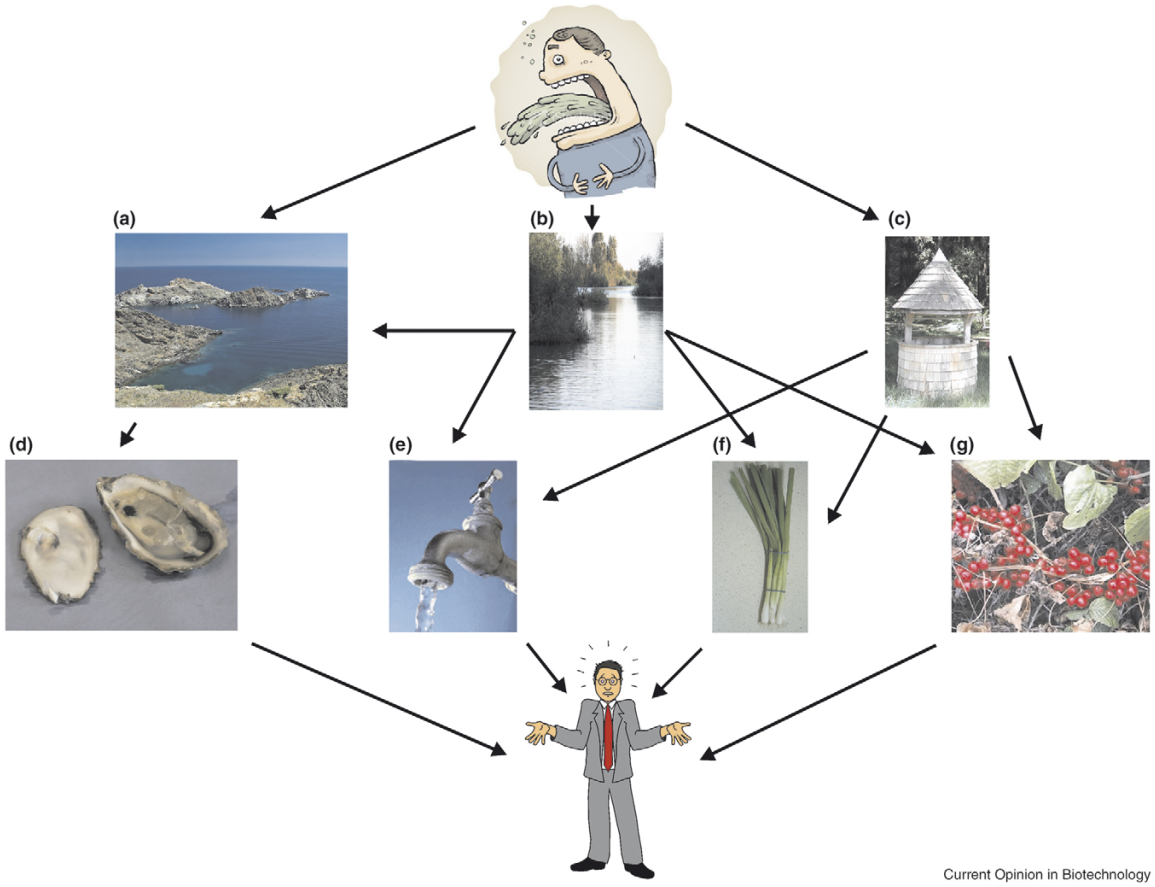

Fig. 1: Possible route of waterborne transmission of enteric viruses. (Reprinted from Ref[22])

2.1.1 Waterborne viruses

In the assessment of drinking or recreational water quality, coliforms[23], enterococci[24] or *E. coli*[25-27] are frequently used as indicators. However, more and more research indicates that bacterial indicators are not effective enough to represent the microbiological quality of water. The risk of infection by consuming drinking water contaminated with viruses is 10 – 10,000-fold greater than that for contamination with pathogenic bacteria at a similar level of exposure[3]. More than 100 human virus species were found in water[8]. Most are nonenveloped and belong to the families of the *Caliciviridae, Adenoviridae, Hepeviridae, Picornaviridae* and *Reoviridae*. Human enteric viruses in water cause several illnesses, such as gastroenteritis, meningitis, hepatitis, *etc*. From the epidemiological reports, many water-associated outbreaks were caused by

transmission of waterborne viruses. On the one hand, the infectious dose for most viruses is quite low, 1 to 10 viral particles are enough to cause illness[4, 5]. On the other hand, the long-term persistence in water and the moderate resistance to disinfection methods are further characteristics of waterborne viruses[6, 7] (Table 1).

Table 1: Human enteric viruses transmitted through drinking water[8, 11]

Viruses	**Disease caused**	**Persistence in water**	**Resistance to chlorine**	**Infectivity**
Noroviruses	Gastroenteritis	Long	Moderate	High
Adenoviruses	Gastroenteritis, respiratory disease	Long	Moderate	High
Rotaviruses	Gastroenteritis	Long	Moderate	High
Poliovirus	Paralysis, meningitis, fever	Long	Moderate	High
Coxsackievirus, A, B	Meningitis, cardiomyopathy, respiratory disease	Long	Moderate	High
Hepatitis A/E	Hepatitis	Long	Moderate	High

Viruses are more resistant to disinfection during water treatment and can be persistent for a longer time than bacteria. Therefore, some viruses are proposed as potential indicators, such as adenoviruses[28-30] and noroviruses[11].

Adenoviruses represent the largest nonenveloped viruses. There are 57 serotypes that have been identified with diameters ranging from 90 to 100 nm and weight around 150 MDa[31] (Table 2). A wide range of illnesses could be due to adenovirus infections. Specifically, adenoviruses 40 and 41 have been recognized as the second most important etiological agents, after rotavirus, for gastroenteritis in children[32]. Adenovirus-associated diseases are transmitted by direct contact, fecal-oral and waterborne transmission. Being

double-strain DNA viruses and having a high molecular weight, adenoviruses are much more resistant to UV disinfection than RNA viruses.

Noroviruses (previously referred to as Norwalk-like caliciviruses or small round-structured viruses) have been found in contaminated water and associated with gastrointestinal disease and endemic cases worldwide[33-36]. From 2007 to 2008, noroviruses alone were responsible for all drinking-water-associated outbreaks caused by waterborne viruses in the US. Murine noroviruses (MNVs) are frequently used as surrogates for human noroviruses since they possess the same characteristics of human noroviruses in diameter (28 to 35 nm), shape (icosahedral), *etc*. (Table 2). Moreover, MNV is the only noroviruses that replicates in cell culture[37, 38].

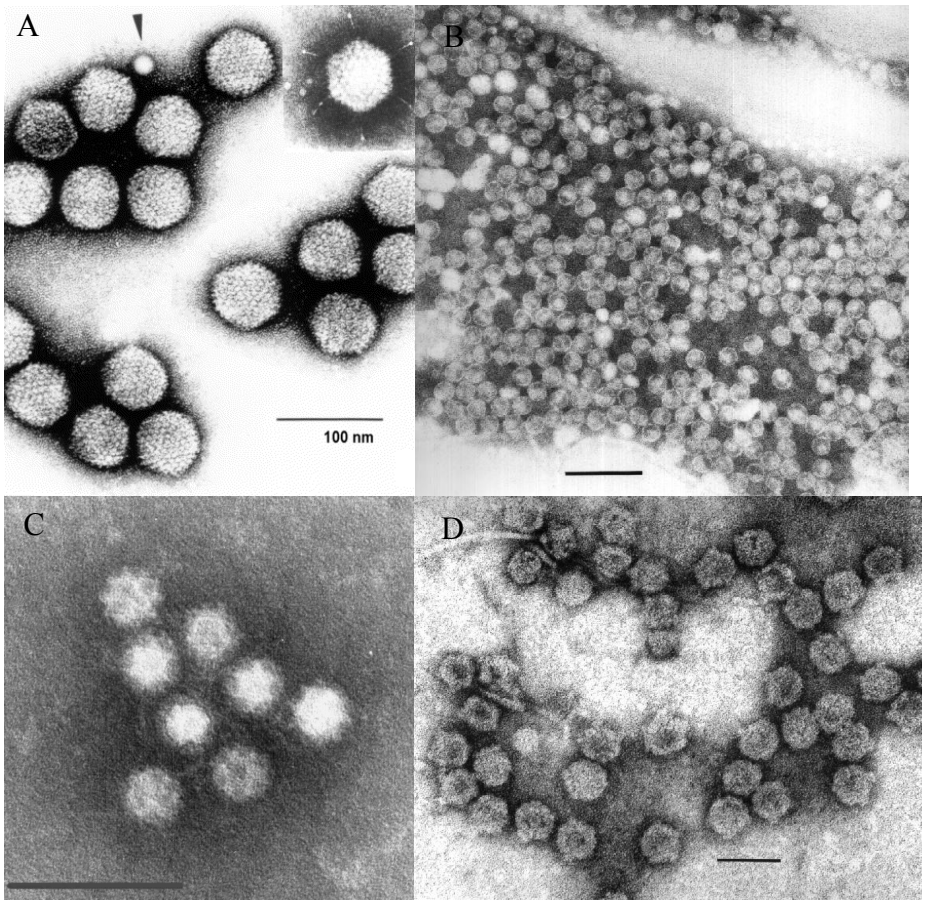

Fig. 2: Electron micrograph of A) human adenovirus; B) bacteriophage MS2; C) human norovirus; D) bacteriophage ΦX174 (adapted from database of International Committee on Taxonomy of Viruses, Columbia University, New York, NY, USA.)

Environmental bacteriophages are viruses that infect microbes in aquatic ecosystems. On the other hand, in terms of size, structure or modes of replication, *etc*., bacteriophages closely resemble enteric viruses[10] (Table 2). Bacteriophages are frequently used as surrogates for human enteric viruses due to the following reasons: presence in water in a higher number than enteric viruses; nonpathogenic; can easily be detected by plaque assay or PCR; only replicate in host; and are not able to multiply in aqueous environments[39-41]. The male-specific bacteriophage MS2, which is an icosahedral, positive-sense single-stranded RNA virus with a diameter of 26 nm[10, 42, 43], is one of the frequently used model viruses. Furthermore, due to its low isoelectric point (IEP), small size and hydrophobicity, MS2 is regarded as the worst-case scenario in membrane filtration[44, 45]. The bacteriophage ΦX174 has a cubic capsid and a circular single-strand DNA. Its diameter is about 24 to 32 nm and its weight is 6.2×10^6 Da[46]. ΦX174 is often used as a model of human enteric virus.

Table 2: Examples of viruses and their characteristics[31, 47, 10, 119, 120, 37, 38, 46]

Viruses	Genome	Size (nm)	IEP	Cultivable
Human noroviruses	ssRNA	28-35	5.5~6	No
Human adenoviruses	dsDNA	90-100	4.5	Yes
MS2	ssRNA	26	2.2~4.0	Yes
ΦX174	ssDNA	24-32	6.0~7.4	Yes

2.1.2 Water matrices containing human viruses

In aquatic environment, the viruses in sewage are the original source of contamination. To protect the water environment, guidelines for sewage discharge were issued in 1991 (Directive 91/271/EEC) in the European Union. Evaluation of chemical and biochemical parameters is required. After treatment, the total phosphorus and the total nitrogen of the incoming wastewater should be reduced by at least 70 - 80%, with

concentrations lower than 2 mg/L of P and 15 mg/L of N. The biochemical oxygen demand without nitrification (BOD) and the chemical oxygen demand (COD) of the incoming wastewater should be reduced by at least 70 - 90%, with concentrations lower than 25 mg/L and 125 mg/L O_2, respectively, before discharge. However, limits for pathogenic viruses are not included.

Surface water may be contaminated by wastewater. Surface water, like lake water, river or canal water may contain a much higher microbial load, suspended solids and a variety of dissolved constituents, like bacteria, viruses, protozoa, chemicals, dust, humid acids and so on. Therefore surface water requires more treatments to meet the standard of drinking water. In general, the most common steps include chemical agglomeration and flocculation, sedimentation, filtration and disinfection by chlorine or UV light.

Due to the filtration effect of soil and rock, ground water is a clear water resource and normally contains a low concentration of microbial agents. It can however be rich in dissolved solids, especially carbonates and sulfates of calcium and magnesium. After reduction of different metal contents and disinfection, it can be acceptable for drinking.

A guideline was adopted in 1998 in the EU (Directive 98/83/EC) concerning the quality of water for human consumption. Monitoring the effect of water treatment, including micropollutants and microbiological quality, is addressed. But the microbiological limits are only given for bacteria. No standard for viruses could be found until now. Similarly, in the guideline for drinking water from the Government of the Federal Republic of Germany (Trinkwasserverordnung – TrinkwV 2013), only limits for occurrences of indicator organisms (0 CFU / 100mL *E. coli* and coliform bacteria, 0 CFU / 250 mL *enterococci*) are listed. Based on quantitative microbial risk assessment, the WHO proposes there should be typically less than one rotavirus per 10^4 - 10^5 liters in drinking water[11, 48]. As recommended by Krauss and Griebler in 2011, large water volumes (> 10 m^3) have to be analysed to fulfil the requirements of the WHO[49].

2.2 Concentration methods for large-volume water samples

The main restriction in direct analysis of viruses in water is that their concentrations are too low to be detected, especially when molecular biological detection methods are employed. In contrast a fast response is important for risk management. Even a quite low number of viruses pose a great threat to human health due to the low infectious dose of pathogenic viruses. Therefore, development of concentration methods to recover the low number of viruses from large volumes of water is important in the virological analysis of water. Viruses are present in various shapes and sizes. Most viruses have a relative molecular mass higher than 10^6 Da and a size between 20 and 300 nm[50]. These features make them suitable to be concentrated by ultrafiltration and ultracentrifugation techniques. On the other hand, as a virion consisting of a protein capsid and a nucleic acid, viruses are highly polar species. The pH-dependent mobility of a virus is the fundamental principle of an adsorption-elution method.

Most concentration methods were developed in the 1980s and were rarely changed. A good concentration method must meet the following criteria referring to practical usage[51, 52]: 1) provide a high concentration factor (have a high virus recovery rate, a small volume of concentrate and be able to process a large volume of water); 2) be fast, simple and inexpensive; 3) simultaneously concentrate a large range of waterborne viruses; 4) be repeatable within a lab and be reproducible between labs. However, there is no single method that can fulfill these requirements.

As a volumetric concentration factor of 10^4 is hardly achievable in a single step, a combination of more than 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 and ultrafiltration techniques are commonly used as a primary concentration step. Secondary concentration methods need to be combinable with primary concentration methods. A last

reduction step is even needed to directly analyze viruses. Using a part of the sample volume for analysis would reduce the sensitivity of the complete analytical method. Possible examples are size-dependent concentration methods such as centrifugal ultrafiltration[53] or ultracentrifugation[54]. On the other hand, the secondary methods additionally serve as purification steps to separate unwanted matrix compounds. Therefore, for this purpose, the most common methods described are immunofiltration[55], immunomagnetic separation[56], precipitation and organic flocculation[57].

2.2.1 Ultrafiltration

Depending on the pore size, membrane separation processes can be classified into microfiltration (MF, pore size 10 - 0.1 µm), ultrafiltration (UF, pore size 0.1 µm - 5 nm), nanofiltration (NF, pore size about 1 nm) and reverse osmosis (RO, pore size < 1 nm) (as shown in Fig. 3)[58, 59]. Viruses, colloids and emulsions are typical examples separated by UF, which is a pressure-driven and size-dependent separation process. The advantage of this method is its applicability without any preconditioning of the sample[60]. A broad range of viruses, as well as pathogenic bacteria and protozoa can be concentrated at the same time[61].

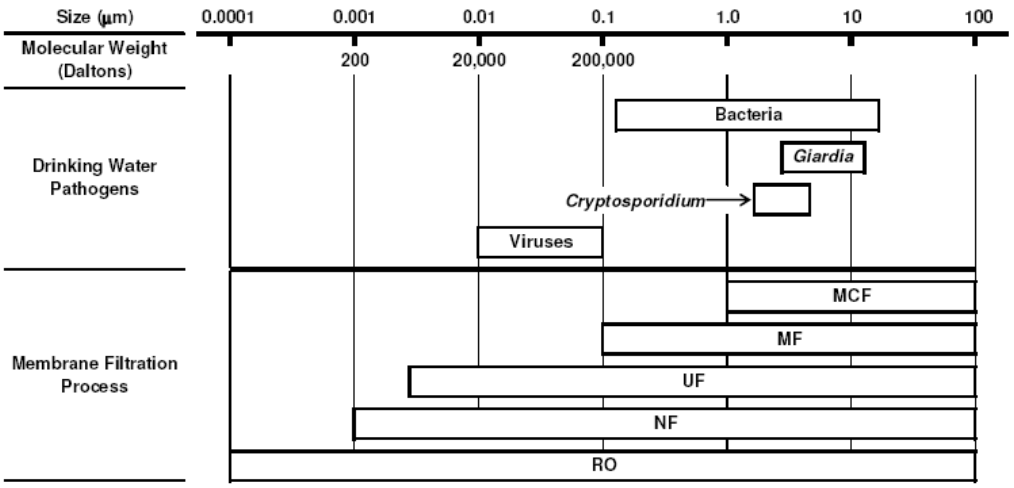

Fig. 3: Filtration application guide for pathogen removal[59]

Most ultrafiltration modules can either be operated in dead-end or crossflow mode. In dead-end mode, particles from the mobile phase are retained on the surface of the membrane. Consequently, a filtration cake may be formed and the filtration flux would decrease. The pressure difference between the feed and the filtrate side is the driving force to pass through the membrane, which is defined as transmembrane pressure (TMP). It can be calculated from the pressure applied on membrane ($P_{RETENTATE}$) and filtrate pressure ($P_{FILTRATE}$):

$$TMP = P_{RETENTATE} - P_{FILTRATE} \qquad \text{Equation 1}$$

In dead-end mode, the relationship between the filtrate rate and the pressure applied on the membrane is usually described by the Darcy equation[62]:

$$J = \frac{TMP}{\mu \cdot R_t} \qquad \text{Equation 2}$$

where J is the filtrate rate, μ is solvent viscosity and R_t is the total resistance including membrane and fouling resistance.

In crossflow mode (also called tangential filtration), the majority of the feed flow passes the membrane surface tangentially instead of going into the membrane. The deposited filter cake could be returned into the feed flux by shear forces. Therefore, the fouling of the membrane can be decreased[63]. In general, the filtrate rate increases linearly with the TMP. The linearity factor is defined as the permeability (P) of the membrane[64].

$$P = \frac{J}{A \cdot TMP} \qquad \text{Equation 3}$$

However, for the same device, operation in dead-end mode could achieve higher flow rates than in crossflow mode[65]. High recovery rates were achieved with crossflow ultrafiltration using sodium polyphosphate precoated hollow fiber dialysis filters made of polysulfone[66, 67]. Echovirus 1 in 100 L of tap water has been concentrated to 400 mL by this method at a flow rate of 1200 mL/min. In combination with a centrifugal ultrafiltration, higher recoveries of viruses were obtained compared to those by the

USEPA VIRADEL method[67]. By using a two-step ultrafiltration procedure, which was based on different sizes of hollow fiber filters, naturally occurring human viruses were targeted. Storm water with volumes up to 100 L was reduced to 1.5 L and then to approximately 50 - 100 mL by two sequential ultrafiltration steps. One out of 61 samples was found to be adenovirus positive. The inhibitory effect in PCR from environmental samples proved to be the main challenge in analysis[68]. Alternatively, the hollow fiber ultrafiltration was combined with a beef extract-celite concentration method, which showed better performance than flocculation and Celite as a secondary concentration method. For the Celite concentration method, the concentrates were amended with beef extract powder and Celite or Celite alone. After pH adjustment, the mixture was stirred and filtered through a glass fiber filter using suction. Then PBS solution was used to elute the viruses from the Celite. For samples spiked with low amounts of poliovirus (7.65×10^1 - 2.47×10^2 PFU/100 L), the highest recovery ($97.0 \pm 35.6\%$) was achieved using a flow rate of 1900 mL/min for ultrafiltration step[69]. Based on these methods, an automated concentration system dealing with an ultrafiltration membrane for use in the field was described in literature[70].

In our previous study, a computer-controlled crossflow microfiltration instrument was built up [64]. Since high volumetric concentration factors were achieved, a multibore ultrafiltration module (Fig. 4) with pore sizes of about 20 nm could be alternatively applied for virus concentration[71].

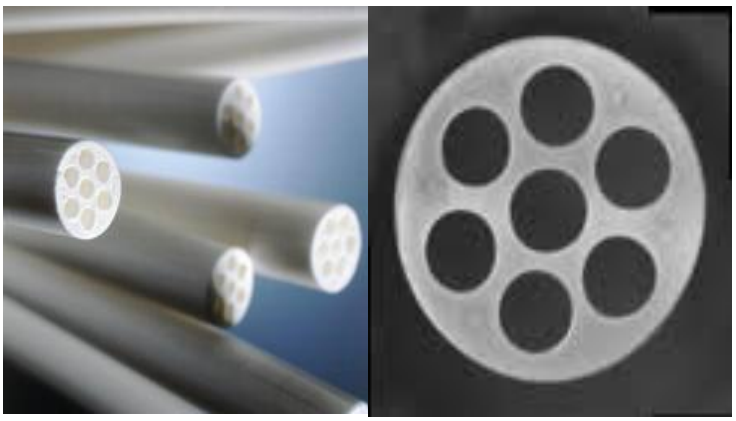

Fig. 4: Light microscope images of the Multibore® membrane[72]

Centrifugal ultrafiltration (CeUF)

A membrane with defined pore sizes can be mounted in a centrifuge tube to form a centrifugal ultrafiltration device. Centrifugal force provides the driving force for filtration. Under strong centrifugal forces, buffers and smaller molecules pass through the membrane while particles and macromolecules larger than the membrane molecular weight cutoff (MWCO) of the membrane are kept in the retentate. Similarly, ultracentrifugation is also a weight or size based separation method driven by centrifugal force. In order to be separated from the matrix, the target particle is forced to sediment into a pellet. For the same analyte, separation by ultracentrifugation requires much higher centrifugal force and much longer centrifugation times. With the help of the ultrafiltration membrane, CeUF is rapid in the order of minutes and needs low centrifugal forces (e.g. 3,000 to 7,500 x g). CeUF is used for separation of biomolecules, such as proteins, nucleic acids, liposomes *etc*. For small volume samples, CeUF is easy to use. The disadvantage of CeUF is clogging of the membrane when processing samples with a high amount of particle loading. Therefore, CeUF is often used as a secondary concentration step in analysis of large volumes of water in combination with ultrafiltration or adsorption-elution methods (more details are shown in Table 3).

2.2.2 Adsorption-Elution

Viruses are highly polar biocolloids (size between 20 and 300 nm), because they are composed of a protein capsid and an enclosed nucleic acid (DNA or RNA). Therefore, the sorption behaviour of viruses is often explained by the theory referring to the interactions between colloidal particles. The most popular one is the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory,

$$W(D)_{DLVO} = W(D)_{vdW} + W(D)_{elec} \qquad \text{Equation 4}$$

which comprises van der Waals and electrostatic interactions between the particles[73, 74]. Both interactions are functions of the distance between the particles. The theory quantitatively explains the aggregation of aqueous dispersions and describes the force between charged surfaces interacting through a liquid medium.

Van der Waals force

Van der Waals force is the total effect of dipole-dipole force, dipole-induced dipole force and dispersion forces, in which dispersion forces are the most important part because they are always present. The van der Waals interaction energy between a particle and a flat surface can be simplified as[75]:

$$W(D) = -\frac{A \cdot R}{6D} \qquad \text{Equation 5}$$

A is the Hamaker constant

R is the sphere radius of the particle

D is the distance between the particle and the surface, D << R

Electrostatic interaction

For a sphere and a shaped surface, the electrostatic interaction can be related to the surface potential Z, the distance between the sphere and the surface D and the Debye length κ via the equation[76]:

$$W(D) \propto Z^2 \exp(-\kappa D) \qquad \text{Equation 6}$$

The inverse Debye length, $1/\kappa$, represents the thickness of defuse electric double layer surrounding the charged particles.

A surface in a liquid may be charged by dissociation of surface groups (e.g. silanol groups for glass or silica surfaces, the charged amino acids of the coat protein of viruses). This results in the development of a surface potential at a wall, which will attract counter ions from the surrounding solution. As a result, protonation of interfacial compounds of organic or inorganic particles in water will lead to the formation of pH-dependent electrically charged surfaces. Fig. 5 sketches a part of a protein and illustrates the origin of its net surface charge, which is because of a superposition of protonated and unprotonated states of functional groups.

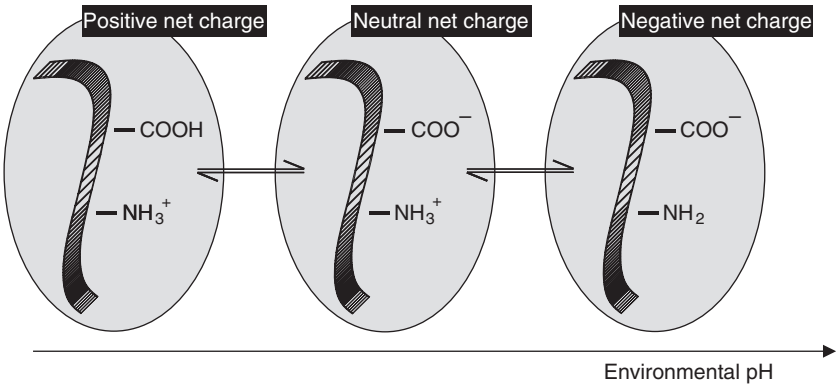

Fig. 5: Schematic showing the influence of environmental pH on the protonation states of charged groups on a protein capsid.

In the explanation referring to the electronic interactions, the isoelectric point is a key parameter. If the net charge of colloids is equal to zero at a particular pH, this electrically neutral state is termed isoelectric point (IEP) [47]. In principle, when the pH of the environment is higher/lower than their IEP, viruses carry negative and positive charges and prefer to adsorb to a solid surface with opposite charge. From reported data, the IEP of viruses are in the range between 1.9 and 8.4 while most are in the range of 3.5

and 7[47], as shown in Fig. 6. For the development of an adsorption-elution method, the surface charges based on the IEPs of the viruses should be taken into consideration during the optimization of adsorbents and elution conditions.

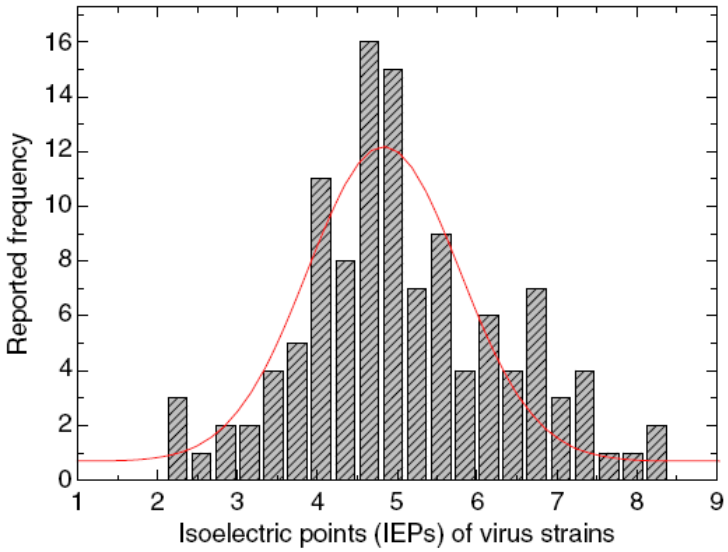

Fig. 6: Isoelectric points of viruses and their reported frequency in literature[47].

The DLVO model has been found to be unable to fully describe biotic and abiotic colloidal behavior in aqueous media. During the last decades it was shown that other types of surface forces are also presented and have to be taken into consideration: e.g., hydrogen bonds and hydrophobic effect.

Hydrogen bond

Hydrogen bonds occur when electronegative atoms bond to hydrogen atoms resulting in an “unshielded” proton, which have an affinity to a group with lone pair electrons. Water and the protein capsid of viruses have many unique characteristics that can contribute to the hydrogen bond with itself or other moieties in solution or on surfaces. Moderate-strength (4 - 15 kcal/mol) hydrogen bonds can form between water and acids, alcohols, or biological molecules[77]. For example, silica has silanol groups (-Si-OH) that may hydrogen bond with water. The oxygen of the water molecule serves

as a proton acceptor interacting with the hydrogen of the silanol groups[78]. It was also observed that water sorbed on polymers by formation of hydrogen binding with the hydroxyl groups on the surface of polymers[79].

Hydrophobic effect

Aqueous colloidal systems have generally been characterized as either hydrophobic or hydrophilic based on their relative affinity for water. The hydrophobic effect has been explained by the decrease in entropy of water molecules associated with cavity formation for the dissolution of hydrophobic moieties, which was not considered by classical considerations of colloid stability (i.e., DLVO theory). Hydrophobic colloids suspended in water result in a discontinuity in the hydrogen-bonded structure of water such that adjacent water molecules become oriented to maximize the number of hydrogen bonds. The water molecules adjacent to the surface thereby become ordered due to the presence of this non-polar surface. This ordering results in a decrease in entropy when compared to bulk conditions. Thus hydrophobic colloids have a tendency to aggregate or bind to a hydrophobic surface in water. However, change in the composition of surface functional groups, e.g., resulting from changes in pH, can result in a change in interfacial polarity[80, 81]. The hydrophobic effect is important in order to understand the structure of proteins in case of protein folding and is considered to play a key role in adhesion and transport of biocolloids, particularly bacteria and virus[82, 83].

Among these interactions, electronic interaction, hydrogen binding and hydrophobic effects are strongly influenced by the pH of the environment. Therefore the sorption processes of viruses onto stationary phase are pH-dependent in an aquatic environment[47]. The pH-dependent mobility of a virus is the fundamental principle of an adsorption-elution method. Viruses in water can adsorb to a solid matrix at a defined pH value. Then the water is discharged and adsorbed viruses can be concentrated when eluted into a small volume of elution buffer with different pHs.

The virus adsorption-elution technique is fundamentally different from other filtration methods used in microbiology. The pore size of filters used in adsorption-elution methods is larger than the size of analytes, i.e. virus particles, while the pore size of that used in size-exclusion based filtration, like ultrafiltration, is smaller than the size of viral particles. Based on the surface properties of media and viruses, different strategies are chosen to maximize the recovery of viruses from large volume of water.

2.2.2.1 Glass wool

Glass wool is a cost-effective choice for concentrating viruses. It was first used by Vilagines *et al*[30, 84] for concentrating various viruses from drinking and seawater. Oiled sodocalcic glass wool (Rantigny 725, Saint Gobain, France) was packed into housings and used as columns (Fig. 7). Viruses adsorb to the surface of glass wool at neutral pH due to the positive charges and hydrophobic binding sites on the surface[10, 30, 85 20](see Table 3).

Fig. 7: Setup of glass wool filtration (by LGA BW, Dr. Fleischer).

In the study to evaluate the performance of glass wool filtration for concentrating viruses, which were on the U.S. Environmental Protection Agency contaminant candidate

list, large volumes of tap or well water (10 to 1,500 L) were filtrated at a high flow rate of 2 - 4 L/min[85]. Captured viruses were eluted by 3% beef extract buffer and further concentrated by flocculation. PCR then was used for quantitative detection. Average recovery rates were 70% for the poliovirus, 14% for the coxsackievirus B5, 19% for the echovirus 18, 21% for the adenovirus 41, and 29% for the norovirus, respectively. Taking glass wool filtration as a sample-processing step[28-30], occurrence of human enteric viruses in European recreational waters was studied. 10-L water samples were collected from 15 surveillance laboratories during the EU bathing season. Adenovirus and norovirus were simultaneously concentrated from freshwater samples and detected by PCR. By glass wool filtration, recovery of adenoviruses in spiked freshwater was 57.1% (range 34.2% - 78.2%). While by nitrocellulose membrane filtration, recovery of adenoviruses in spiked artificial seawater was 35.4% (range 22.5% - 43.8%). 553 out of 1410 samples were positive for one or more pathogenic viruses, which entailed a possible public health risk for bathing.

On the other hand, the efficiency of glass wool is severely affected by the pH of the water, water matrix and type of viruses[85]. By using glass wool filtration, the recovery rate of MS2 coliphage from 5 L dechlorinated tap water was 1.1% (range from 0.3 to 1.8%)[10]. The recovery of feline calicivirus F9 was 0.5%, much lower than that by membrane filter (75%) [86].

2.2.2.2 Zeta Plus 1MDS

The Virosorb 1MDS filter (CUNO Inc.) is an electropositive surface-modified fiberglass-cellulose pleated cartridge filter with a pore size of 0.2 μm (Fig. 8), which has been recommended by the United States Environmental Protection Agency (USEPA) to recover enteric viruses from drinking water[59].

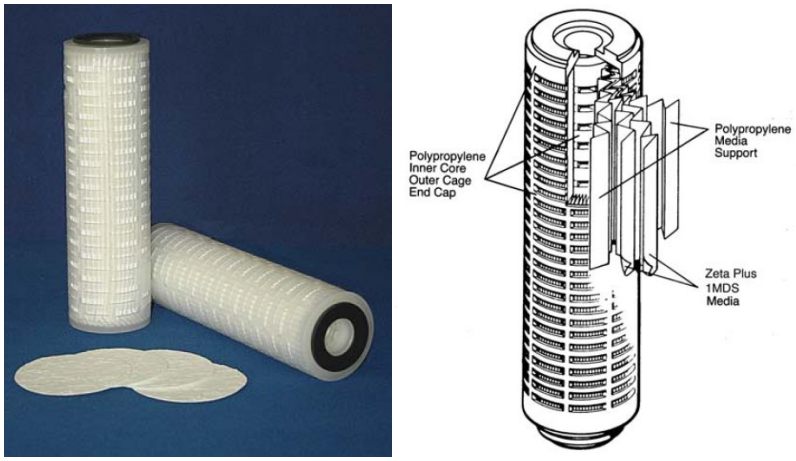

Fig. 8: Image of 1MDS cartridges and disk filters (left) and structure of cartridge filter (right).

Virosorb 1MDS filters are available in both two-layer cartridges and one-layer disk forms (see Table 3). In a study of Polaczyk *et al*[42, 87], the performance of cartridge filters and disk filters were evaluated by simultaneously recovering multiple microbe classes from tap water. 24.5-cm 1MDS cartridge filter and 142-mm 1MDS disk filters were tested. Both MS2 and ΦX174 showed a higher breakthrough in the flat filter experiments (0.02% and 5.6%) than in the cartridge filter experiment (< 0.01% and 3.0%). Cartridge filters could bear a higher flow rate (2700 mL/min) than flat disk filters (160 mL/min). However, mean recoveries for both phages achieved by cartridge filter (32 (± 13)% and 37 (± 26)%) from 20-L samples were significantly lower than those by flat filters (92 (± 10)% and 82 (± 17)%) from 1-L samples. The differences could be caused by differences in set-up and flow regime of the two kinds of filters.

1MDS filters were successfully applied to confirm the presence of pathogenic agents responsible for outbreaks of gastrointestinal illness. Ground water samples of an average volume of 1448 L were filtrated by a 1MDS cartridge filter following the standard concentration method. The eluate from the 1MDS filter was further concentrated by flocculation or polyethylene glycol[88]. The viruses in the concentrates were analyzed by PCR and identified by nucleotide sequencing. 7 of 30 samples were positive for

enteroviruses and one of these samples was positive for the infectious echovirus 18[89]. In another study, viruses from a 2010-L well water sample were concentrated into 80 mL by a 1MDS cartridge filter and reconcentrated by a Celite column[90]. The concentrates were further purified by ultracentrifugation and centrifugal ultrafilters to remove PCR inhibitors. Human caliciviruses were found by PCR detection in concentrates and confirmed by sequencing analysis[91].

The presumed advantage of positively charged filters is that they can handle large volumes of fluid without pretreatment. However, in concentrations of the poliovirus from tap water, the recommended working range for 1MDS is between pH 3.5 and 7.5. The adsorption rate of the poliovirus decreased in tap water of pH higher than 7.5, because the surface charge of 1MDS became negative when the pH increased[92]. Therefore monitoring and adjustment of pH during filtration are also necessary to achieve a high recovery.

2.2.2.3 NanoCeram

NanoCeram (Argonide) is a cheaper alternative to 1MDS. It is a non-woven medium and formed by microglass fibers (~ 0.6 μm in length), which is grafted with nanoalumina fibers (~ 2 nm in diameter and 0.2 - 0.3 μm in length) (Fig. 9). Due to a large external surface area of the nanoalumina fibers (~500 m^2/g), this medium has an extensive surface area for adsorption of viruses. It is available with pore size ranging from 1 to 30 μm. The pore size of media used for drinking water purification is about 2 μm[93].

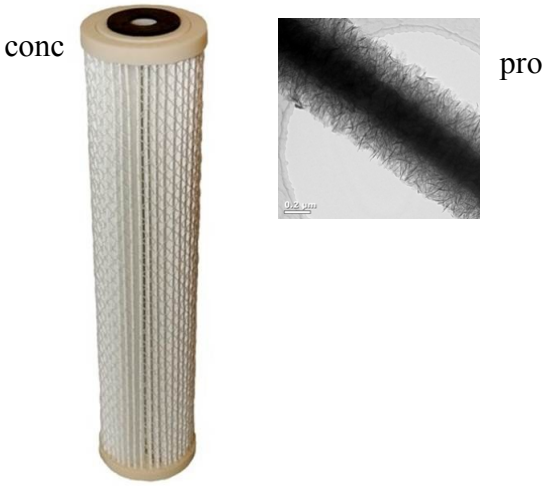

Fig. 9: NanoCeram 0.2 micron pleated filter (a); nanoalumina on microglass fibers (b)[93].

NanoCeram samplers have been used for the concentration of viruses spiked in tap water[94] (see Table 3). 20 L of seeded tap water was processed at a flow rate of 2.5 L/min. The adsorbed viruses were eluted with 420 mL elution buffer composed of 1.0% sodium polyphosphate solution and 0.05 M glycine. Both cell culture and PCR assay were applied for quantification. The recovery efficiencies of viruses were 69% for poliovirus 1, 134% for echovirus 1, 72% for coxsackievirus B5, 39% for adenovirus 2 and 86% for MS2 coliphage. Afterwards, the volume of eluate was further reduced to 3.3 mL by centrifugal ultrafiltration (Centricon Plus-70). The overall recovery efficiencies of these two-step concentrations were 66% for poliovirus 1, 83% for echovirus 1, 77% for coxsackievirus B5, 14% for adenovirus 2, and 56% for MS2 coliphage.

Another study reported that 84% of polioviruses seeded in 100-liter tap water samples were retained on NanoCeram filters[95], which was comparable to the reported adsorption rate by 1MDS (62 - 79%)[96]. The recovery efficiencies of poliovirus, coxsackievirus B5, and echovirus 7 by NanoCeram were 54%, 27%, and 32%, respectively. Differences of viruses in electronegativity were supposed to cause various binding efficiencies and recoveries. However, virus recovery was not intensively influenced by pH (6 - 9.5) and flow rate (5.5 L/min - 20 L/min). Based on the established

protocol, poliovirus recovery on NanoCeram filters (51 (± 26)% from tap water and 38 (± 35)% from river water) were similar to that on 1MDS (67 (± 6)% from tap water and 36 (± 21)% from river water). Recoveries of Norwalk virus by NanoCeram filters (3.6 (± 0.6)% from tap water and 12.2 (± 16.3)% from river water) were higher than that by 1MDS (1.2 (± 1.4)% from tap water and 0.4 (± 1.8)% from river water). As they followed the inhibitor removal process described by Fout *et al*.[97], inhibitors for PCR detection accumulated during filtration procedures were effectively removed.

In an evaluation of the performance of NanoCeram for filtration of seawater, norovirus and mail-specific coliphage (Q_β) were recovered from 40 L of seawater[98] with high efficiencies (> 96%). While 1MDS is rarely used for the concentration of viruses from seawater, as salts in the sample (higher than 0.01 M) interfere with the adsorption of viruses onto 1MDS under neutral and acidic conditions[99]. Although both 1MDS and NanoCeram are normally classified to be positively charged filters, the high recovery rate by NanoCeram was attributed to its unique surface properties. There are not only electrostatic interactions, which would be disturbed by the presence of salts in seawater, but also hydrophobic interactions, which are enhanced by high concentration of salts in water, contributing to the efficient adsorption of viruses. In the same research, adenoviruses were recovered with much lower efficiency (< 3%) by NanoCeram from seawater. This fact implies that physical entrapment is another viral retention mechanism.

2.2.2.4 HA

Viruses could be retained on negatively-charged surfaces by the adsorption-elution method based on flat-disk cellulose nitrate HA membranes (Millipore)[100, 101]. The pore size (0.45 μm) is much larger than the size of viruses. The adsorption rate could be enhanced either by altering the surface charge with addition of cations (Al^{3+} or Mg^{2+})[99, 102-104] or adjusting the pH of samples near the isoelectric point of viruses (see Table 3).

For concentrating polioviruses from 1 L artificial seawater, samples without

pretreatment were filtrated by HA filters with a diameter of 47 mm at a flow rate of 100 mL/min. Filters with retained viruses were rinsed with H_2SO_4 before being eluted by 5 mL NaOH[102]. A recovery rate of 61% was achieved by this method, which was much higher than that with a positively charged 1MDS membrane filter (6%) tested in parallel. However, 1MDS recovered almost all polioviruses seeded in pure water. Multivalent salts in seawater and the acid rinse step before elution enhanced the adsorption of viruses onto the negatively charged filter (HA), but not onto the positively charged one (1MDS). This fact is consistent with the strategy to acidify the sample to low pH when using a negatively charged filter[102]. The developed method was further used in a weekly survey of noroviruses and enteric adenoviruses in river water during a one year-long time period. 1-L river water samples were concentrated to 1 mL by a HA filter and centrifugal concentrator. Real-time PCR was used for detection. 54%, 63% and 44% of 52 samples were positive for Noroviruses GI, GII and adenoviruses, respectively. The number of acute infectious gastroenteritis cases in the upper river basin showed a strong correlation with detected virus concentrations in the lower river basin[105].

In the 14-month survey of tap water in Japan, norovirus was the microbial target. A large volume of adequately treated tap water (100 - 532 L) was continuously filtrated by two HA filters with diameters of 47 mm and 293 mm. Filters were pretreated with $AlCl_3$ solution and rinsed with H_2SO_4 before elution to remove aluminum ions and other inhibitory substances. TaqMan PCR was used for detection. Although tap water samples met the standard of drinking water in Japan (containing high enough concentrations of chlorine), noroviruses of genotype 1 and genotype 2 were found in 4.1% and 7.1% of 98 samples. The frequency of occurrence for detectable noroviruses in winter was higher than in summer, which was consistent with epidemiological reports[106].

Water samples can be processed at a higher flow rate without pretreatment by positively charged cartridge filter (e.g. 1MDS and NanoCeram), while the negatively charged membrane (e.g. HA) is more cost-effective and easy to use. Acidification or

addition of cations is needed to ensure the high efficiency by negatively charged membranes. This could be facilitated by inserting an in-line fluid proportioner for pH and ionic strength adjustment[107, 108]. Processing at high flow rate requires an increase in the area and volume of the filter, which also leads to a large volume of eluate. A second or third step of further concentration is needed before quantification by molecular biological, antibody-based or cultivation based methods. CeUF, flocculation, immunoseparation and other techniques are used to reduce the volume of final concentrates (see Table 3).

2.2.3 Other concentration methods

Flocculation is another method based on the sorption behavior of viruses, which recovers viruses by adsorption of viruses to flocculants, such as aluminium hydroxide[109], iron oxide, ammonium sulphate[110], skimmed milk[194], casein[111] or beef extract[85, 88]. The viruses in solution are adsorbed to flocs, which could be separated by centrifugation. For flocculation with beef extract, flocs could be formed either by acidification or PEG, which was compared in the study by Borchardt *et al*[88]. In the acidification protocol, 1-L beef extract solutions were adjusted to pH 3.5 to trigger flocculation. The mixture was stirred for 30 min and centrifuged at 2,500 × g for 15 min. The pellet was dissolved in 30 ml of 0.15 M Na_2HPO_4 and centrifuged again for 10 min before analysis. Flocculation with PEG was accomplished by adjusting the beef extract pH to 7.5 with HCl, then flocculated with polyethylene glycol 8000 (8% [wt/vol]) and NaCl. After overnight incubation, the mixture was centrifuged at 4,200 × g for 45 min. The pellet was resuspended in 3 to 6 ml of 0.15 M Na_2HPO_4. The PEG method yielded a smaller final concentrated sample volume and higher concentration factor than the acidification method.

Alternative concentration methods for viruses in water are, for example hydro-extraction[112], immunofiltration[55], immunomagnetic separation[56], freeze-drying[113].

In a hydro-extraction method, water samples are filled in a dialysis bag, exposing it

to hygroscopic solid (such as polyethylene glycol (PEG) or sucrose) and leaving it at 4 °C for several hours[112]. Water is drawn through the semipermeable membrane by the solid, while viruses and other macro solutes remain in the bag. The shortcomings are the limited sample volumes (up to 1 L) and co-concentrated component from matrix, which could contain inhibitors for following analysis.

Immunofiltration and immunomagnetic separation are used for the concentration or purification of human enteric viruses. The advantage of the antibody-based capture is the high specificity. The main purpose is to remove the inhibitors for following PCR detection. Due to the high cost of antibodies, the sample volume is limited to some millilitres[114] or microlitres[115]. Therefore, these immunological methods were not applied to raw water directly, but to purified concentrates after several concentration or purification steps.

Freeze-drying is a technique to forcibly remove water from samples. Higher amount of rotavirus were recovered by freeze-drying method (29 - 45%) than by PEG precipitation method (16 - 17%)[116], which was important for detection of rotavirus by ELISA. Limited sample volume (7.5 mL) and relative long waiting time (4 h) made it more suitable to combine with a primary concentration method.

Table 3: Typical techniques for concentrating virus from large volume of water

Primary concn. method	2ary concn. method	3ary concn. method	Water type	Initial volume (L)	Final volume (mL)	Volumetric concn. factor	Recovery	Target viruses	Cost	Time
UF[64]	UF		Storm	100	50 - 100	10^3	9.7% - 98%	Human enterovirus Adenovirus	High	2.5 h
UF[65]	Beef extract–celite		Tap	100	35	2.8×10^3	65% - 99%	Poliovirus	High	2 h
UF[63]	CeUF		Tap	100	1 - 6	$1 \times 10^5 \sim 2 \times 10^4$	60% - 100%	Echovirus 1 MS2, ΦX174	High	2 h
Glass wool[80]	FL**		Tap/Well	10 - 1500	2	$7.5 \times 10^5 \sim 5 \times 10^3$	14% - 70%	Poliovirus, Coxsackievirus B5, Echovirus 18, Adenovirus 41, Norovirus	Low	Overnight
Glass wool[24]	FL**		Recreational	10	10	10^3	22% - 78%	Adenoviruses Noroviruses	Low	1 h
HA [106]	HA	CeUF	Tap	532 - 100	1	$5.9 \times 10^5 \sim 1.1 \times 10^5$	Unknown	Noroviruses	Medium	Unknown
HA[100]	CeUF		River	1	1	10^3	Unknown	Adenoviruses Noroviruses		
HA[97]	CeUF		Sea	1	5	2×10^2	38% - 89%	Polioviruses		

Primary concn. method	2ary concn. method	3ary concn. method	Water type	Initial volume (L)	Final volume (mL)	Volumetric concn. factor	Recovery	Target viruses	Cost	Time
1MDS[83]	FL** / PEG		Ground	1500	6~30	2.5×10^5 – 5×10^4	Unknown	Enteroviruses	High	Overnight
1MDS[86]	Celite column		Well	2010	80	2.5×10^4	Unknown	Human caliciviruses		Overnight
1MDS[82]			Tap	20	Unknown	Unknown	30%	MS2, ΦX174		0.5 h
NanoCeram[93]			Sea	40	Unknown	Unknown	3% - 96%	Norovirus, Qβ, Adenovirus		0.5 h
NanoCeram[89]	CeUF		Tap	20	3	6.7×10^3	14% - 83%	Poliovirus, Coxsackievirus B5, Echovirus 7	Medium	1 h

***HA filter with diameter of 293 mm for the primary step; with diameter of 47 mm for the secondary step.**

****FL: flocculation**

2.3 Monolithic column for concentrating viruses from water

2.3.1 Brief introduction about monolithic column

Research on monoliths as a separation support began in the late 1980s[117]. In comparison with a traditional packed column, a monolith is a single block of porous polymer[118]. The common characteristics of various monoliths are their interconnected pores and high porosity (see Fig. 10)[119, 120]. The pore size of packed columns is between 10 and 100 nm, while the pore size of a macroporous monolith is bigger than 1000 nm. Large pores enable macromolecules to more easily access the surface[121, 122]. This feature offers a monolithic column higher capacity for binding macromolecules even when its inner surface is lower than that of a packed column[123, 124]. For purification of medium-sized proteins (less than 7 nm), conventional stationary phases with a narrow pore size distribution were much better than monoliths (20 – 30 g/L for BSA)[122]. However, for adenovirus with a diameter of about 100 nm, the predicted binding capacity on monolith was about 50 g/L (1.8×10^{14} virus particle/mL), which is 4 times greater than that observed on a packed column. The porosity of monoliths, which is defined as the ratio of the volume of void-space to the total volume of material, is in the range of 0.25 - 0.73, while that for packed columns is limited to 0.4. The increase of porosity from 0.4 to 0.9 results in a 410-fold backpressure decrease[125]. High flow rate with low backpressure is the precondition to process large scale samples[126,127].

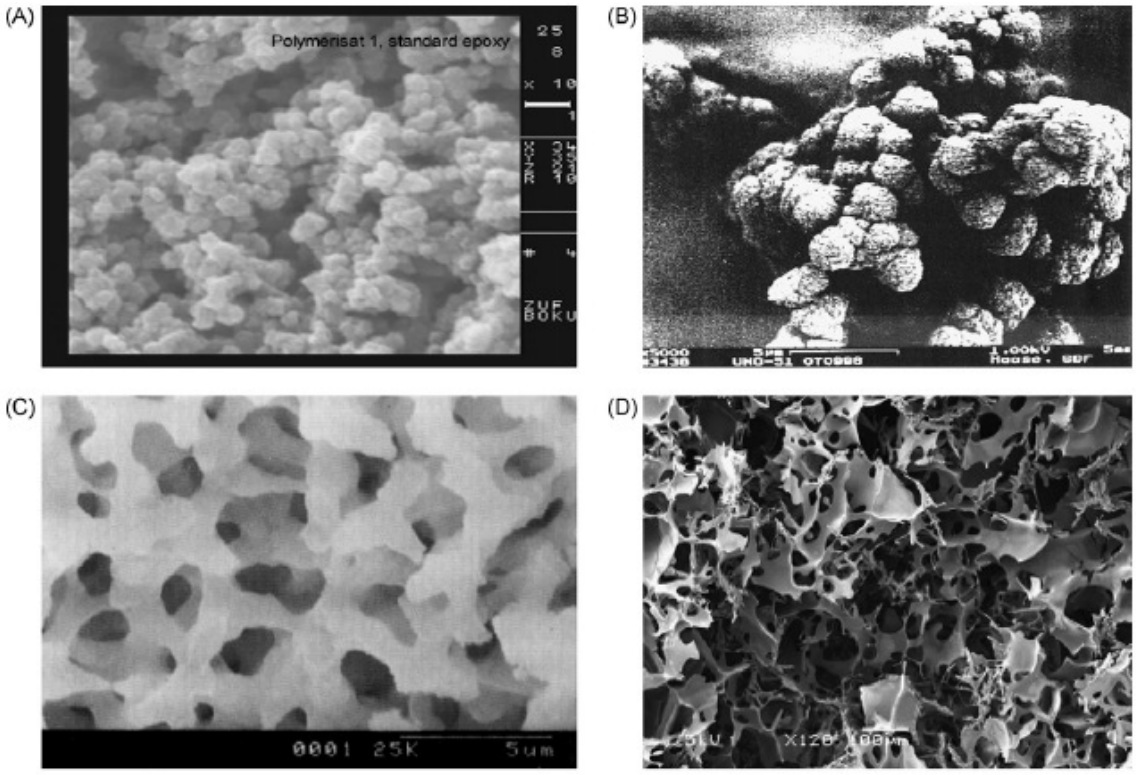

Fig. 10: Electron micrographs of monoliths of (A) polymethacrylate, known as CIM, (B) polyacrylamide UNO column from Bio-Rad, (C) silica-based monoliths from Merck, (D) cryogel[123]

Properties of monoliths, e.g. porosity, pore size, surface area, are a direct consequence of their preparation method. Monoliths can be prepared from inorganic materials, natural polymers or synthetic polymers[123, 128-132] (shown in Fig. 10). Various monomers are used in polymerization, e.g. styrenedivinylbenzene[133, 134], methacrylates[123, 135], vinyl acetate[136]. The desired surface chemistry for special usage could be obtained by selection of monomers or surface modification. For example, epoxy groups are easily modified to various functional groups. Monomers, e.g. glycidyl methacrylate (GMA), containing epoxy groups are widely used in preparation of monoliths[137, 138]. The epoxy groups can be ring-opened by hydrolyzation or secondary amines, which are essential for following surface modifications, such as immobilization of the ligand or antibodies[139-141]. As polymerization is carried out in a molding process[141-143], monoliths could be prepared

in any demanded shape using molds of various sizes, from microfluidic channels to large flat sheets[144-147]. The bottleneck in preparing large volume monoliths is that heat is problematic because of the highly exothermal polymerization reaction. Increased heat leads to an inhomogeneous structure. The shrinkage of monolith in different matrices is unavoidable. In preparation of epoxy-based monoliths, the shrinkage could be decreased from 28% to 5% by using toluene/MTBE as porogen instead of MTBE/dioxane[141]. Due to the shrinkage of monoliths, the 'wall channel' problem in a plastic housing results in the liquid bypassing the monoliths[123]. To solve this problem, most monoliths were prepared with bonding to the glass wall or sealing tightly to the wall. Therefore the preparation procedure of an individual column is labor-intensive and may lead to possible reproducibility problems, which are the disadvantages of monolith columns.

2.3.2 Applications

In most cases, monolithic columns are used for analytical purposes to separate organic compounds or large biomolecules such as proteins, DNA or viruses. Due to its unique structure, a monolith column offers a fast analytical method for biomolecules having high molecular mass and low diffusivity. A Protein A immobilized monolithic disk was used for analysis of immunoglobulin G (IgG). The quantification of IgG was accomplished within 5 min from purified samples as well as crude cell supernatant. The linear concentration range was found to be from 4 to 1000 μg/mL[148]. Similarly, peptide immobilized monolithic columns were selected for the analysis of IgM. LODs for IgM in buffer and in cell culture supernatant were 51.69 μg/mL and 48.40 μg/mL, respectively. The analysis could be done within 2 min[149].

Separation of large molecules by monolithic columns is enhanced by the unique structure. But because of the restriction in preparation, applications with a large volume monolith as adsorbent are relatively few. For preparative purpose, the majority production procedures are based on polymethacrylate monoliths. Commercially available monoliths are called Convective Interaction Media (CIM; BIA Separations, Ljubljana,

Slovenia). By different surface treatment technologies, the monoliths are also modified as ion exchangers or media for hydrophobic interaction chromatography. Purification of proteins is the standard application of monolithic columns. Monolithic column were used for separation of polyclonal IgM, IgG, enzymes and other proteins[150-152]. The processing of plasmid DNA by CIM monoliths could be done within 0.6 h, with a capacity of 6.3 g/L[153]. The productivity of plasmid DNA, i.e. the amount of pDNA that can be processed in a certain time range with a certain volume of chromatographic support, could be 8.7 g pDNA/(L·h), which is 15 times higher than the conventional method based on packed bed. In gene therapy related fields, viruses and virus-like particles are widely used as delivery vehicles. Separation of viruses from cell culture matrix requires sophisticated purification procedures. Purification of viruses by monolith columns was studied by Tomato mosaic virus (ToMV) as a model, which is nonpathogenic for humans[154]. The separation was based on a CIM disk with a diameter of 12 mm and a length of 3 mm. The best recovery was achieved by using sodium acetate as loading buffer and high salt buffer for elution with a flow rate of 6 mL/min. Compared with the traditional procedure for purification, the isolation procedure was reduced from 5 days to 2 h. The viral purity achieved by this method was up to 90%. Purification methods based on monoliths were also evaluated by using T4 bacteriophages and mumps viruses as models[155, 156].

Because our aim is to concentrate viruses from water, we were especially concerned with the removal of viruses and virus-like particles from a large volume water matrix. Taking the advantages in fast and efficient separation of macromolecules, monoliths are used as adsorbents for concentration or separation of microorganisms. Anion- and cation-exchange CIM disks have been tested for concentrating rotaviruses from tap water[157]. Rotaviruses bind to anion-exchangers (CIM QA) efficiently, due to their negative charge in a neutral environment. Rotaviruses spiked in 1 L tap water were loaded on an 8-mL monolithic column at a flow rate of 100 mL/min and eluted by 15-mL elution buffer containing 1 M NaCl. RT-PCR was used for detection. Almost all seeded

viruses were recovered, resulting in concentration factors ranging from 55 to 65. For concentrating viruses in river water, concentration factors ranging from 20 to 40 were achieved.

A similar method based on CIM QA is used for concentration of the hepatitis A virus (HAV) and the feline calicivirus (FCV, a surrogate of the norovirus)[158]. In this method, centrifugal ultrafiltration was used as a secondary concentration step in order to further reduce the sample volume. After two concentration steps, the sample volume could be reduced from 15 mL to 150 μL. The result of PCR showed that 37.5% seeded HAV was recovered, which was much better compared with the methods based on positively charged membranes. However, in the case of FCV, a positively charged membrane followed by direct lysis of viruses on a membrane, achieved higher recovery than the CIM based method. But better performance could be expected from virus concentration by CIM when using the same RNA extraction method.

Likewise, a CIM QA with a diameter of 12 mm and length of 3 mm was used for concentration of the plant virus (tomato mosaic virus)[159]. The monolithic disk was placed in a special housing and connected to an HPLC system. 0.5 L tap water spiked with virus was loaded on the column at a flow rate of 8 mL/min. The bound viruses were eluted by a high salt elution buffer containing 20 mM sodium acetate and 1.5 M NaCl at flow rate of 6 mL/min. Following such a concentrating step, the detection limit by ELISA was decreased and a concentration factor of about 500 was achieved.

In concentration of the potato spindle tuber viroid (PSTVd)[160], which is a circular RNA molecule with about 360 nucleotides, the C4 monolithic column with butyl functional groups exhibited higher efficiency than the diethylamine (DEAE) monolithic column. The difference was assumed to be the weaker binding on C4 matrix by hydrophobic interaction than that on DEAE by electrostatic interaction, which is easier to be eluted. 70% of PSTVd seeded in 1-L water sample could be recovered by 1 mL C4 monolithic tube with a concentration factor of two orders of magnitude.

2.3.3 Monolithic columns developed at IWC

A flow-through concentration technique, called monolithic adsorption filtration (MAF), was developed at the Chair of Analytical Chemistry, at Technische Universität München[141, 161]. A macroporous epoxy-based monolithic column was prepared by direct polymerization in a small glass column to achieve a covalent binding to the glass surface. Polymyxin B as affinity ligand was immobilized on the monoliths to concentrate *E.coli* with high efficiency[141]. 97% of seeded bacteria were recovered in 200 μL carbonate buffer, which is compatible for biological detection. An antibody-immobilized column was used for enrichment of *Staphylococcus aureus*[100], which is an important food contaminant. The detection limit was decreased to 42 *S. aureus*/mL after concentration.

Although high efficiencies were achieved by MAF, a series of modification steps are still needed and the target analyte is limited to the used specific affinity receptors. The objective of this work is to develop a low-cost but highly efficient monolithic column to simultaneously concentrate viruses in water. To simulate the surface property of glass wool, the epoxy-based monolithic column was hydrolyzed in sulfuric acid. As mentioned above, electrostatic and van der Waal interaction are not the only factors to control the adsorption behavior of biotic particles, hydrogen bonding should be accounted for as well. As the polymer chains contain a lot of polarized electron rich alkyl oxygen atoms with lone pairs of electrons[162], which can serve as good hydrogen bond acceptors, hydrogen bonding could be the dominant factor for capturing viruses[163]. On the other hand, the protein capsid of a virus has many amino acid functional groups, which can make the surface of a virus to be acidic or basic depending on the pH value of the environment. It is known that the unshielded protons from carboxylic acid groups and ammonium groups have high affinities toward electron rich lone pairs. Thus, we can expect strong hydrogen bonding between viruses and a monolithic column under acidic conditions. The pH-dependent adsorption-elution mechanism is illustrated in Fig. 11.

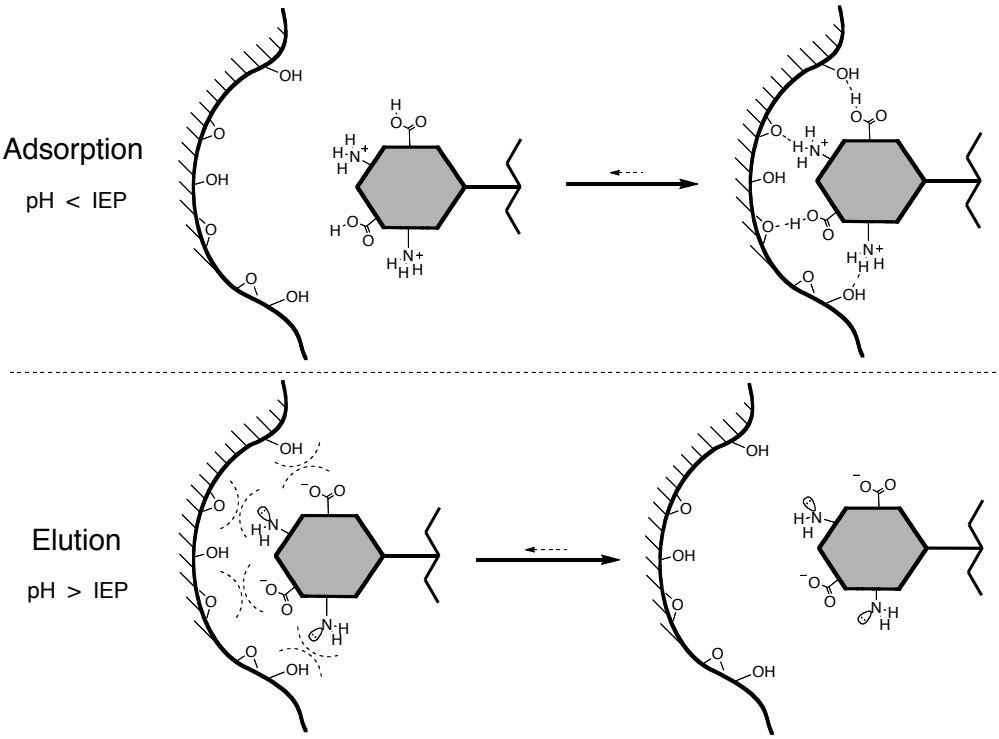

Fig. 11: pH-dependent hydrogen bond involved adsorption-elution mechanism between monolithic column and viruses.

As illustrated in Fig. 11, in the adsorption stage, amino acids on the surface of a virus are protonated due to the low pH value of the aqueous environment. Both carboxylic acid and ammonium groups are good hydrogen bond donors compared with a water molecule. As a result, the virus can be tightly bound onto the surface of the column through the hydrogen bond between alkyl oxygen and protonated amino acid groups of the virus. During the elution stage, the pH is adjusted to a higher value than the IEP of the virus. Both carboxylic acid and ammonium groups on the surface of the virus will be deprotonated. Consequently, the hydrogen bonding formed under acidic condition is dramatically diminished, and the interaction forces between the surface of the column and that of virus become repulsive. Although the hydroxyl group on the surface of the column could form a week hydrogen bond with the deprotonated carboxylic group and amino group as well, it is not competitive with water molecules[162]. As a result, the viruses are released back into the elution buffer under basic conditions.

2.4 Analytical methods for water-borne viruses identification

2.4.1 Cell culture assay

Cell culture is the most widely used method for detection of viruses prior to the development of molecular biological techniques[1]. It is based on the infectiosity of viruses and done by inoculation of cell cultures with samples containing the virus[52]. Therefore, it is still the most reliable method to determine the infectivity of viruses in an environmental sample, which is crucial for risk assessment. However, the cell culture assay is laborious and time-consuming. Days or weeks are required to obtain results. Furthermore, many enteric viruses, e.g. noroviruses or rotavirus, are not able to be cultured on established cell lines[106].

Titers of phages are quantitatively determined by the plaque assay, which was first published by Adams[164]. A certain volume of sample containing phages is mixed with molten agar and the host bacterium. Then the mixture is poured on top of bottom agar in a petri dish. During incubation, phages adsorb to the host, infect and lyse the cells. Then in the following days, plaques within the lawn of bacteria can be observed by the naked eye (as shown in Fig. 12). It is assumed that one plaque is the progeny of one infectious unit. The number of phages in a sample is counted in plaque-forming units (PFU)[10, 52].

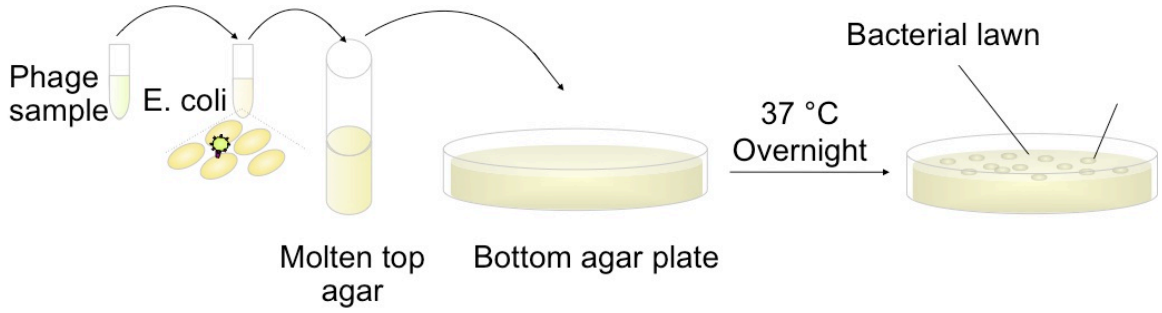

Fig. 12: Illustration of double layer plaque assay for detection of bacteriophage MS2.

2.4.2 Molecular biological methods

2.4.2.1 Polymerase chain reaction (PCR)

To shorten the time needed for detection, new techniques have been developed. PCR-based viral assays are the most important molecular biological technique. Analysis of a targeted organism by PCR is based on the detection of a special region of the viral genome, which is highly specific and conserved for the target viruses. The target DNA fragment is exponentially amplified during PCR. PCR contains several repeated heating and cooling cycles. In each cycle, there are three discrete temperature steps, namely the denaturation step, annealing step and elongation step (Fig. 13). Denaturation steps result in DNA melting to form single-stranded DNA. In annealing steps, primers anneal to the single-stranded DNA and the polymerase binds to the primer-template hybrid. Then the elongation of the primers begin resulting in a new DNA strand complementary to the DNA template. If the analyte is an RNA virus, the RNA of interest needs to be reverse transcribed into complementary DNA (cDNA) before PCR.

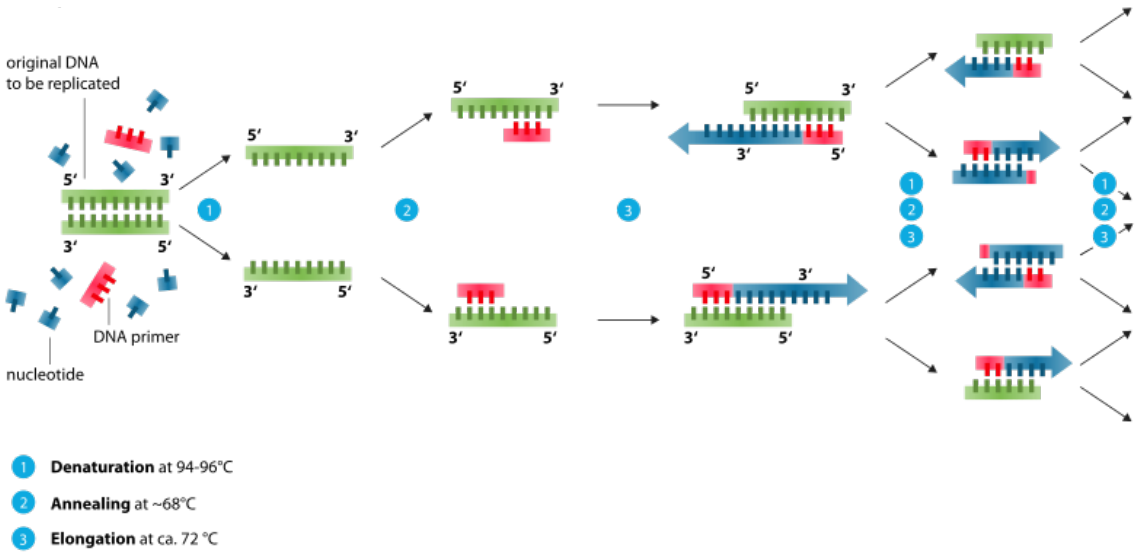

Fig. 13: Illustration of a thermal cycle in PCR[165]

Compared to cell culture assay, PCR is rapid and more specific. The specificity of a PCR assay could be optimized by well-designed primers, which can target the whole virus class, a single type of virus or virus genotypes[166, 167]. PCR techniques have been used for detecting various enteric viruses, especially those who are not cultivable in established cell lines. However, the sensitivity of a PCR assay is much easier to be influenced by contaminations in environmental samples, such as humic acids, heavy metals, complex polysaccharides or proteins[168, 169]. Inhibitors could interact with the DNA or bind to DNA polymerases[170]. Several manipulations have been carried out to reduce the inhibitory effect, including additional purifying steps by resin or column and modification of nucleic acid extraction methods.

Table 4: Comparison of different PCR methods for detection of viruses in water

Method	**Advantages**	**Disadvantages**
PCR	Rapid; increased sensitivity and specificity compared to cell culture	Presence or absence only (nonquantitative); inhibitors present in environmental samples may interfere with PCR amplification; infectivity cannot be determined
Nested PCR	Increased sensitivity compared to conventional PCR; can replace PCR confirmation steps, such as hybridization	Potential risk of carryover contamination when transferring PCR products
Multiplex PCR	Several types, groups or species of viruses can be detected in a single reaction; saves time and cost	Difficult to achieve equal sensitivity for all targeted virus species, groups, or types; may produce nonspecific amplification in environmental samples
Real-time PCR	Provides quantitative data; confirmation of PCR products is not required (saves time); can be done in a closed system, which reduces risk of contamination compared to nested PCR	Expensive equipment; occasionally less sensitive than conventional PCR and nested PCR
ICC-PCR	Improves detection of infectious viral pathogens compared to conventional cell culture; detects viruses that do not produce CPE in cell culture; provides results in half the time required for conventional cell culture	Less time-efficient and more costly than direct PCR detection; carryover detection of DNA of inactivated viruses inoculated onto cultured cells is possible

To improve the sensitivity of PCR and to quantify the numbers of genome detected, methods with some modifications to traditional PCR have been developed, such as nested PCR, multiplex PCR, integrated cell culture PCR (ICC-PCR), real-time PCR (Table 4). In nested PCR, internal primers were included. While in ICC-PCR, samples were cultured in cell line at first but measured by PCR before CPE is noted. It was shown that nested PCR and ICC-PCR have increased sensitivity but also some drawbacks, like carryover contaminations or reduced time-efficiency. Real-time PCR offers the possibility to quantify the genome copies of the target virus during amplification by including a fluorescent dye, which will specifically bind to an amplified DNA or cDNA strain. Alternative fluorochrome-tagged probes are used which are only detectable if they are bound to the amplified target region. As identification of the sequence by agarose gel electrophoresis is omitted, the time needed for one assay is dramatically reduced. The qPCR were used to detect viruses in aquatic environments, such as adenoviruses[17, 18], noroviruses[19] and enteroviruses[20] (Table 5). In general, the sensitivity of qPCR is about 1 - 10 gene copy (GC) per PCR reaction. The volume of DNA template used in a single PCR reaction is mostly limited to 10 μL. However, concentrations of some viruses are lower than the sensitivity of qPCR even in raw sewage. The viruses in surface water, which are transmitted from treated sewage, are even lower. But the infectious dose of viruses is also very low. Therefore, in order to have a rapid analysis method by PCR for the viruses in source and finished drinking water, a previous enrichment step is needed.

Table 5: qPCR for viruses in water

Viruses	Gene amplified	LOD GC per reaction	Positive %	Concentration found / GC L^{-1}	Assay
Adenovirus	Fiber gene[17]	10 / 5 µL	61% river water	10^3 - 10^5 / L	Taqman
	Hexon gene[18]	10 / 12 µL	100% sewage	10^6 - 10^8 / L	Taqman
			100% effluent	10^3 / L	
Norovirus	Polymerase[19]	20 / 5 µL	16% sewage	$\leq 10^7$ / L	SYBR Green
			7% effluent	$\leq 10^5$ / L	
			11% marine	$\leq 10^4$ / L	
			12% river	$\leq 10^3$ / L	
Enterovirus	5' noncoding region[20]	10 / 50 µL	3% marine/ lake	10^5 - 10^7 / L	RT-qPCR
			100% river	10^6 / L	
			100% sewage	10^8 / L	
			100% effluent	$\leq 10^7$ / L	

DNA microarrays are based on the microdispension of nucleic acids to immobilize them on a surface within a defined pattern. A combination of microarray techniques and PCR amplification offers a chance for multiplex detection of various species. DNA microarrays for multiplex detection of viruses have been reported. The eBiochip enables the simultaneous detection of the Epstein–Barr virus, herpes simplex virus, and cytomegalovirus in blood samples. The ViroChip is immobilized with a high-density 70-mer oligonucleotide and able to target all viral families. Most methods were used to detect pathogens in clinical samples. To make it more practical in analysis real water samples, previous enrichment methods are needed to increase the sensitivity. In our group, an oligonucleotide microarray was developed for the quantification of MS2, ΦX174, and adenoviruses on the multiplex analysis platform MCR 3[171]. The efficiency and selectivity

of DNA hybridization resulted in higher signal intensities and lower cross-reactivities of PCR products from other viruses. LODs were calculated to be 6.6×10^5 GU/mL for MS2, 5.3×10^3 GU/mL for ΦX174, and 1.5×10^2 GU/mL for adenoviruses, respectively, similar to results by quantitative PCR. The total analysis time from nucleic acid extraction to microarray detection was within 4 - 4.5 h. For detection of naturally occurring viruses in water matrices, effective concentration methods are needed that are combined with microarray techniques to achieve necessary sensitivities.

2.4.2.2 Sequencing

Sequencing is used to determine the order of nucleotides in DNA or RNA, which emerged in the 1970s. The first-generation sequencing, e.g. automated Sanger sequencing, was based on chain-terminating inhibitors[172]. The techniques of next generation sequencing (NGS) are low cost but high-throughput. Different strategies are used for clonal amplification and sequencing, including cyclic reversible termination, sequencing by ligation, single-nucleotide addition (pyrosequencing) and real-time sequencing[173].

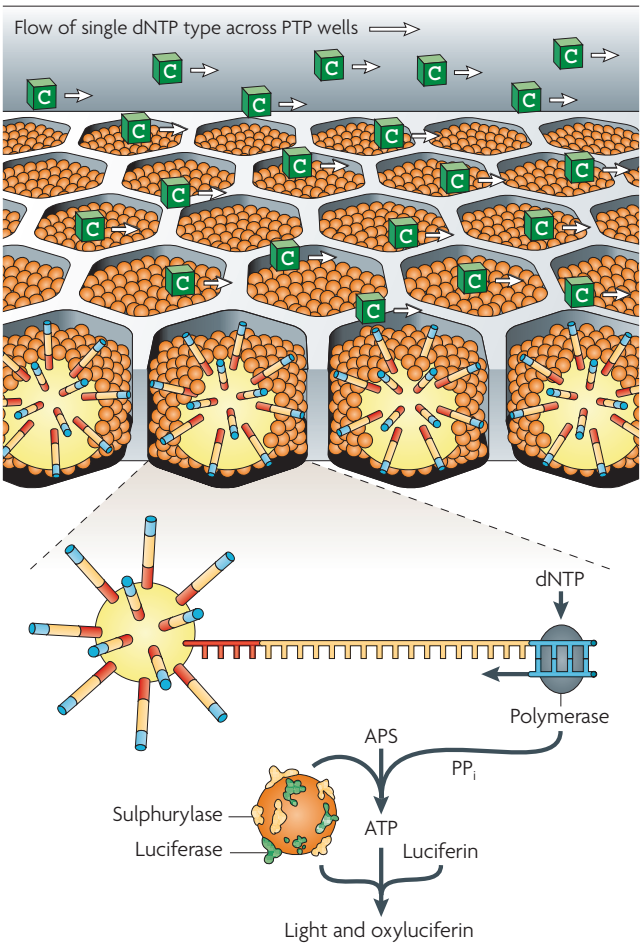

Fig. 14: Pyrosequencing using Roche/454's Titanium platform. Reprinted from ref [173]

Pyrosequencing is used in our research. Roche/454 is the commercially available platform (as shown in Fig. 14). The DNA is firstly amplified by emulsion PCR. Amplified products are bound to a sequencing primer immobilized on a bead and separated in to wells on the picotitre plate, which is large enough for a single bead. 2'-deoxyribonucleoside triphosphates (dNTPs) are added into wells in a predetermined sequential order. When the right dNTP is incorporated, pyrophosphate (PPi) is released. PPi will react with adenosine 5' phosphosulfate (APS) into adenosine triphosphate (ATP) by ATP-sulfurylase. ATP-depending conversion of luciferin by luciferase generates light, which is recorded. The analysis of output data files from sequencing is challenging,

including mapping the reads from NGS to reference or assemblies. These could be done by software based on certain algorithms. These approaches are used in virology for sequencing full viral genomes, seeking out resistance profiles to drugs and discovering of new pathogenic viruses[88, 174].

In PCR and microarray methods, the target analytes could only be microorganisms with well-known sequence information. But sequencing is not limited to this, it is also able to detect novel pathogens. Sequencing was used to identify pathogens responsible for outbreaks, such as enteroviruses and infectious echovirus 18 in ground water[89] or human caliciviruses in well water[91]. In the metagenomic detection of environmental samples, it was able to identify sequences of clostridium, mycobacterium, parechovirus, coronavirus, adenovirus, aichi and herpes virus in wastewater biosolid[175, 176], bacteriophages, plant viruses and invertebrate picornaviruses in reclaimed water[177]. But most of the viral sequences have no significant similarity with known sequences, which indicates towards the high abundance of unknown potential viruses[178].

The sample volumes in molecular biological assays are limited to milliliter range. However the concentration of viruses in source and finished drinking water are too low to be detected directly. Therefore, methods able to rapidly concentrate and purify various viruses are important for rapid, multiplex, high-throughput detection, which are essential for water quality and for health risks assessment.

Results and Discussion

3 Results and discussion

3.1 Development of concentration method based on monolithic adsorption filtration (MAF) and its application in combination with crossflow ultrafiltration (CF-UF)

In most cases, glass wool is the first choice for concentrating viruses, because it is cost-effective and simple to use. Oiled sodocalcic glass wool was packed into housings and used as columns. However, glass wool filters were rarely commercially available. The preparation by hand could result in loose structure and highly variable performances as reported in many publications[28, 85]. Lack of reproducibility is the restriction for it to be a reliable concentration method. Monoliths with a defined network of pores could overcome this problem. The first aim of our research was to replace glass wool filtration by MAF.

The monolithic columns used in our research were prepared by self-polymerization of polyglycerol-3-glycidyl ether (R9)[141]. Compared to polymethacrylate-based or polyacrylamide-based monolithic column, the preparation of epoxide monolith is very simple and fast. The epoxy-based monolithic polymer is optimal for surface functionalization and prevents unspecific matrix effects. The structure with macropores is more stable to pressure than monolithic cryogels with similar surface areas and also results in low backpressure at high flow rate. The interconnected channels and the highly porous structure of the epoxy-based monolithic columns enable a good performance in separation of macromolecules.

In comparison with glass wool, the defined network of pores provides the potential for high reproducibility and low volumes of eluates. Therefore high concentration factors can be achieved. Additionally, the combination with bioanalytical detection methods is promising. The combination of MAF with qPCR or cell cultivation assays was applied as

a first principle study to quantify viruses in water.

3.1.1 MAF column for water samples < 100 mL

Figure 15 describes the reaction mechanism of polyglycerol-3-glycidyl ether. The Lewis acid BF_3 was used as initiator to activate the epoxy groups of the monomer for a nucleophilic attack.

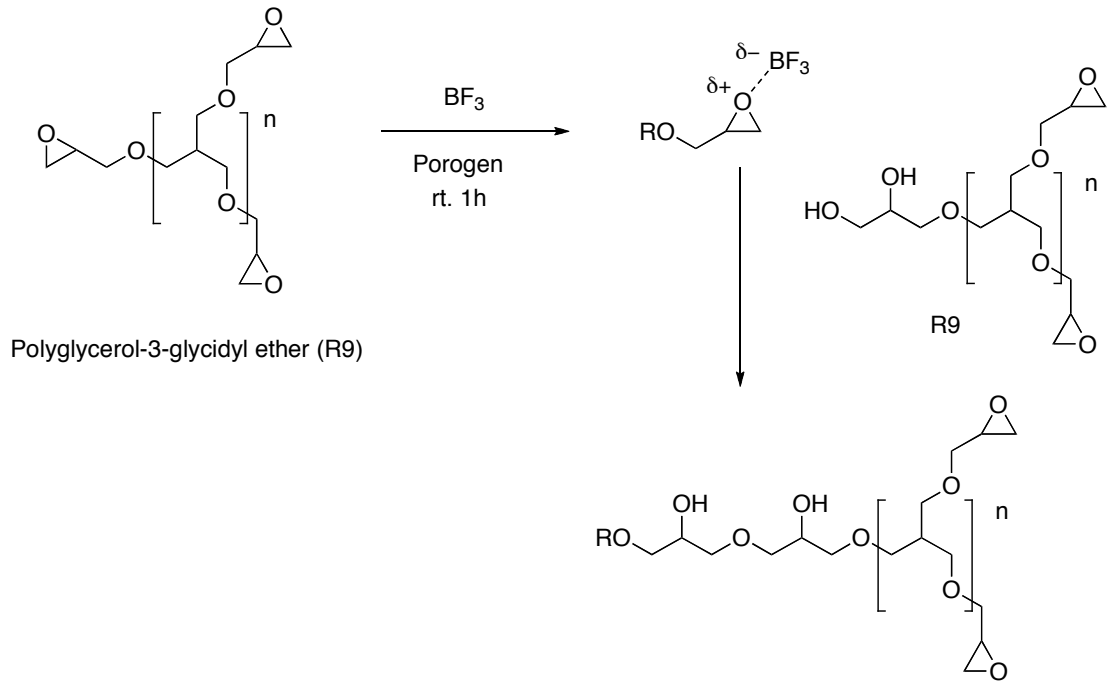

Fig. 15: Reaction for the polymerization of polyglycerol-3-glycidyl ether (R9)

A mixture of toluene and mTBE (60:40, v/v) was used as porogen. To achieve a high porosity and a sufficient rigidity, the porogen/monomer ratio was selected to be 80:20 (v/v). The two-component porogen resulted in a high porosity of 79%. The reaction was carried out at room temperature and completed after 1 h. The homogenous macropore structure with an average pore size of 21 µm under scanning electron microscopy (SEM) was published by Peskoller *et al.*[64].

The polymer was prepared in glass syringes (ID 4.5 mm) creating a covalent bonding to the glass wall. Therefore, an effective sealing of the monolith in glass columns can be achieved. As shown in Fig. 16, the inner wall of glass columns was silanized by

3-Glycidyloxypropyltrimethoxysilane (GOPTS) as a first reaction step producing initiating epoxy-groups on the surface. The hydroxyl groups from glass react with the methoxy groups of GOPTS after treatment under highly basic conditions. The epoxy groups of GOPTS could form covalent bonds with the polyepoxide R9 during the polymerization reaction.

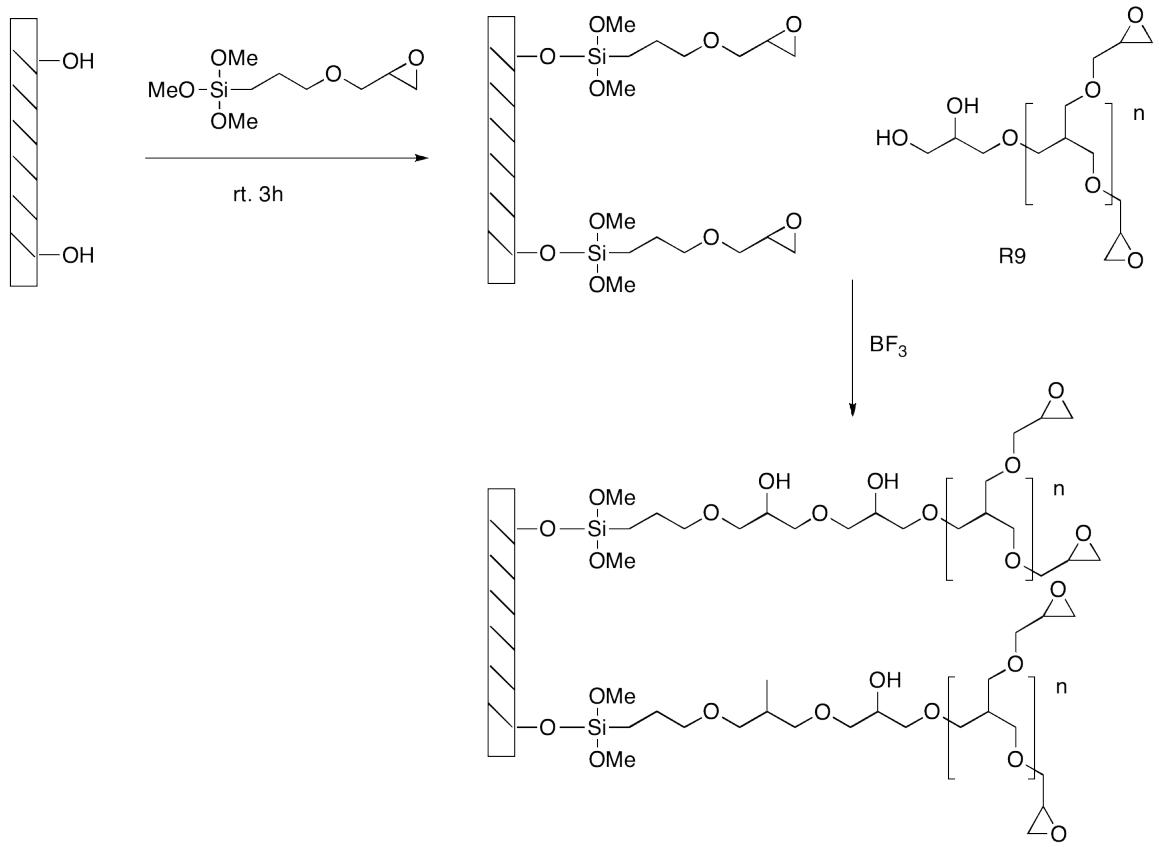

Fig. 16: Schematic description of silanization of glass wall with GOPTS and its reaction with R9

The epoxy groups of the polymerized monolith were hydrolyzed with sulfuric acid at 60 °C for 3 h. The formed hydroxyl groups should be similar to activated glass wool, which is used for concentration of viruses by glass wool filtration as standard adsorption – elution method for environmental samples.

The pore size of the polyepoxide-based monolith is about 21 µm, which is much

larger than the size of viruses and most of the environmental matrix. The adsorption-elution mechanism is preferred. Specifically, the sample was pumped through the monolithic column after acidification. The filtrate was discharged. The captured viruses were eluted into a small volume elution buffer with a different pH. Viruses in the original sample were concentrated and purified. Proposed scheme and monolithic column used in this work are shown in Fig. 17.

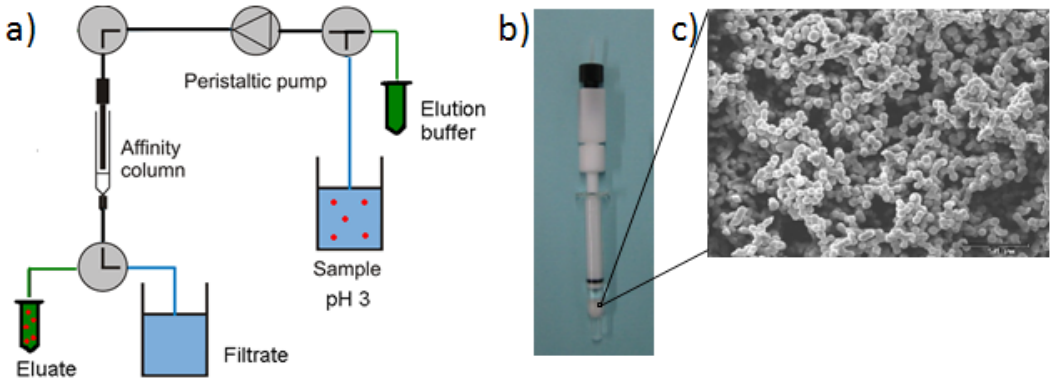

Fig. 17: Schematic diagram of the MAF system (a), image of the MAF column (b) and scanning electron micrograph of polyepoxy-based monoliths with 500-fold magnification (c).

3.1.1.1 Optimization of conditions

Evaluation of effect of pH

The dependency of the adsorption efficiency of MS2 to the pH value was examined (Fig. 18a) to determine if the surface of the monolithic column is comparable to the negatively charged glass wool at low pH and if MAF is able to capture positively charged viruses [179].

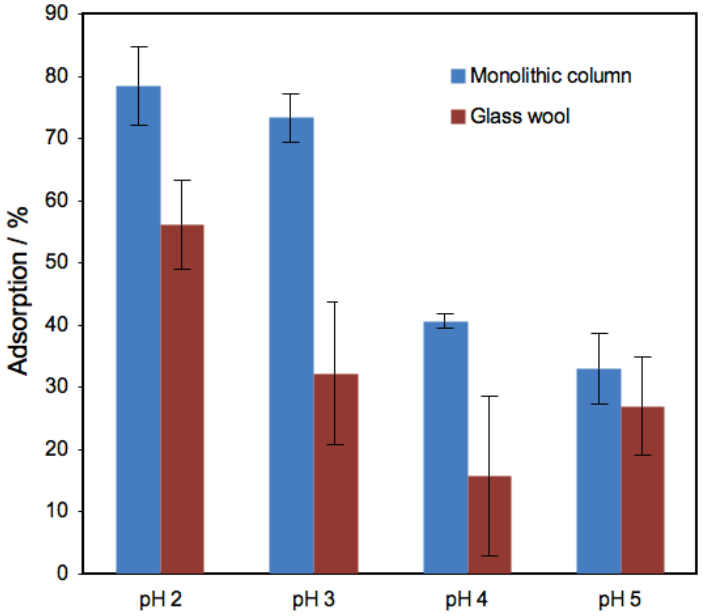

Fig. 18: Comparison of MS2 adsorption rates for MAF and glass wool filtration at different pH (n = 4, m = 3).

100 mL tap water was adjusted to pH 2, 3, 4, or 5 using 1 M HCl. The samples were spiked with 10^4 PFU of bacteriophage MS2. The water samples were subsequently pumped through activated MAF and glass wool columns (both 0.017 g) at a flow rate of 10 mL/min. Captured viruses were eluted using 1 mL BEG buffer of pH 9.5. The highest adsorption, with 78.5 (± 10.7)% for monolithic columns and 56.2 (± 7.2)% for glass wool columns, respectively, were determined at pH 2 (see Table 6). 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[47], indicating the contribution of electrostatic interaction to the adsorption process. Glass wool filtration is an accepted method and is able to concentrate multiple types of viruses. However, the results have confirmed (see Table 7) that the developed MAF-based concentration method is promising because higher adsorption efficiency per gram can be achieved.

Table 6: Adsorption rates and recoveries of MS2 at different pH by MAF (m = 3)

MAF	V /mL	Seeded C(MS2) / PFU mL^{-1}	Uncaptured C(MS2) / PFU mL^{-1}	Eluted C(MS2)/ PFU mL^{-1}	Adsorption / %	Recovery / %
pH 2	96	4.0×10^2	8.6×10^1	2.6×10^4	78.5 ± 10.7	62.1 ± 8.9
pH 3	99	4.2×10^2	1.1×10^2	4.2×10^4	73.3 ± 6.3	111.8 ± 16.3
pH 4	96	3.8×10^3	2.2×10^3	3.7×10^3	40.6 ± 1.1	1.0 ± 0.9
pH 5	98	4.3×10^3	2.9×10^3	3.7×10^3	33.0 ± 5.6	1.0 ± 0.9

Table 7: Adsorption rates and recoveries of MS2 at different pH by glass wool (m = 3)

GW	V /mL	Seeded C(MS2) / PFU mL^{-1}	Uncaptured C(MS2) / PFU mL^{-1}	Eluted C(MS2)/ PFU mL^{-1}	Adsorption / %	Recovery / %
pH 2	99	2.6×10^3	1.1×10^3	1.4×10^4	56.2 ± 7.2	5.1 ± 1.8
pH 3	98	3.4×10^3	2.3×10^3	4.4×10^4	32.2 ± 11.5	13.6 ± 4.3
pH 4	102	3.8×10^3	3.2×10^3	1.9×10^3	15.8 ± 12.8	0.5 ± 0.5
pH 5	100	4.3×10^3	3.1×10^3	9.3×10^2	27.0 ± 7.9	0.2 ± 0.2

For the elution step, the retained MS2 phages on the columns were recovered by beef extract glycine (BEG) buffer (pH 9.5), which is a high-ionic-strength protein solution[180]. However, the recovery of MS2 in tap water at pH 2 (62.1(± 8.9%)) was much lower than at pH 3 (111.8 (± 16.3)%) (Fig. 19).

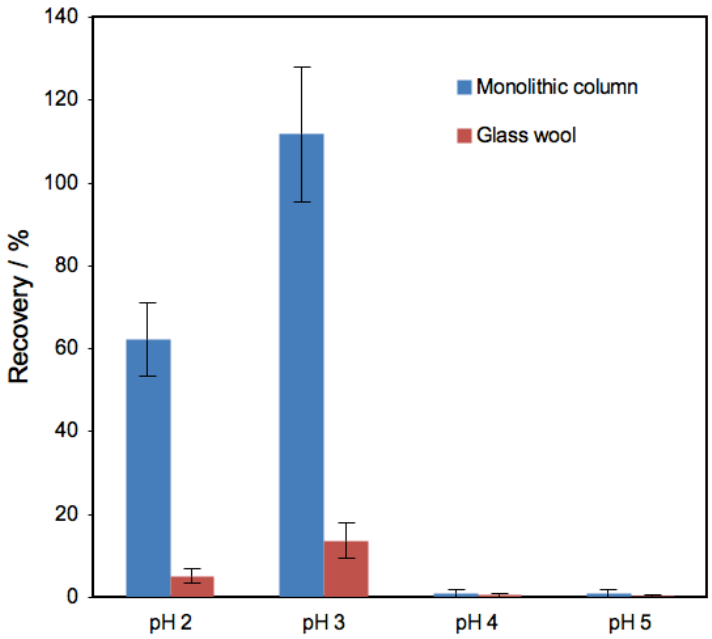

Fig. 19: Comparison of MS2 recoveries by MAF and glass wool filtration (n = 4, m = 3)

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 (containing NaCl, $MgSO_4 \cdot 6H_2O$, TRIS-HCl and gelatine, pH 7.5) and tap water of pH 3, were kept at room temperature for 1 h. Concentrations of 1.73 (± 0.09) × 10^3 and 1.65 (± 0.10) × 10^3 PFU/mL (n = 4) were determined by plaque assays. No inactivation effect was observed at pH 3. As MS2 were captured and recovered with high efficiency, pH 3 was chosen for acidification of water samples for the MAF process.

Recoveries of occasionally more than 100% may be explained by disaggregation of microbial aggregates during the filtration procedure, although vigorous vortexing was involved to disperse the microbial stocks during serial dilution procedure. As microbes aggregate differently at different pH values[181], samples differing in pH were adjusted to neutral 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%)[10]. The fact that with the MAF procedure nearly 100% of seeded MS2 can be recovered in tap water shows the promising high potential of the macroporous epoxy-based polymer material.

Binding capacity of monolithic column

The binding capacity of the monolithic column for MS2 bacteriophages was evaluated by using 1-L samples spiked with MS2 (c = 316 ± 16 PFU mL^{-1}, pH 3). The water samples were pumped through activated monolithic column at a flow rate of 10 mL/min. To create a profile of the binding procedure, aliquots of input and filtrate solutions were collected at certain time points and enumerated by plaque assay (see Table 8). A saturation of the monolithic affinity column (0.017 g) was observed after passing through of 300-mL sample (Fig. 20). The binding capacity of the MAF column was determined to be 5.6×10^6 PFU g^{-1} for MS2 in tap water.

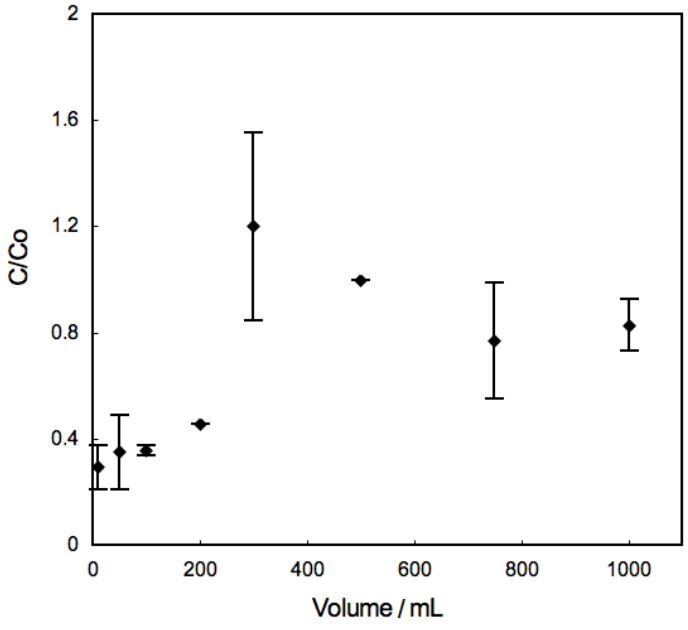

Fig. 20: Binding capacity of the monolithic column for MS2 in tap water (m = 3).

Table 8: Binding capacity of the monolithic column for MS2 in tap water (m = 3)

Time /min	Volume /mL	Seeded C(MS2) / PFU mL^{-1}	Uncaptured C(MS2) / PFU mL^{-1}	c/co
1	10	1.7 (± 0.4) × 10^2	5.0 (± 1.4) × 10^1	0.29 ± 0.08
5	50	4.0 (± 2.8) × 10^2	1.4 (± 0.6) × 10^2	0.35 ± 0.14
10	100	7.0 (± 2.0)× 10^2	2.5 (± 0.1)× 10^2	0.36 ± 0.02
20	200	2.2 (± 0.9) × 10^2	1.0 (± 0.0)× 10^2	0.45± 0.00
30	300	2.0 (± 0.6) × 10^2	2.4 (± 0.7) × 10^2	1.2 ± 0.35
50	500	1.8 (± 0.9) × 10^2	1.8 (± 0.0)× 10^2	1.0 ± 0.00
75	750	3.9 (± 1.0) × 10^2	3.0 (± 0.9) × 10^2	0.77 ± 0.22
100	1000	2.9 (± 0.7) × 10^2	2.4 (± 0.3) × 10^2	0.83 ± 0.10

3.1.1.2 Validation of the method

Validation of MAF by tap water spiked with various amounts of MS2

After optimization of the parameters, the MAF method was examined with samples of various concentrations (Fig. 21). 300-mL samples spiked with MS2 (final concentration = 23 - 1100 PFU mL^{-1}, pH 3) were tested (see Table 9). The water samples were pumped through activated monolithic column at a flow rate of 10 mL/min. Captured viruses were eluted using 1 mL BEG buffer. The maximum amount of MS2 eluted from the column in 1 mL was 1.0 (± 0.2) × 10^5 PFU, which is consistent with the binding capacity determined above. For samples containing MS2 below the capacity limit high recovery rates of 110 ± 19% (n = 5, m=3) were obtained.

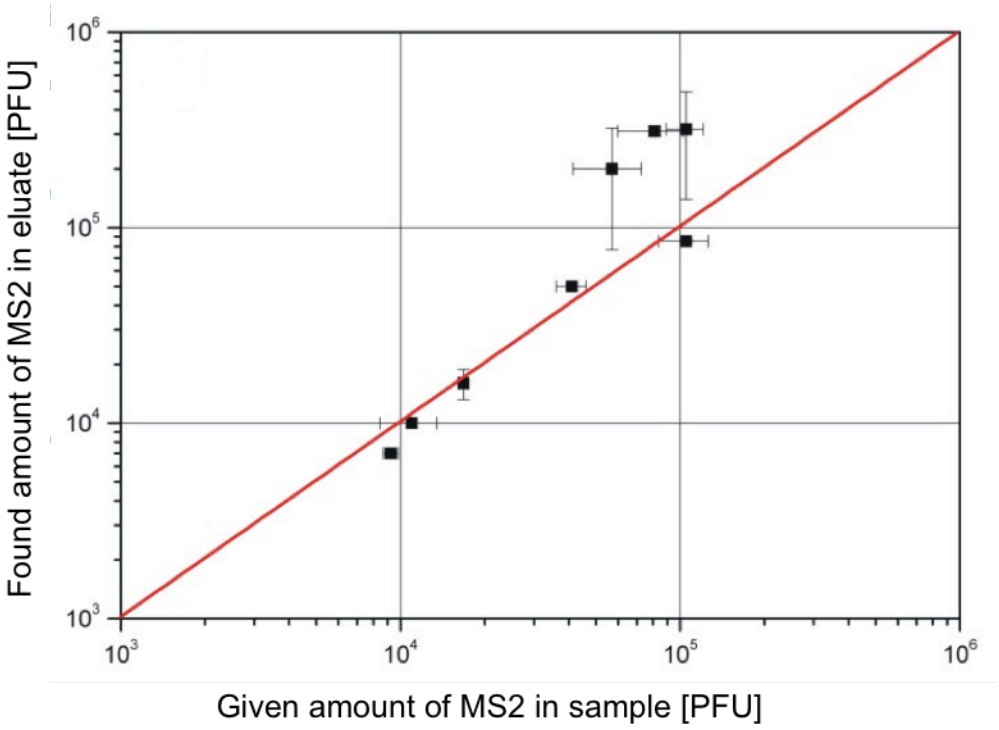

Fig. 21: Recovery experiments for concentrating of MS2 from 300 mL samples by MAF methods (m = 3). (Data points are shown with standard deviations. The line represents fictive recoveries of 100%).

Table 9: Recovery experiments for concentrating of MS2 by MAF methods (m = 3)

Seeded Nr (MS2) / PFU	**Recovered Nr (MS2) / PFU**	**Recovery /%**
$7.0 (\pm 0.0) \times 10^3$	$9.2 (\pm 0.6) \times 10^3$	132
$1.0 (\pm 0.0) \times 10^4$	$1.1 (\pm 0.3) \times 10^4$	110
$1.6 (\pm 0.3) \times 10^4$	$1.7 (\pm 0.1) \times 10^4$	105
$5.0 (\pm 0.0) \times 10^4$	$4.1 (\pm 0.5) \times 10^4$	82
$8.5 (\pm 0.2) \times 10^4$	$1.0 (\pm 0.2) \times 10^5$	123
$3.2 (\pm 1.8) \times 10^5$	$1.0 (\pm 0.2) \times 10^5$	33
$2.0 (\pm 1.2) \times 10^5$	$5.7 (\pm 1.6) \times 10^4$	29
$3.1 (\pm 0.2) \times 10^5$	$8.1 (\pm 2.1) \times 10^4$	26

Validation of MAF by real viruses and other surrogates

The small volume of the macroporous monolith (100 µL) allowed the elution of MS2 into a volume of 1 mL, which can be directly analyzed by RT-qPCR or plaque assay. MS2 in 100 mL tap water were concentrated in 11 min by the MAF process, which results in a concentration rate of 9.1 min^{-1}. The aim to establish a rapid concentration method was achieved. Furthermore, human adenoviruses and murine noroviruses, as surrogates for human noroviruses, were examined (tested by Dr. Hartmann Marten Nils). Under the optimized conditions described above, average recoveries of 42.4 (± 3.4)% (n = 3, m = 3) and 42.6 (± 1.9)% (n = 4, m = 3) were achieved for human adenovirus type 2 and for murine norovirus type 1, respectively. Recoveries of adenoviruses and noroviruses using monolithic columns were lower than those for bacteriophage MS2. Definitely, the MAF process was more effective than glass wool filtration published elsewhere[16].

3.1.2 Two-step concentration system: CF-UF-MAF

3.1.2.1 Description of the two-step concentration system

The two concentration methods were combined with rapid entrapment of viruses in a first step by CF-UF, followed by concentration and purification of the viruses in a second step by MAF. In the first step, the 10-L sample was concentrated by entrapment on a multibore hollow fiber module with pore sizes of 20 nm, and 100-mL concentrate was eluted into a sterile beaker. After acidification to pH 3, bacteriophages were captured on the macroporous hydrolyzed epoxy-based polymer. The small volume of 100 µL of the macroporous monolith has allowed the elution of MS2 into a small volume of 1 mL, which can be directly analyzed by PCR or plaque assay. As the aim was to establish a rapid analysis method for viruses, we wanted to show that the combined concentration and purification method is compatible with fast detection by RT-qPCR. In this regard, the secondary MAF should separate the inhibiting compounds, which were co-eluted in the

first step.

3.1.2.2 Improvement of LOD of PCR by the two-step concentration system

For quantification of the bacteriophage MS2 with RT-qPCR, a dilution series of standard MS2 RNA was measured. The resulting Cp values were plotted against the concentration of standard MS2 RNA, given in genomic unit (GU) per mL. With RT-qPCR the bacteriophages MS2 could be quantified in a range of $4 \times 10^3 - 4 \times 10^{11}$ GU/mL[183].

To determine the effectivity of the CF-UF-MAF/RT-qPCR method, the limits of detection (LOD) of the molecular detection method itself and of the combined analysis method were determined (in this cooperation, CF-UF was done by Dr. Martin Rieger and PCR detection was done by Sandra Lengger). Therefore, as published recently[184], the resulting fluorescence signal gained at a predetermined PCR cycle was referred to the given concentration. The cycle was selected so as to include most data points within the linear range of the sigmoidal calibration curve. In Fig. 22, the resulting calibration curves with or without pre-concentration of the phages are shown. For the curve without pre-concentration (analyzed at cycle 40), a series of MS2 dilutions, spiked in tap water, were measured by RT-qPCR. The LOD was determined to be 79.5 GU/mL. For generating the curve for the combined method (analyzed at cycle 42), five 10-L tap water samples were concentrated by CF-UF-MAF, followed by RT-qPCR detection. A LOD was calculated to be 0.0056 GU/mL. This data shows that the sensitivity of the bacteriophage MS2 quantification assay was improved by a factor of at least 1.4×10^4.

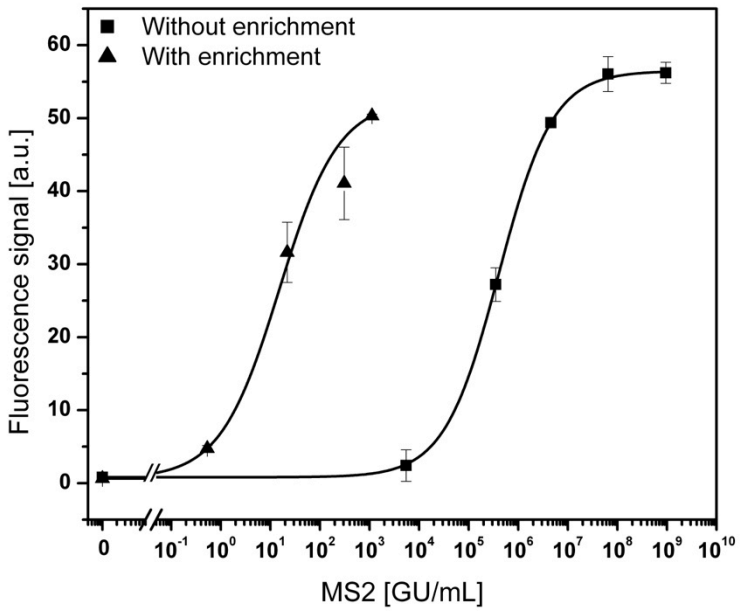

Fig. 22: Dose-response curves for bacteriophage MS2 in water samples measured with RT-qPCR (n = 11, m = 3).

3.1.2.3 Characterization of the two-step concentration system

To characterize the combined concentration system, spiking experiments with initial concentrations of MS2 between 0.53 and 1120 GU/mL were conducted (Table 10). In pure tap water no MS2 was found. At low concentrations of bacteriophage MS2 (< 1 GU/mL) the recovery was nearly 100% and a concentration factor of 10^4 was achieved. Therefore, the first concentration step by CF-UF was highly efficient at low concentrations. Lower recovery efficiencies analyzed by plaque assay could partially be caused by agglomeration of bacteriophages during the CF-UF 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 CF-UF at high concentration levels and the limited capacity of the monolithic columns, which were determined to be 10^5 PFU for MS2. Correspondingly, when the concentrations of MS2 in 10 L water were lower than 1 GU/mL, 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 CF-UF units and monolithic columns will be discussed in the next sections, so that better recovery at larger numbers of viruses and wider working ranges with high efficiencies can be expected.

Table 10: Concentration factors and recoveries of the RNA bacteriophage MS2 in samples subjected to the CF-UF-MAF/RT-qPCR combination system (m = 3)

Initial MS2 concentration /[GU/mL]	**Recovery**	**Concentration factor**
0	-	-
0.53	97.2 ± 28.9	1.0×10^4
2.20×10^1	56.6 ± 8.1	6.0×10^3
3.10×10^2	9.4 ± 3.2	9.4×10^2
1.12×10^3	10.6 ± 5.0	1.1×10^3

3.1.2.4 Process of environmental samples by CF-UF-MAF

Moreover, the CF-UF-MAF/RT-qPCR method was further tested with environmental water samples in order to show its ability for analyzing viruses 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 because of 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 during the CF-UF process. Fig. 23 illustrates the challenge, which environmental waters pose to the two-step concentration process. The high amount of matrix components co-eluting with the CF-UF could severely inhibit sensitive molecular detection methods, such as the enzyme reactions of the RT-qPCR. Therefore, the separation of viruses from other matrix components is necessary before performing the RT-qPCR. During the MAF process, most matrix components passed through the

columns. Purified eluates of 1 mL were generated and analyzed by RT-qPCR. 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) x 10^3 GU/mL. Furthermore, it can be stated that PCR-inhibiting compounds, like humic acids, were discharged after MAF.

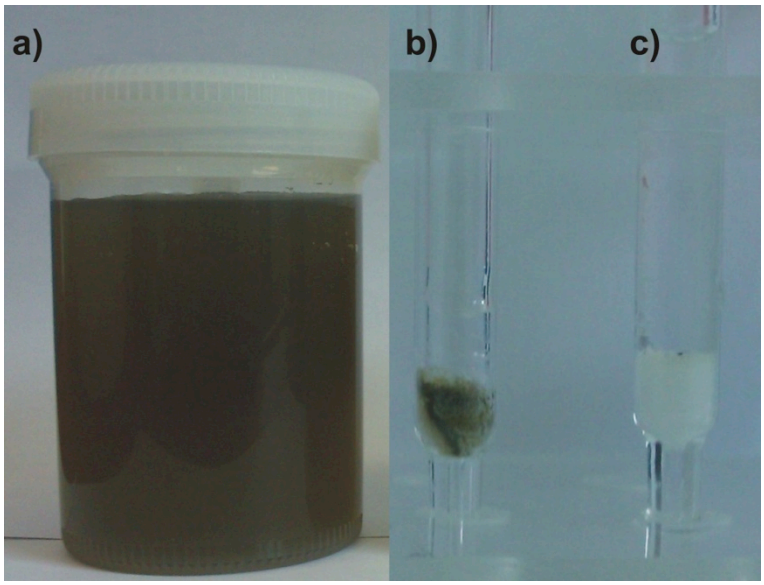

Fig. 23: Image of the 100-mL eluate after concentration of a 10-L sample from urban river water by CF-UF (a) and image of a monolithic column after (b) and before (c) processing the 100- mL eluate.

In comparison to the urban river, the alpine river water displayed a lower turbidity. 10-L water samples were collected after a wastewater treatment plant. Here, a corresponding concentration range of 8.6 ± 2.5 GU/mL was determined.

3.1.3 Summary

A rapid and effective adsorption-elution method was developed based on 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 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 CF-UF. Because of the adsorption of the examined viruses to the macroporous surface of the MAF column at pH 3, concentrated matrix components by CF-UF can be discharged. Bacteriophages MS2 were spiked in tap water and concentrated by the new CF-UF-MAF concentration method with a volumetric factor of 10^4 within 33 min. Furthermore, the detection limit for quantification of bacteriophage MS2 by quantitative reverse transcriptase PCR (RT-qPCR) could be improved from 79.47 to 0.0056 GU mL^{-1} by a factor of 1.4×10^4. The two-step concentration and purification method combined with RT-qPCR detection, allows fast quantification of MS2-like bacteriophages in surface water within 3 – 4 h.

3.2 Upscaling of monolithic column and its application in environmental samples

The volume of water samples required to be concentrated for virus detection depends on several parameters, e.g. the recovery rate, concentration factor and the source of the environmental water sample. In groundwater and drinking water only a low amount of pathogenic viruses could be expected due to filtration effects of soil and sediments or of water treatment. For these samples, volumes of more than 100 L have to be processed. 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 wastewater. Therefore, the processing of 10-L samples of surface or seawater will most likely be sufficient to gain detectable concentration levels of viruses.

Our previous study showed that filtration methods based on monolithic materials were very effective for concentrating viruses in water[183]. Almost all seeded viruses could be recovered. However, due to the limited flow rate, MAF(Small) is not able to process the required volume directly (~ 10 L). To compare with the mostly used concentrating methods and to explore its potential in effectively handling larger volume of water samples (~ 10 L), scaling-up of the monolithic column was necessary.

One of the challenges in preparing a large volume monolith is that the heat is problematic because of the highly exothermal polymerization reaction. Increased heat leads to an inhomogeneous structure. Another problem is because of the shrinkage of monolith in different matrix, which becomes more serious in monoliths with larger diameter. Due to the shrinkage of monolith, the 'wall channel' problem in a plastic housing results in the liquid bypassing the monolith and the recovery efficiency decreasing dramatically. The methods used to address these problems will be discussed.

3.2.1 MAF for water samples > 1 L (MAF(Big))

To achieve higher flow rates and to increase the binding capacity, the volume of column needs to be enlarged. In a first experiment, the upscaled column was prepared in the same way as the small one (diameter of 4.5 mm) in a glass syringe with an inner diameter of 26.7 mm (see Table 11). However the time needed for surface activation of the glass was much longer and the cost for one column was much higher than those of the small one, making it unsuitable for batch production. Furthermore, due to the small outlet of the glass syringe, the flow rate was limited to 0.4 L/min, which was much lower than the expected 1.0 L/min. To have a cost-effective method, a disposable syringe made of polypropylene (PP) was selected as the housing of the monolithic column. A disk-shape monolith was prepared in a PTFE mold. The desired dimension can be achieved by using a mold with a defined size. The proposed column consists of 5 parts: the commercial PP housing, the PTFE holder with bore holes (2 mm in diameter), the monolithic disk, the fitting and the blocker (see Fig. 40 in Experimental section). Compared to preparation in glass housing, this method is less laborious and less time-consuming (Table 11). With the column prepared in such a way, the expected flow rate of 1 L/min can be achieved. Filtration methods based on this upscaled column will be referred to as MAF(Big) while the methods based on the column with a diameter of 4.5 mm will be referred to as MAF(Small) (Fig. 24).

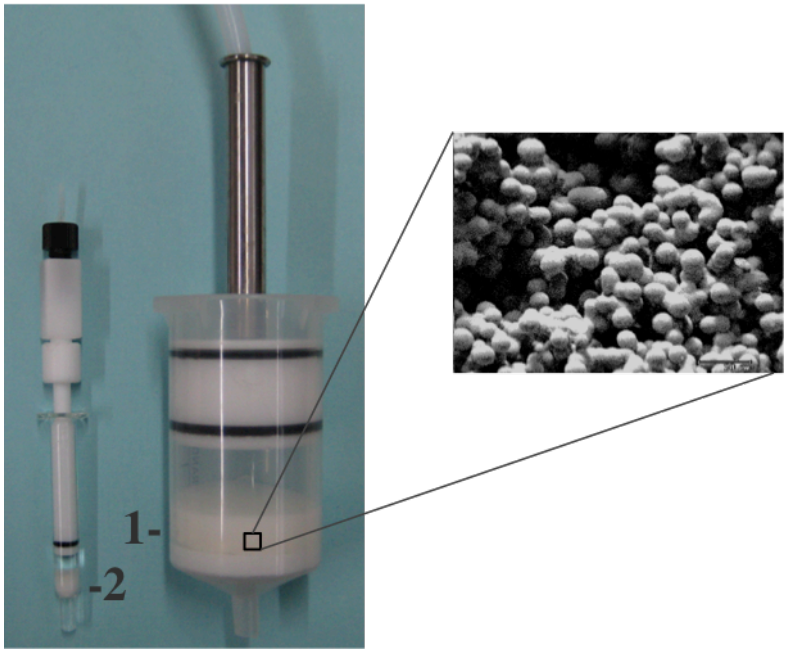

Fig. 24: Dimension of MAF(Big): 6 mm length and 35.5 mm diameter (1) and the respective scanning electron micrograph (by Christine Sternkopf, IWC, TUM); MAF(Small): 8mm length: and 4.5mm diameter (2)

Table 11: Main factors of columns prepared in different housings

Column	Material of housing	Diameter [mm]	Cost [€/column]	Time for preparation [h]	Max flow rate [L/min]
a	Glass	4.5	4.15	0.45	0.04
b	Glass	26.7	>40	1.5	0.4
c	PP	35.5	1.77	0.2	>1.0

3.2.1.1 Optimization of MAF conditions

Optimization of dimensions of the monolithic disk

In the next step, the dimensions of a single monolithic disk were optimized. In general, to solve the 'wall channel' problem in a plastic housing, the diameter of the monolithic disk was designed to be somewhat larger than the inner diameter of the housing, because the swelling of wetted MAF disks could serve to compress the gap between the polymer and the inner side of the housing. The appropriate length of the

monolithic column could be achieved by disk-stacks. In the preparation step, to design an appropriate length of a single monolithic disk, the exothermic nature of the polymerization should be taken into consideration. For example, when the length of a single monolithic disk was 20 mm (about twice of the optimum), the temperature in the polymerization mixture increased from 40 to 80 °C within 5 min, which led to a non-homogeneous structure. The details of the influence of the diameter and the length of a single monolithic disk on adsorption rate, recovery as well as elution efficiency are described in the following sections.

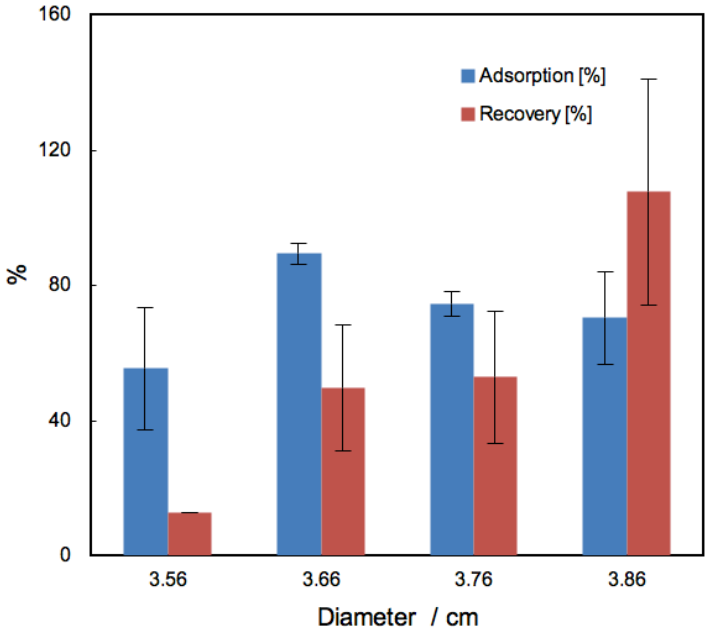

Fig. 25: Adsorption and recovery of MS2 by monolithic columns of the same volume (n = 4, m = 3)

While keeping the same column volume (12 mL), the diameter of the monolithic disk was adjusted to decrease the 'wall channel'. Monolithic disks with four different diameters, ranging from 3.56 cm (the inner diameter of the housing) to 3.86 cm (the maximum that can be squeezed into the housing), were produced (Fig. 25).

Table 12: Adsorption and recovery of MS2 by monolithic columns of the same volume (n = 4, m = 3)

Diameter	Seeded C(MS2) / PFU mL^{-1}	Uncaptured C(MS2) / PFU mL^{-1}	Eluted C(MS2)/ PFU mL^{-1}	Adsorption / %	Recovery / %
3.56	9.6×10^2	4.3×10^2	5.6×10^4	55.5 ± 18.1	12.8
3.66	1.5×10^3	1.6×10^2	3.5×10^5	89.4 ± 3.2	49.7 ± 18.5
3.76	8.8×10^2	2.2×10^2	2.7×10^5	74.6 ± 3.7	52.8 ± 19.5
3.86	6.4×10^2	1.9×10^2	4.1×10^5	70.3 ± 13.6	107.6 ± 33.3

10-L tap water samples (containing 10^3 PFU/mL of MS2 bacteriophages) were filtrated at a flow rate of 1 L/min. Subsequently, the adsorbed MS2 was eluted by a 20-mL elution buffer. It was shown that the highest recovery of 107 (± 33)% (adsorption rate 70 (± 14)%) was achieved with a MAF-disk of 3.86 cm in diameter and 1.0 cm in length (Table 12). The recovery was decreased to 13% (adsorption rate 55 (± 18)%) with a lower diameter (3.56 mm) because of the gap between MAF-disk and housing.

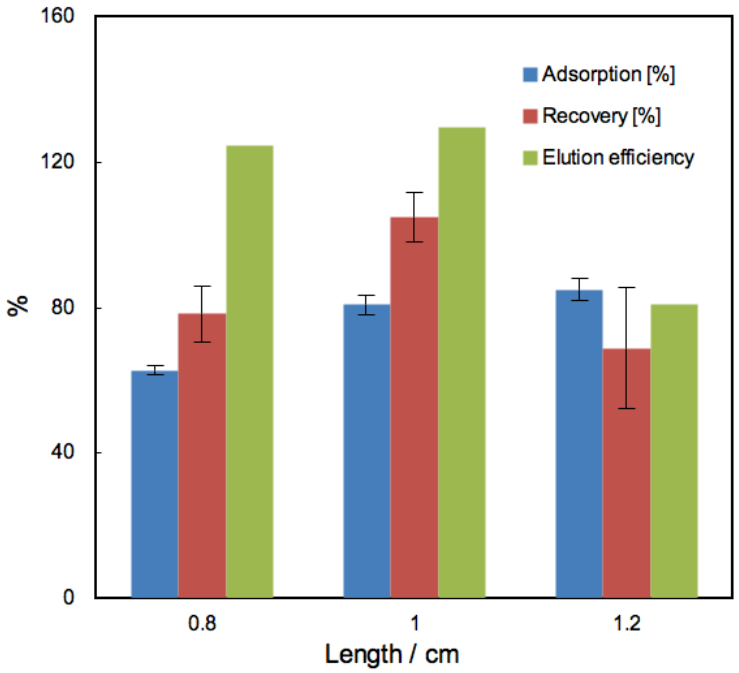

Fig. 26: Adsorption and recovery of MS2 by monolithic columns of the same diameter (n = 3, m = 3)

Furthermore, disks of the same diameter (3.86 cm) but with different lengths (0.8 cm, 1.0 cm and 1.2 cm), were also compared (Fig. 26). While a reduced height of 0.8 cm led to decreased adsorption rate (62 (± 1)%) and recovery rate (78 (± 8)%), an increased height (1.2 cm) gave a comparable high adsorption rate (85 (± 3)%) but a reduced recovery (69 (± 17)%) (see Table 13). It can be explained by the decrease of elution efficiency from 130% to 81%. To keep the volume of the elution buffer as small as possible, the MAF disks with dimensions of 3.86 cm in diameter and 1.0 cm in height were synthesized and used throughout the following studies.

Table 13: Adsorption and recovery of MS2 by monolithic columns of the same diameter (n = 3, m = 3)

Length / cm	**Seeded C(MS2) / PFU mL^{-1}**	**Uncaptured C(MS2) / PFU mL^{-1}**	**Eluted C(MS2) / PFU mL^{-1}**	**Adsorption / %**	**Recovery / %**	**Elution efficiency / %**
0.8	2.7×10^3	9.9×10^2	8.5×10^5	63 ± 1	78 ± 8	125
1.0	3.0×10^3	5.7×10^2	1.4×10^6	81 ± 3	105 ± 7	130
1.2	3.4×10^3	5.0×10^2	1.0×10^6	85 ± 3	69 ± 17	81

3.2.1.2 Characterization of MAF(Big)

Characterization of MAF(Big) by tap water spiked with various amount of MS2

The MAF disk module was characterized by acidified (pH 3) 10-L tap water samples spiked with different amounts of MS2 ranging from 2.2 (± 0.5) × 10^3 to 1.9 (± 0.2) × 10^8 PFU. Almost all of the seeded MS2 was recovered (recovery rates of 102 (± 23)%, n = 10, m = 3) in 15 min. 10-L water samples were pumped through an activated monolithic column at a flow rate of 1 L/min. Captured viruses were eluted by 20 mL BEG buffer. The maximum amount that could be eluted from the column was 1.5 × 10^8 PFU and the corresponding concentration of phage in initial sample is much higher than the acceptable detection limits of plaque assay (see Fig. 27). Therefore the binding capacity for the

viruses on MAF disks would not be a limiting factor. Compared to the crossflow ultrafiltration method, which was used for the same scale of sample, the concentration factor was improved from 58 to 500 by MAF(Big) due to its low elution volume and high recovery.

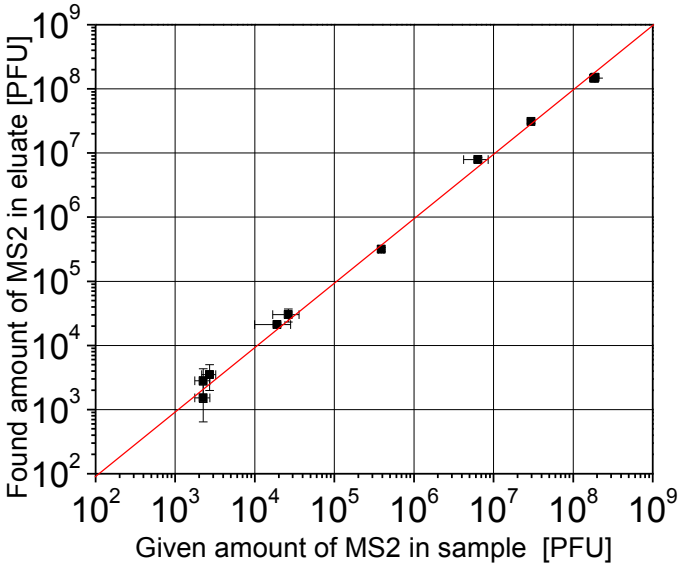

Fig. 27: Recoveries of MS2 at different spiked levels. Data points are shown with standard deviations. The red line represents fictive recovery of 100% (n = 10, m = 3).

Characterization of MAF(Big) by real virus and other surrogates

The established method was tested with bacteriophage MS2, ΦX174, human adenoviruses and murine noroviruses (as surrogates for human noroviruses) by our project partners (Dr. Hans-Christoph Selinka and Dr. Nils Marten Hartmann from UBA). 10 L of acidified tap water was spiked with a mixture of MS2, ΦX174, human adenoviruses and murine noroviruses. Captured viruses were eluted using 20 mL BEG buffer (pH 9.5). 106.2% of bacteriophage MS2 and 40 (± 17)% of ΦX174 were recovered which were quantified by plaque assay, respectively. Human adenoviruses and murine noroviruses were recovered with efficiencies of 67 (± 59)% and 12 (± 6)%, shown by

qPCR, respectively (Table 14). These results proved the applicability to simultaneously concentrate various viruses by MAF(Big).

Table 14: Recovery of human and animal viruses by MAF(Big). Bacteriophage MS2 and ΦX174 were tested by plaque assay. Human adenoviruses and murine noroviruses were detected by TaqMan qPCR (m = 3).

	MS2	Murine noroviruses	ΦX174	Human adenoviruses
Recovery (%)	106.1	67.2±58.8	40.2±17.0	12.2±5.7

3.2.2 MAF(Big) - centrifugal ultrafiltration (CeUF) - RT-PCR for environmental samples

In contrast to tap or drinking water, which was treated before consumption to fulfill the required parameters in acceptability, chemical and microbial aspects, the environmental samples are more complicated. In the aquatic environment, the viruses in sewage are the original source for contamination, which may be transmitted into surface water. Surface water, like lake, river or canal water, may contain high microbial load, suspended solids and a variety of dissolved constituents, like bacteria, viruses, protozoa, organic and inorganic hydrocolloids, dust, humic acids and so on. Therefore, for the analysis of viruses in environmental samples, on one hand, the volume of samples should be reduced to be compatible with molecular biological detection methods. On the other hand, the problems during enrichment and detection caused by components in the matrix need to be addressed.

3.2.2.1 Combination of MAF(Big), CeUF and RT-PCR for environmental samples

Improvement of MAF(Big) for environmental samples

At first, the MAF(Big) was tested with water samples from the Havel river (Berlin-Spandau). 10 L acidified river water was spiked with a mixture of MS2 (4.0×10^6

PFU/mL), ΦX174 (4.9×10^5 PFU/mL), human adenoviruses (2.49×10^6 CP/mL) and murine noroviruses (6.03×10^5 CP/mL). MS2 and ΦX174were measured by plaque assay while hAdV2 and muNV were quantified by qPCR. The experiments were done by our project partners Dr. Hans-Christoph Selinka and Dr. Nils Marten Hartmann from UBA.

Table 15: Recovery efficiencies of phages and viruses in tap and river water (m = 2)

	Recovery [%]	
	Tap water	River water
ΦX174	40.2	8.6
MS2	106.1	3.0
hAdV2	12.2	5.1
muNV	67.2	9.6

Compared with the results in tap water, the efficiencies of MAF(Big) for all viruses were dramatically decreased (Table 15). The reasons could be: 1) the enlarged ‘wall channel’ between the monolithic disk and the housing due to the increased backpressure caused by the blocking of pores. It was indicated by the color change on both the top as well as on the side face of the disk (see Fig. 42 in Experimental section). River water and elution buffer bypassing the disk led to reductions of both adsorption rate and elution efficiency. 2) the competitive attachment of viruses onto both column and suspended solid in river water. To solve these problems, the setup of MAF was modified in the following aspects: 1) a bouncy O-ring was inserted between the polymer and housing to block the ‘wall channel’; 2) two monolithic disks were stacked to increase column length and the stacked disks were eluted separately in order to maintain the high elution efficiency achieved by 20 mL of elution buffer.

CeUF as secondary concentration method:

Because the volume of eluate in the MAF step (20 mL) is still higher than that needed in molecular biological detection, further concentration was needed. MAF(Small)

showed good performance as a secondary concentration method in combination with crossflow ultrafiltration. But if MAF(Big) was used as the primary step, the viruses are concentrated in a protein buffer. The protein in the buffer, which facilitated the desorption procedure in the primary step, would disturb the adsorption procedure onto the MAF(Small) in the secondary step. Therefore, a concentration method based on a different mechanism is preferred, e.g. size-exclusion or flocculation. Size-exclusion based centrifugal ultrafiltration (CeUF) was selected in our study, because it was less laborious and more robust. During flocculation, inhibitory components would get coprecipitated, which would result in more inhibitory effects for the PCR detection. In CeUF, those components could be partly discharged by choosing an appropriate membrane molecular weight cutoff (MWCO). MS2 is a soft particle and relatively small, with a molecular weight of 3.5×10^6 g/mol. Centrifugal filters with 50,000 MWCO are promising in respect to rapidness and purification of analytes. The combination of the MAF(Big) and CeUF method will be used for 10-L scale environmental samples.

Description of MAF(Big)-CeUF procedure

The scheme of improved setup for the two-step concentration method involving MAF(Big) and CeUF is illustrated in Fig. 28..

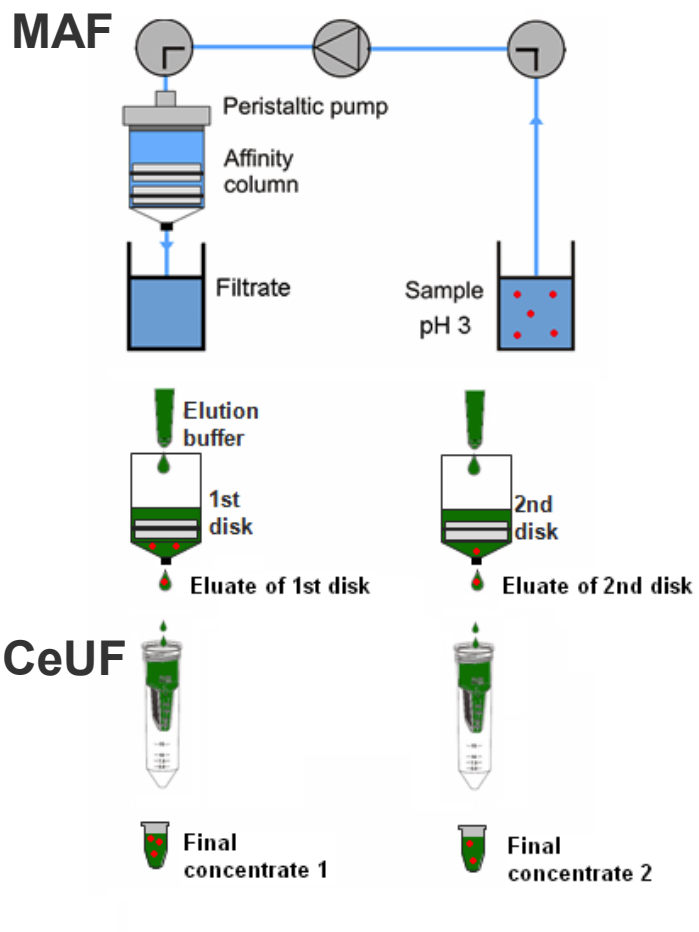

Fig. 28: Schematic diagram of the improved MAF(Big)-CeUF system

In the set-up of modified MAF(Big), stacked monolithic disks (two pieces) were used for filtration. These two monolithic disks were eluted in parallel, using a 20-mL elution buffer for each. Eluting two monolithic disks in parallel offered the chance to probe the distribution of various constituents, e. g. viruses and other components, on the two monolithic disks. These two eluates from the MAF(Big) step were processed separately by two centrifugal filters in the CeUF step. After centrifugation, two 1-mL final concentrates were obtained. Aliquots from input sample, eluates of MAF(Big) and final concentrates from CeUF were collected and quantified by RT-qPCR.

RT-PCR was used, instead of plaque assay, for rapid and specific quantification of MS2 in environmental samples. Moreover, to improve the specificity, the annealing

temperature during PCR was increased from 50 ºC to 60 ºC. To establish the calibration curve for MS2, a RNA standard (4×10^{14} GU/mL) was diluted by ten-fold in series and measured by PCR. The Cp values obtained from PCR were plotted against the given concentrations of standard MS2 RNA (see Fig. 29). The bacteriophages MS2 could be quantified in a range of $1.0 \times 10^4 – 1.0 \times 10^{10}$ GU/mL (n = 7, m = 3).

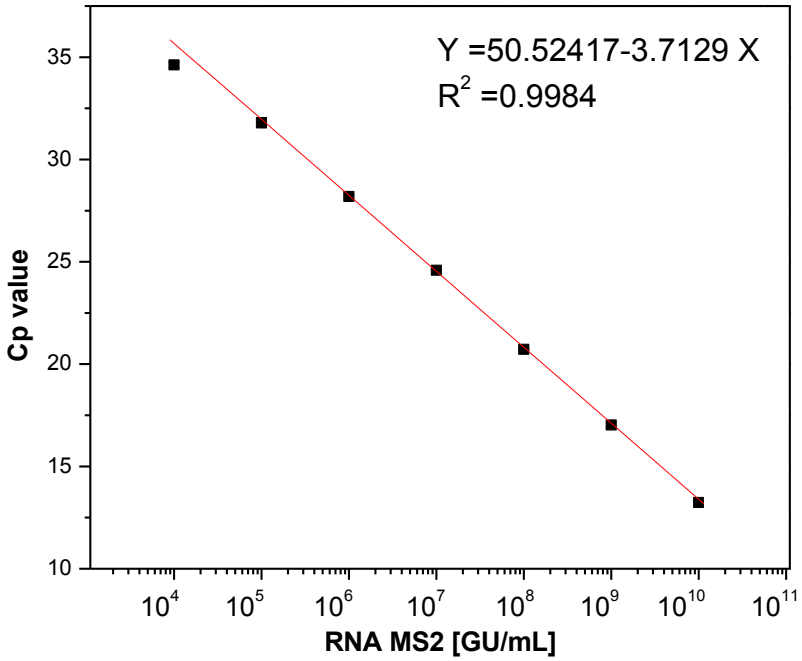

Fig. 29: RT-qPCR calibration curve for bacteriophage MS2 referred to standard MS2 RNA at annealing temperature of 60 ºC. (n = 7; m = 3).

3.2.2.2 Test with lake water and treated wastewater

Test with lake water

The improved analytical method MAF(Big)-CeUF-RT-qPCR was tested with lake water. 10-L samples from Starnberg lake were acidified to pH 3 and filtrated at a flow rate of 0.5 L/min. Captured viruses were eluted as illustrated in Fig 28. Aliquots from the

input sample, eluates of MAF(Big) and final concentrates from CeUF were collected. The amount of naturally occurring MS2 was enumerated by RT-qPCR and shown in Table 16. Before enrichment, the concentration of naturally occurring MS2 in the lake water was too low to be detected by RT-qPCR. After MAF, the concentration of MS2 in the eluate of the top disk was detectable (8.1 (± 2.0) × 10^3 GU/mL). But after CeUF, in the final concentrate 1, nothing was found by PCR. In contrast, the concentration in the eluate of the bottom disk was still not detectable after MAF, but after CeUF, it was detectable and resulted in high concentration (1.2 (± 0.2) × 10^4 GU/mL). This fact indicated, by MAF(Big) or MAF(Big)-CeUF, that the concentration of naturally occurring MS2 could be enriched to a detectable level. But further investigation was needed to find out the disturbing factors.

Table 16: Concentrations of naturally occurring MS2 in Starnberg lake water during enrichment procedure

	Input	**Eluate of disk 1 [GU/mL]**	**Eluate of disk 2 [GU/mL]**
MAF(Big)	N.D.	8.1 (± 2.0) × 10^3	N.D.
CeUF	----	N.D.	1.2 (± 0.2) × 10^4

N.D.: not detectable

Test with treated wastewater

To solve the problem, treated wastewater was tested as a worst-case scenario. Treated wastewater was taken from a wastewater treatment plant. To ensure that the concentration of MS2 in each step was detectable, an acidified wastewater sample (pH 3) was spiked with MS2 stock solution to reach a concentration of 6.1 (± 0.5) × 10^5 GU mL^{-1}. Then the sample was filtrated by MAF(Big) at a flow rate of 0.5 L/min. Then two monolithic disks were eluted and CeUF was carried out as described in Fig. 28. After that, aliquots from the input sample, eluates of MAF(Big) and final concentrates from CeUF were collected. The amount of MS2 was enumerated by RT-qPCR and shown in Fig. 30. Corresponding recoveries and concentration factors achieved for each step are shown in

Table 17.

Table 17: Recovery and concentration factor for different steps after quantification with RT-qPCR (Input concentration of MS2 6.1 (± 0.5) × 10^5 GU mL^{-1})

	MAF(Big)		**MAF(Big) +CeUF**	
	Eluate of 1st disk	**Eluate of 2nd disk**	**Final concentrate 1**	**Final concentrate 2**
Concentration / GU mL^{-1}	3.4 (± 0.02) × 10^7	7.2 (± 0.4) × 10^7	3.1 (± 0.8) × 10^4	1.2 (± 0.01) × 10^9
Recovery / %	28 ± 3	59 ± 6	0.1	64±6
Concentration factor	56 (± 5)	1.2 (± 0.1) × 10^2	0.05±0.01	1.9 (± 0.3) × 10^3

By the MAF(Big) step, in the elutes from the top and the bottom disks, 28 (± 3)% and 59 (± 6)% given MS2 were recovered, respectively (Table 17). By the CeUF step, almost all viruses in the eluate of the bottom disk were recovered in the final concentrate 2. It yielded a recovery rate of 64 (±6) % and a concentration factor of 1.9 (± 0.3) × 10^3 by this MAF(Big) and CeUF combination system. However, only 0.1% seeded MS2 could be found in the final concentrate 1, which was sourced from the eluate of the top disk. The disappearance of a signal in the final concentrate 1 was consistent with what was observed before (Table 16).

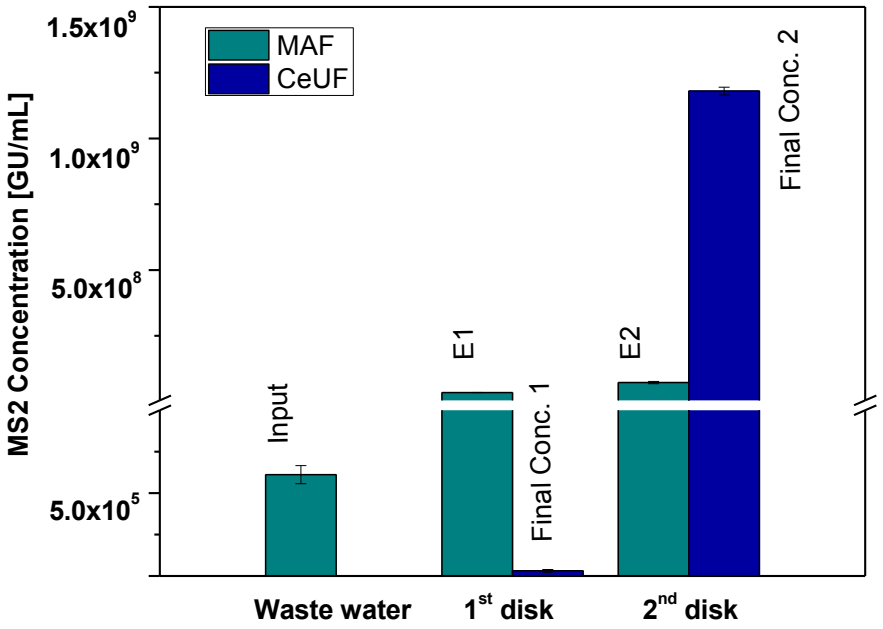

Fig. 30: Concentration of MS2 at different steps (n = 1, m = 3). The 1st disk and the 2nd disk represent the top and the bottom disk of the monolithic column, respectively, as shown in Fig 28.

3.2.2.3 Removal of PCR inhibiting substances in matrix

Inhibitory effect for PCR

To find the fundamental factors, control experiments were carried out. Wastewater, which was sampled at the same time, was precessed by the MAF(Big)-CeUF method as described above. Aliquots from the input sample, eluates of MAF(Big) and final concentrates from CeUF were collected. Instead of spiking them into the initial sample, the same amounts of MS2 stock were spiked into these aliquots and an equal volume of ultrapure water before nucleic acid extraction. The ultrapure water sample was used as a positive control. Concentrations of MS2 were calculated from the calibration curve

(shown in Fig. 29). In principle, the same spiking amount should result in an equivalent concentration of MS2 in every sample. But the signals observed by RT-qPCR were quite distinct (see Fig. 31). The initial wastewater showed a comparable signal with the positive control, while the inhibitory effect in other samples could be clearly observed. Eluate 1 of the top filter contains more inhibitors for RT-qPCR than eluate 2. One reason is that all bigger particles of the matrix are forming a cake layer on the top of the first MAF disk. The inhibitors in eluate 1 are further concentrated by CeUF. The extremely low concentration of MS2 found by RT-qPCR in final concentrate 1 indicated the strong inhibitory effect, which result in a reduced amplification. In contrast, nearly no inhibitors were found in eluate 2. The final concentrate 2 results in similar recoveries as for eluate of disk 2. This fact indicates that there are more inhibitory components retained on the top disk. These inhibitors are of a larger size, which could not go through the membrane of CeUF modules. Co-concentrated inhibitors in final concentrate 1 showed a much higher inhibitory effect, which could cover the concentration effect by CeUF. The purification by CeUF could only be exhibited by final concentrate 2. The relative concentration of MS2 indicated in Fig. 31 is consistent with the changing trend of MS2 concentration in Fig. 30 and Table 16. The increase of inhibitory effect in final concentrate 1 in Fig. 31 could explain the underestimated MS2 concentration in final concentrate 1 and corresponding sharply decreasing recovery in Fig. 30.

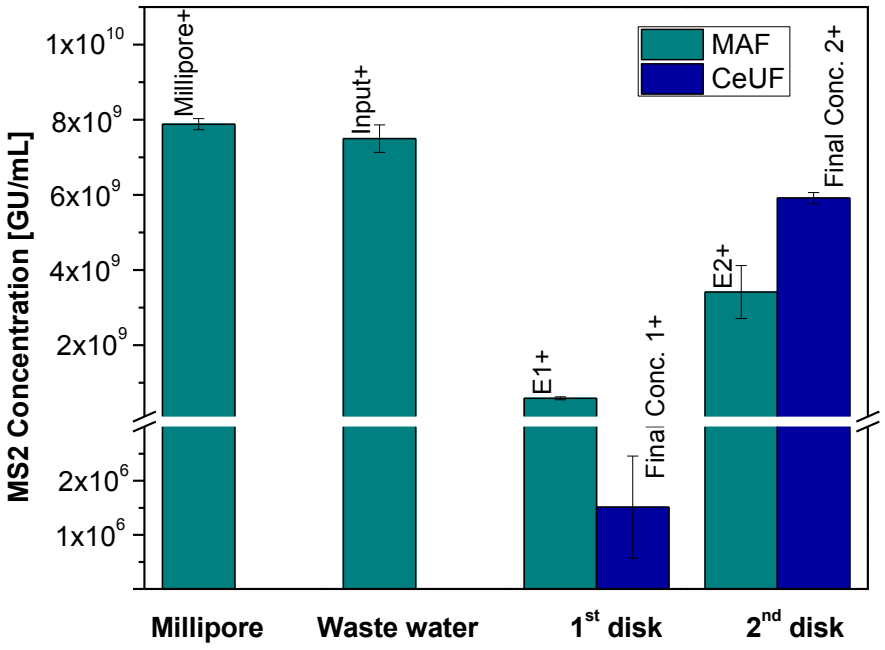

Fig. 31: Concentrations of MS2 calculated from calibration curve. Samples were spiked with the same amount of MS2 stock (n = 6; m = 3). The 1st disk and the 2nd disk represent the top and the bottom disk of the monolithic column, respectively, as shown in Fig. 28.

Removal of inhibitors: comparison of two nucleic acid extraction strategies

The inhibitory effect from the matrix leads to an underestimation of viruses. It could be caused by co-concentrated compounds, including humic acid, fulvic acid and cations such as calcium and iron[129, 130]. To decrease the inhibitory effect, several actions were taken, e.g. pre-filtration, pH adjustment, centrifugation, *etc*. But these strategies led to a very limited improvement. Then the nucleic acid extraction method was re-estimated. A sufficient nucleic acid extraction should optimally recover the target and remove amplification inhibitors[185]. At the beginning of our research, the ViralXpress Nucleic Acid Extraction kit (Millipore,Germany) was selected due to its low cost and rapidity. The NucliSens Magnetic Extraction kit (Biomerieux, France) was included for

comparison. The extraction chemistry of NucliSens is adapted from the Boom method[186], but also facilitated by magnetic silica beads. Nucleic acids were released by guanidine thiocyanate and bound to magnetic silica beads. After successive washing steps, it was eluted into a small volume. The final concentrate 1 from wastewater using the MAF(Big)-CeUF method, which showed the highest inhibitory effect, was tested as a worst case scenario. Of the final concentrate and ultrapure water, 150 µL were spiked with the same amount of MS2. Nucleic acids were extracted by the ViralXpress Nucleic Acid Extraction kit and the NucliSens Magnetic Extraction kit in parallel according to the instructions of the manufacturers. The following cDNA synthesis and the PCR amplification were carried out in the same way as before.

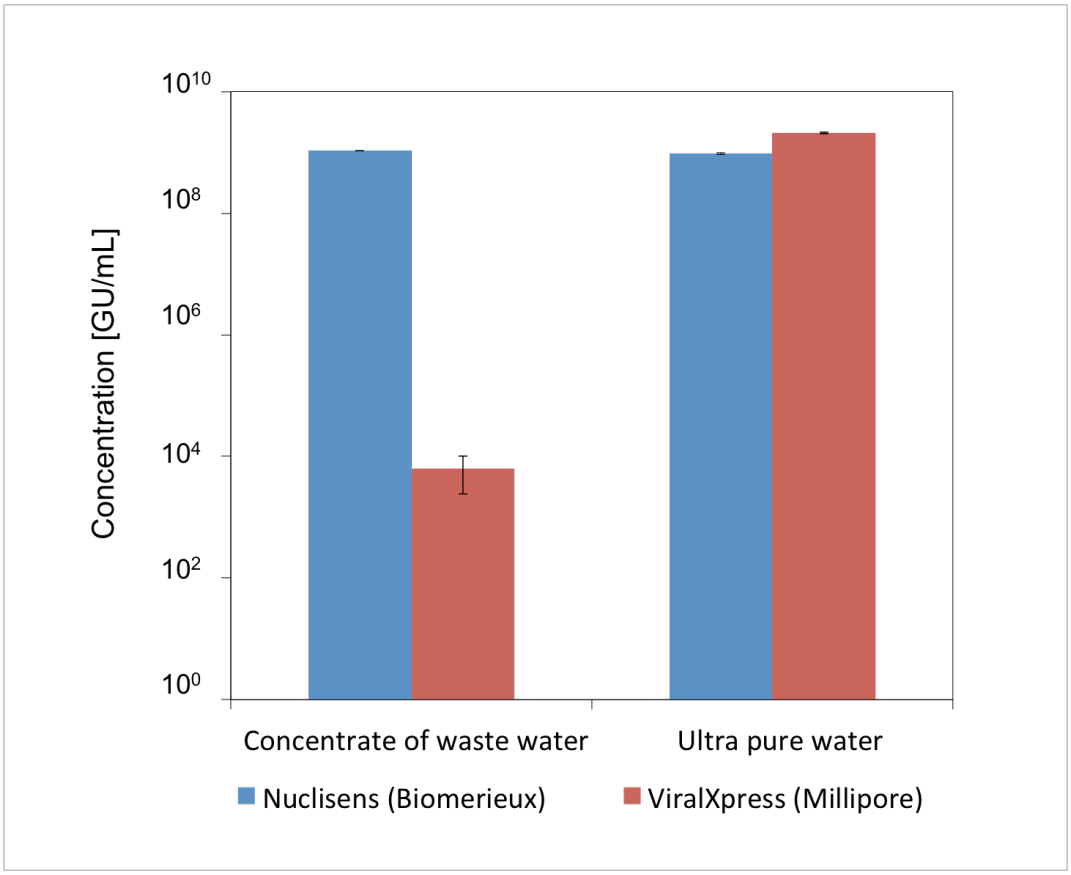

Fig. 32: Quantitative results of the same amount of MS2 from RT-qPCR with different nucleic acid extraction methods in different matrices (m = 3)

The results from PCR are shown in Fig. 32. The same amount of nucleic acid should be present in the sample due to the same spiking amount. However, the quantities of nucleic acid shown by RT-PCR vary considerably depending on the matrix and the extraction method used. In ultrapure water, recovered concentrations of MS2 RNA are comparable using Nuclisens and ViralXpress ($9.2\ (\pm 0.3) \times 10^8$ GU/mL and $2.01\ (\pm 0.06) \times 10^9$ GU/mL, respectively). In final concentrate 1 of wastewater, similar amounts of MS2 were recovered using Nuclisens ($1.06\ (\pm 0.01) \times 10^9$ GU/mL), while much lower values were found using ViralXpress ($2.4\ (\pm 0.3) \times 10^3$ GU/mL), which showed inhibitory effect of $6\log_{10}$ steps. These results prove that the inhibitory effect from environmental samples could be eliminated using the NucliSens extraction method. Drawbacks of Nuclisens are its higher cost and hands-on time per sample. But this could be partly compensated by using an automated extractor. Therefore the underestimated recovery of the MAF(Big)-CeUF enrichment system could be corrected and higher efficiency for viruses from environmental samples could be expected.

3.2.3 Summary

Upscaled monolithic columns (diameter: 3.86 cm) were prepared in a cost-effective way (< 2 euro/disk) and successfully achieved the expected high flow rate (> 1 L/min) and binding capacities ($>10^8$ PFU). Even larger binding capacities could be obtained by the stacking of monolithic disks. The corresponding filtration method, MAF(Big), is simple, rapid and effective. Almost all seeded bacteriophage MS2 in 10-L tap water could be recovered in 15 min resulting in a concentration factor of 500. 40.2% of ΦX174, 12.2% of hAdV2 and 67.2% could be recovered at the same time. By a two step concentration system MAF(Big)-CeUF, a volumetric concentration factor of 10^4 could be achieved. In analysis of the virus from environmental samples by MAF(Big)-CeUF/PCR, the main obstacle was found to be the inhibitory effect in PCR caused by co-concentrated components. We proved that the inhibitors can be effectively removed by a nucleic acid

extraction method, e. g. Nuclisens Magnetic Extraction kit. Therefore, with such a nucleic acid extraction method, the efficiency for MAF(Big)-CeUF in concentrating of viruses from environmental samples could be appropriately assessed and higher recovery rates can be expected in the future.

3.3 Fast and efficient concentration of viruses from large volumes of water by a three-step system

Waterborne diseases arise from the contamination of water, either by pathogenic viruses, bacteria or protozoa. In most cases, concentrations of viruses are very low in the ambient environment and even lower than the LODs of detection methods. However, viruses are 10 - 10,000-fold more infectious than pathogenic bacteria at similar exposures. For example, exposure to 10 viral particles is enough to cause illness for a child and only 1 infectious unit of rotavirus is enough to cause infection for an adult with no antibodies against this virus. Moreover, the long-term persistence in water and the moderate resistance to disinfection methods are further characteristics of waterborne viruses. Treatment facilities often fail to ensure the complete disinfection of viral pathogens. Human enteric viruses in water cause several illnesses, such as gastroenteritis, meningitis, hepatitis, *etc*. From the epidemiological reports, many water-associated outbreaks were caused by water-transmitted viruses. As proposed by the WHO, there should be typically less than one organism per 10^4 - 10^5 liters in drinking water[11]. However, quantification methods for viruses are only developed for small volumes and presently the enrichment systems dealing with such large volumes of water are still missing.

In processing of 10 m^3 water to 1 mL, a volumetric concentration factor of 10^7 needs to be achieved. Thus, a combination of more concentration steps is necessary. For this purpose, we designed a three-step enrichment system combining ultrafiltration (UF), monolithic adsorption filtration (MAF) and centrifugal ultrafiltration (CeUF). The schematic principle is shown in Fig. 33. In our previous studies, the combination of ultrafiltration and MAF(Small) was shown to be very effective and promising in dealing with water samples of about 10 L. To process large-volume water samples (> 10 m^3), UF(Small)-MAF(Small) have to be up-scaled. In this up-scaled three-step concentration

method, primary concentration is processed by ultrafiltration, whereby all particles larger than the pore size of the membrane (20 nm) are enriched within the retentate. In the secondary step, viruses are captured on to a monolithic column, while other components in the matrix are discarded after passing through the column. Centrifugal ultrafiltration (CeUF) is selected as a third step to achieve a final volume of 1 mL, due to its rapidity and robustness. A proof-of-principle study was carried out to test the route for concentrating viruses from large-volume water sample.

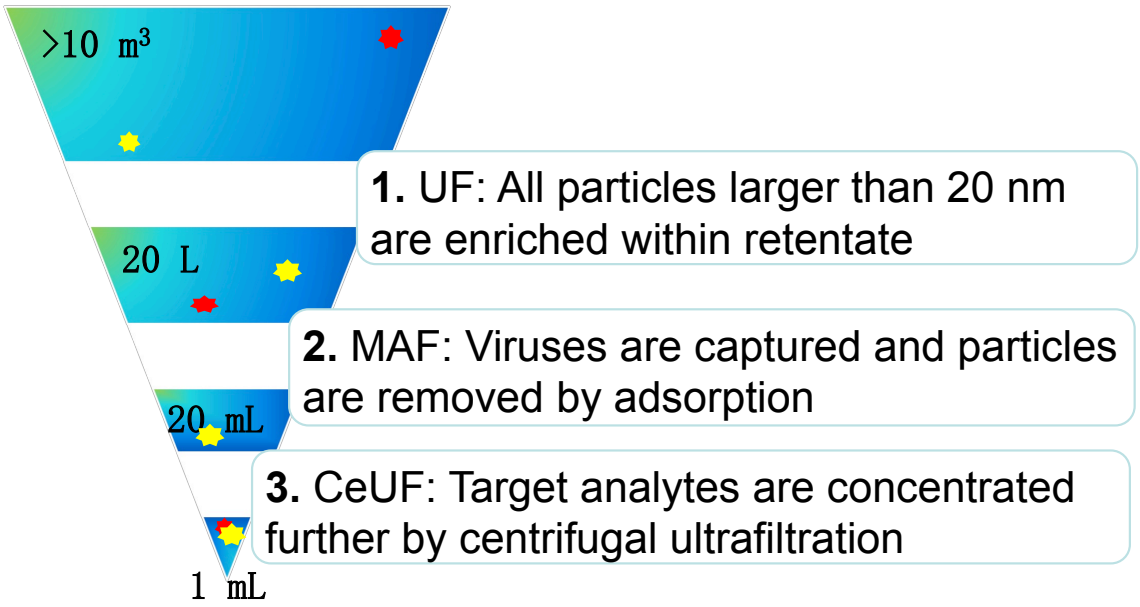

Fig. 33: Schematic diagram of the three-step concentration route

3.3.1 Description of the 3-step concentration route

In order to rapidly reduce large volumes of tap water (> 1 m^3), a ultrafiltration instrumentation for automated sampling was established. The instrumentation was developed in the PhD work of Dr. Martin Rieger[187]. The used ultrafiltration module was a multibore hollow fiber membrane with a nominal pore size of 20 nm and a membrane area of 6 m^2. The permeability of this module was about 1000 L/m^2. The retentate containing the concentrated viruses was eluated by backflushing at 2.5 bar into a volume of 20 L, which was 1.4 fold of the dead volume (14 L) in the closed loop of the UF

system. Having its own power generator on board and being transportable with a truck palette, the system can be set up on site. It can be operated in crossflow (CF-UF) as well as dead-end mode (DE-UF). For water with high turbidity (e.g. surface water), which may easily cause clogging of the membrane, operation in crossflow mode will reduce the risk[69]. However, in processing of water with low turbidity, e.g. tap water, a flow rate of 1724 L/h was achieved by DE-UF, which is much higher than by CF-UF (984 L/h). The rapidity of an analytical method is important for carrying out corresponding action at the early stage of water treatment, which is considered to be the most effective way to minimize microbial risk in consumption of drinking water[48]. On the other hand, a longer dwell time while processing large volume of sample would also lead to inactivation of microorganisms. Therefore, for low turbidity water (like tap water), DE-UF mode is preferred, while for high turbidity water, CF-UF mode is better.

In the first step, sample volume was reduced dramatically to 20 L. All particles larger than the pore size of 20 nm were kept in the retentate. To get the microorganisms and the viruses out of these components of the matrix, an adsorption-elution mechanism based MAF(Big) was selected as a secondary step. The MAF(Big), consisting of monolithic disks with a diameter of 3.86 cm and a length of 1.0 cm, was suitable for concentrating viruses from a 20-L concentrate of UF. Viruses were captured and the matrix was discharged. Captured viruses were eluted by 20 mL of BEG buffer (pH 9.5). The MAF step served not only as a further concentration but also as a purification step. In the third step, CeUF was selected due to its rapidness and robustness. As described previously, centrifugal filters with 50,000 MWCO promised a fast and effective method to purify the analytes.

3.3.2 Preliminary test

10 m^3 of tap water spiked with MS2 stock solution was continuously concentrated

by this route. The initial concentration of MS2 in 10 m^3 water was lower than the LOD of RT-qPCR which was 79.5 GU/mL. The MS2 concentration of 1.35 GU/mL was calculated from the concentration in the stock solution and the dilution factor. At first, the water was processed by DE-UF at a flow rate of 1700 L/h. The concentration of MS2 in the eluate of UF was still lower than the LOD of RT-qPCR. After applying the MAF(Big) step, it became detectable. A concentration of 1.21×10^5 GU/mL was quantified by RT-qPCR. After CeUF, an increased concentration (3.26×10^5 GU/mL) was found. As shown in Table 18, the increase of MS2 concentration after each enrichment step in this combined concentration method is in consistence with the efficiency of every step when tested separately. MS2 was continuously concentrated by this route from 10 m^3 to a final volume of 1 mL in 7 hours. The whole concentration and detection procedure was finished within 11 h. By this three-step enrichment route, a recovery rate of 11.2 (±3.1)% and a concentration factor of 2.4×10^5 were achieved. The main features of each enrichment step are shown in Table 18.

Table 18: Summary of the consecutive concentration of MS2 by CUF, MAF, and CeUF.

	Sample volume [L]	Input concentration [GU/mL]**	Elution volume [L]	Elution concentration [GU/mL]**	Time [h]	Recovery in total [%]	Enrichment factor in total
UF	10,000	1.35	20	N.D.	5.9	-	-
UF+MAF	20	N.D.	0.027	1.21×10^5	0.5	24.1 ±8.3	9.0×10^4
UF+MAF+CeUF*	0.01***	1.21×10^5	0.001	3.26×10^5	0.5	11.2 ±3.1	2.4×10^5

*: Centrifugal ultrafiltration (Amicon Ultra-4, 50kDa NMWL, Millipore);
**: quantified by RT-qPCR;
***: a portion of all from MAF step (39.2 mL). N.D.: not detectable

Compared to the concentration route consisting of UF, CF-UF(small) and MAF(small) established in our previous study, the recovery rate in total was highly improved from 0.1%[187] (by UF-CF-UF(small)-MAF(small)) to 11.2 (±3.1) % (by

UF-MAF(Big)-CeUF). On the one hand, compared to MAF(Big), the lower binding capacity of MAF was supposed to be a limiting factor in UF-CF-UF(small)-MAF(small). On the other hand, in the UF-CF-UF(small)-MAF(small), two steps of ultrafiltration were used continuously. Both of them are based on a size-exclusion mechanism. Microorganisms were agglomerated together with particles during UF procedure, which could form particles larger than 100 μm[188]. Particles bigger than the pore size of the monolith (21 μm) were kept on the top of the column. These particles would block the column and microorganisms agglomerated inside failed to be captured. Both factors would decrease the efficiency of the MAF(Small) step and the total recovery. In the UF-MAF(Big)-CeUF route, there is an adsorption-elution based enrichment between two ultrafiltration steps. Aggregation of viruses could be reduced during the MAF(Big) procedure[183]. Therefore in these combined concentrating routes, the 'sandwich' structure showed better performance than continuous repetition of the same mechanism.

Although in this preliminary test the enrichment method was tested using MS2 bacteriophage as a surrogate, it can be applied to other viruses. MAF(Big) also worked in the enrichment of adenoviruses and murine noroviruses. UF and CeUF are size-exclusion methods. All microorganisms larger than the pore size of the membrane would be concentrated. These properties of such a combination method enable simultaneous enrichment of various organisms. In other words, the diversity of organisms in the original sample remains in the final 1-mL eluate. This is important for high-throughput detection methods, e.g., microarray technology, and also in pathogenic virus identification.

3.3.3 Testing real samples in the field

All concentration systems instruments were transported to our project partners from the German Federal Environment Agency (Umweltbundesamt, UBA) to test under real conditions, e. g., sampling outdoor, working with high turbidity water and real viruses.

Fig. 34: UF instrumentation at the artificial stream and pond simulation system in Marienfelde, Berlin.

3.3.3.1 Challenges of water with high particle loading in outdoor

Primary filtration by UF

The concentration system was tested with not only the MS2 bacteriophage but also other viruses. 35m^3 of processed ground water (manganese and iron eliminated) floating in an outdoor channel was spiked with 0.01% (3 L) mechanically treated sewage and stock solutions of murine noroviruses. Water was firstly filtrated with a pre-filter equipped with nylon meshes of 25 µm to remove big particles and suspended matter. The ultrafiltration was operated in CF-UF mode due to the high turbidity of the water (Table

19). However, the flow rate kept decreasing during the filtration procedure. At the beginning the flow rate was 1.15 m^3/h. When 7 m^3 of water was filtrated, the flow rate decreased to 0.41 m^3/h (Fig. 35). In this case, backflushing (2.5 bar) was used twice to remove the particles attached on the membrane (Eluate 1 and Eluate 2 were obtained). Afterwards, 3 m^3 was processed additionally by CF-UF, but due to high backpressure from the blocked membrane, the pump was overloaded. Then the system was backflushed twice (Eluate 3 and Eluate 4 were obtained). The limitation of CF-UF in processing water of high particle loading (turbidity ≥ 0.3) was 10-m^3. Eluates of UF were further concentrated.

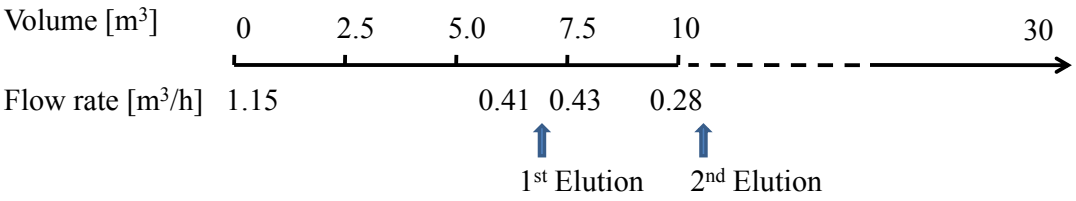

Fig. 35: Experiment process of the experiment in UBA

The turbidity values are shown in the Table 19. The turbidity was tested one week later due to technical problems. To avoid the formation of colloid during this time, samples were acidified. In such a case the turbidity values were underestimated. But the turbidity of water in the channel was still 10 times higher than the tap water in Munich. The high turbidity of input sample led to blockage of the membrane of UF, which accounted for the decrease in flow rate. It also resulted in an even higher turbid eluate from UF and more trouble in the following steps, such as blocking of pores of MAF(Big), competition in binding sites and inhibitory effect in PCR detection, *etc*.

Table 19: Turbidity values of samples

	Turbidity*(FNU/NTU)
Tap water	<0.01
Water in channel	0.29 ± 0.07
Concentrate from 1st Elution	19 ± 4
Concentrate from 2nd Elution	24.6 ± 0.5

*: Measured at pH 3

Further enrichment by three concentration methods in comparison

To meet these challenges, two more concentration methods were also included. Descriptions of these three concentration methods are listed in Table 20.

Table 20: Concentration methods involved

	Composition	**Abbreviation**
Concentration method 1 (CM1)	Ultrafiltration – Monolithic adsorption filtration – Centrifugal ultrafiltration	UF-MAF-CeUF
Concentration method 2 (CM2)	Ultrafiltration – Glass wool filtration – Flocculation	UF-GW-FL
Concentration method 3 (CM3)	Ultrafiltration – Glass wool filtration – Monolithic adsorption filtration – Centrifugal ultrafiltration	UF-GW-MAF-CeUF

At the beginning, concentration method 1 (CM1) was used. Eluate 1 was pumped through a 3-disk monolithic column at a flow rate of 0.5 L/min. But after 2L of eluate passing through, the column was blocked by particles. These three disks were eluted separately by 20-mL beef extract-glycine buffer. The eluates of MAF were further concentrated by centrifugal filter.

In the concentration method 2 (CM2), the rest of Eluate 1 and 10 L out of Eluate 3 were pumped through the first glass wool column. However, the glass wool column was also blocked. Therefore, a second glass wool column was used for the rest of Eluate 3. The eluates of the glass wool column were further concentrated by flocculation.

With the concentration method 3 (CM3), the filtrate of the first glass wool column was concentrated by a 2-disk monolithic column. These two monolithic disks were eluted separately. The eluates of MAF were further concentrated by a centrifugal filter.

3.3.3.2 Results and discussion: comparative study of three concentration methods

CF-UF-MAF-CeUF (CM1)

The CM1 was designed for the concentration of viruses in tap water. Because of high amounts of particles contained in the eluate of CF-UF, in this test, only 2 L was filtrated by MAF(Big) (Table 21, 22). As the sample volumes were reduced during the three-step procedure, the corresponding concentrations increased, resulting in increasing enrichment factors. With UF the recovery rate for murine noroviruses (49.48%) was higher than human adenoviruses (1.29%). In contrast, with MAF(Big), the recovery rate for human adenoviruses (24.16%) was higher than murine noroviruses (9.97%). CeUF showed the highest efficiency (99.21% and 97.41%). Almost all seeded viruses could be recovered. Differences of noroviruses and adenoviruses in size and surface properties would account for different efficiencies in each step. This fact proved the importance of including concentration techniques of different mechanisms in one concentration system, which can compensate the drawbacks of a single method in concentrating a wide range of viruses.

Table 21: Main features of human adenoviruses in concentration method CF-UF-MAF-CeUF (CM1) (m = 1, n = 3)

	Sample volume [L]	**Input concentration [GU/mL]**	**Elution volume [L]**	**Elution concentration [GU/mL]**	**Recovery of single step [%]**	**Recovery in total [%]**	**Enrichment factor in total**
CF-UF	7,000	2.65	20	12.0	1.29	1.29	4.5
MAF	2	12.0	0.06	96.6	24.16	0.31	51.5
CeUF	0.06	96.6	0.003	1.92×10^{3}	99.21	0.31	9.53×10^{2}

Table 22: Main features of murine noroviruses in concentration method CF-UF-MAF-CeUF (CM1) (m = 1, n = 3)

	Sample volume [L]	**Input concentration [GU/mL]**	**Elution volume [L]**	**Elution concentration [GU/mL]**	**Recovery of single step [%]**	**Recovery in total [%]**	**Enrichment factor in total**
CF-UF	7,000	5.49	20	950	49.48	49.48	1.73×10^{2}
MAF	2	950	0.06	3.16×10^{3}	9.97	4.93	1.03×10^{3}
CeUF	0.06	3.16×10^{3}	0.003	6.15×10^{4}	97.41	4.81	1.64×10^{4}

CF-UF-GW-FL (CM2)

In CM2, all 38-L eluates from CF-UF were processed by glass wool filtration (Table 23, 24). Similar to the result by MAF(Big), the recovery rate of adenoviruses (1.3%) was also higher than that of murine noroviruses (0.58%) with glass wool (-FL). This indicates the similarity of surface properties between glass wool and monolithic columns. But the recovery efficiencies by MAF were much higher. It could be caused by the difference in structure of materials. Although a larger volume was processed by CM2, the concentration factor (542.32) in total was still lower than that by CM1.

Table 23: Main features of human adenoviruses in the concentration system CF-UF-GW-FL (CM2) (m = 1, n = 3)

	Sample volume [L]	**Input concentration [GU /mL]**	**Elution volume [L]**	**Elution concentration [GU /mL]**	**Recovery of single step [%]**	**Recovery in total [%]**	**Enrichment factor in total**
CF-UF	10,000	2.65	40	6.58	0.99	0.99	2.5
Glass wool	38	6.58	0.04	77.7	1.30	0.01	29.28

Table 24: Main features of murine noroviruses in the concentration system CF-UF-GW-FL (CM2) (m = 1, n = 3)

	Sample volume [L]	Input concentration [GU /mL]	Elution volume [L]	Elution concentration [GU /mL]	Recovery of single step [%]	Recovery in total [%]	Enrichment factor in total
CF-UF	10,000	5.49	40	557	40.61	40.61	101.5
Glass wool	38	557	0.04	2.98×10^3	0.58	0.23	542.32

CF-UF-GW-MAF-CeUF (CM3)

While most particles were largely retained on the glass wool column, viruses were not effectively recovered by glass wool. The idea to use glass wool as a pre-filter for MAF(Big) was tested in CM3. From this point of view, the amount of viruses attached on glass wool column should be estimated at first. In Table 25, the concentrations of viruses in input, e.g. eluate of CF-UF, and in the filtrate of glass wool filtration are compared. Surprisingly, the concentrations in the filtrate (10.3 cp/mL for hAdV2, 2.06×10^3 cp/mL for MNV) are higher than in input (6.58 cp/mL for hAdV2, 557 cp/mL for MNV), which resulted in a negative adsorption rate. The reason could be that large amounts of particles including inhibitors were retained on the column. There were less inhibitors for PCR in filtrate of glass wool. With lower inhibitory effect, a higher number could be shown by PCR when the actual amounts are the same or even less. This fact indicates the purification effect of the glass wool column as a pre-filter.

Table 25: Glass wool as a pre-filter to remove particles

	Sample volume [L]	Input concentration [GU/mL]	Flow-through concentration [GU/mL]	Adsorption rate
hAdV2	38	6.58	10.3	-56.5%
muNV	38	557	2.06×10^3	-269.8%

Including glass wool as a pre-filter of MAF(Big), much larger volume of eluate from UF could be processed by CM3 (30 L) than that by CM1 (2 L) (Table 26, 27). Moreover, much higher concentration factors were achieved by CM3 (1.68×10^3 for hAdV2 and 6.93×10^3 for muNV) compared to CM2 (29.28 for hAdV2 and 542.32 for muNV). These improvements benefit from glass wool as pre-filter for particle removal, which increased the filtration volume of CM3.

Table 26: Main features of human adenoviruses in concentration system CF-UF-GW-MAF-CeUF (CM3) (m = 1, n = 3)

	Sample volume [L]	**Input concentration [GU /mL]**	**Elution volume [L]**	**Elution concentration [GU /mL]**	**Recovery of single step [%]**	**Recovery in total [%]**	**Enrichment factor in total**
CF-UF	10,000	2.65	40	6.58	0.99	0.99	2.5
MAF	30	10.3	0.04	60.5	0.61	0.01	35.2
CeUF	0.04	60.5	0.002	3.19×10^3	263	0.03	1.68×10^3

Table 27: Main features of murine noroviruses in concentration system CF-UF-GW-MAF-CeUF (CM3) (m = 1, n = 3)

	Sample volume [L]	**Input concentration [GU /mL]**	**Elution volume [L]**	**Elution concentration [GU /mL]**	**Recovery of single step [%]**	**Recovery in total [%]**	**Enrichment factor in total**
CF-UF	10,000	5.49	40	557	40.61	40.61	101.5
MAF	30	2.06×10^3	0.04	8.84×10^2	0.06	0.08	2.94×10^2
CeUF	0.04	8.84×10^2	0.002	2.36×10^4	133	0.10	6.93×10^3

Comparison of three concentration system in recovery and concentration factor

To compare the efficiencies of three concentration systems, concentration factors and recoveries are shown in Fig. 36 and Fig. 37. From the charts, it can be concluded that

the CM1 is the best in recovery. CM1 and CM3 achieved similar concentration factors, which were higher than CM2. Compared to CM3, CM1 is a simple and rapid concentration route. For all three routes, recoveries of murine noroviruses are better than human adenoviruses.

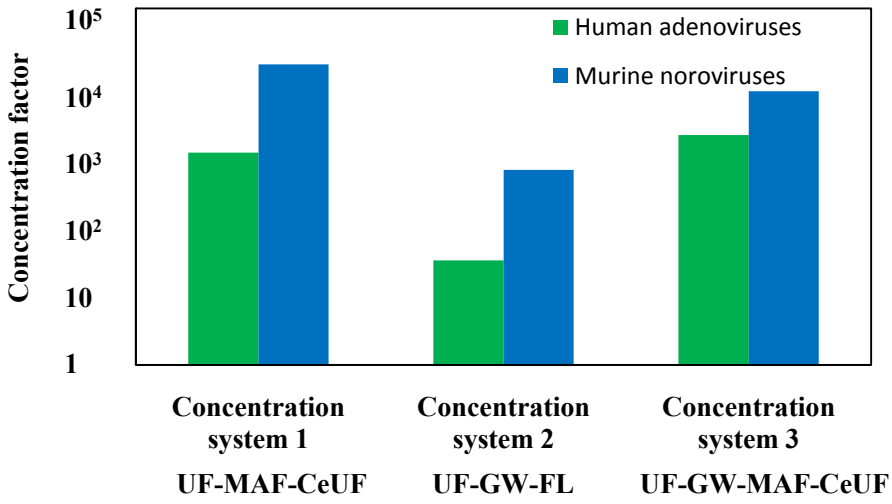

Fig. 36: Comparison of concentration factors of three concentration methods.

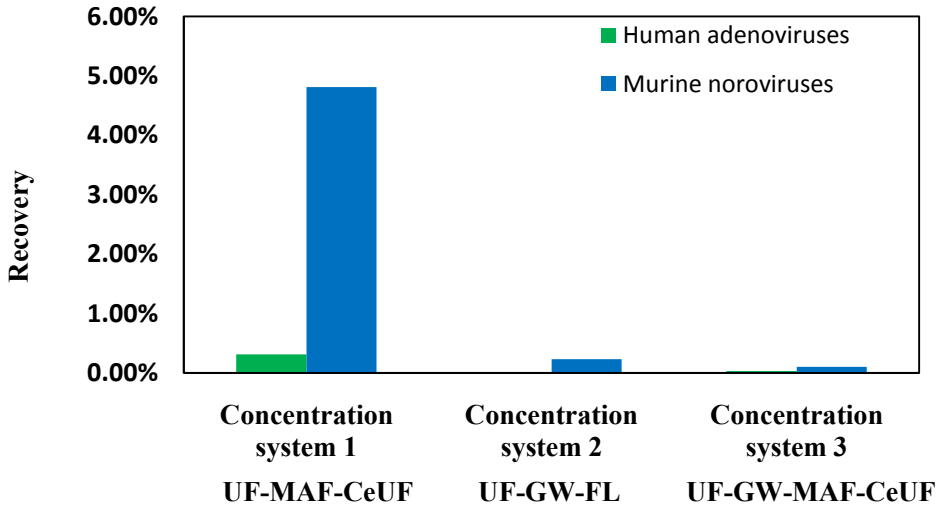

Fig. 37: Comparison of recoveries of three concentration methods.

3.3.3.3 Summary

It was proven that various viruses could be simultaneously concentrated by the combination route UF-MAF(Big)-CeUF. Among the three tested routes, MAF-CeUF is the best to combine with UF due to its rapidity and effectiveness in purification. In this test, the concentration system designed for tap water was tested by water with extremely high turbidity, which is more than ten times higher than that in tap water. Accumulated particles were shown to be a limiting factor in processing water of large volumes.

3.3.4 Concentration of viruses from 30-m^3 tap water and ground water

From the last experiment, it had been proven that various viruses could be simultaneously concentrated by the concentration system UF-MAF(Big)-CeUF. The diversity of the original sample would be retained in the final eluate. To simulate the real conditions and to sample diverse viral particles, 30m^3 of tap water and ground water were reduced to about 1 mL and analyzed by pyrosequencing.

3.3.4.1 Process of 30-m^3 tap and ground water by UF-MAF(Big)-CeUF

Tap water and ground water were taken from IWC, TUM. UF was operated in DE-UF mode at a flow rate of about 1700 L/h. 30m^3 of water was reduced into 1 mL by the developed three-step concentration method within 20 hours. 1 mL out of 20 mL eluate of UF-MAF(Big) and 1 mL of final concentrate after UF-MAF(Big)-CeUF were collected for pyrosequencing analysis (Sample 1 and Sample 2). In the experiment with ground water, two monolithic disks were used in the MAF(Big) step and eluated separately, as described in Fig 28. After CeUF, The final concentrate 1 and 2 were collected for following pyrosequencing analysis (Sample 3 and Sample 4). The ground water, concentrate from UF and flow through of MAF were also collected to measure amounts of metallic elements by atomic absorption spectrometry (as shown in Table 28).

Table 28: Concentration of metallic elements in ground water, concentrate from UF and filtrate of MAF

Conc. [mg/L]	Ground water	Eluate of CF-UF	Flow-through of MAF
[Mg]	20.5	19.9	19.6
[Ca]	78.9	77.6	74.8
[Mn]	0.0095	0.0306	0.00683
[Cr]	0.0006	0.0023	0.001
[Al]	0.00668	0.061	0.019
[Zn]	0.00398	0.0454	0.031
[Cu]	0.00336	0.0384	0.00524
[Fe]	0.037	0.522	0.0717

Compared to surface water, ground water is a clear water resource due to the filtration effect from soil and rock. But it can be rich in in dissolved solids. The changing of concentrations of metallic elements in ground water, concentrate from UF and filtrate of MAF showed similar trends. The concentrations of magnesium and calcium remained at the same levels. However the concentrations of other elements, e.g. Mn, Cr, Al, Zn, Cu and Fe, increased in eluate from UF and decreased in the filtrate of MAF. This is similar to the behavior of the biocolloids, e.g. viruses, during this concentration route. The results lead to the assumption that some inorganic colloids or particles larger than the pore size of the UF module were concentrated during UF and also stopped on the monolithic column. Therefore not only biocolloids but also inorganic colloids were concentrated by this route.

3.3.4.2 (RT)-PCR and Next Generation Sequencing analysis

Reduce inhibitory effect by dilution

To examine the inhibitory effect, different nucleic acid extraction methods were used,

e.g. the ViralXpress Nucleic Acid extraction kit (Millipore, Schwalbach, Germany) and the Viral RNA Mini Kit (Qiagen, Hilden, Germany). Using the ViralXpress kit**,** 150-µL portions were taken out of four samples and spiked with MS2 stock solution to test the matrix effect from concentrated tap water and ground water. Ultrapure water spiked with the same amount of MS2 was used as a positive control. SYBR Green RT-qPCR was used for quantification of MS2. Using the Viral RNA Mini Kit, nucleic acids were extracted according to the manufacturer's instructions (analyzed by Dr. René Kallies, Bonn University). From each column, nucleic acids were eluted in 2 × 50 µl buffer. Each of the samples (195 µl) was spiked with 5 µl (containing approx. 100,000 copies) of Culex-Y-virus (CYV) RNA. The same amount of CYV RNA was spiked into 195 µl of molecular grade water that was used as a control. CYV RNA was detected using a one-step TaqMan real-time RT-PCR assay[189]. Different signals from these four samples and positive control by these two analysis methods are shown in the following chart Fig. 38 and Fig. 39.

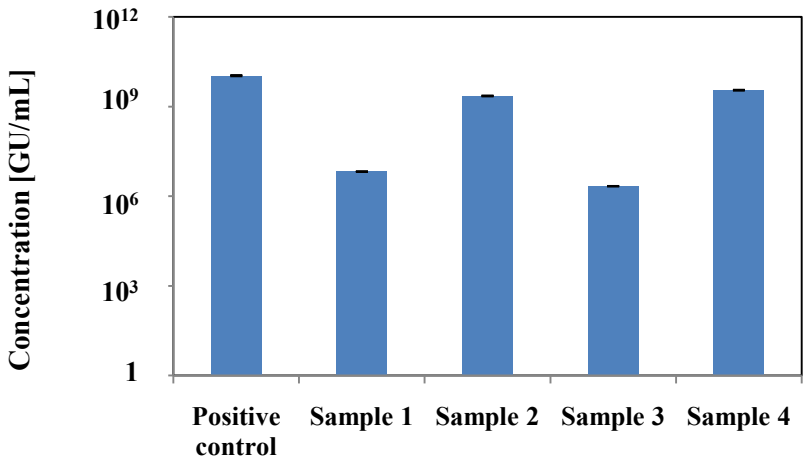

Fig. 38: Quantitative results of the same amount of MS2 from SYBR Green qPCR in different matrixes using ViralXpress nucleic acid extraction methods

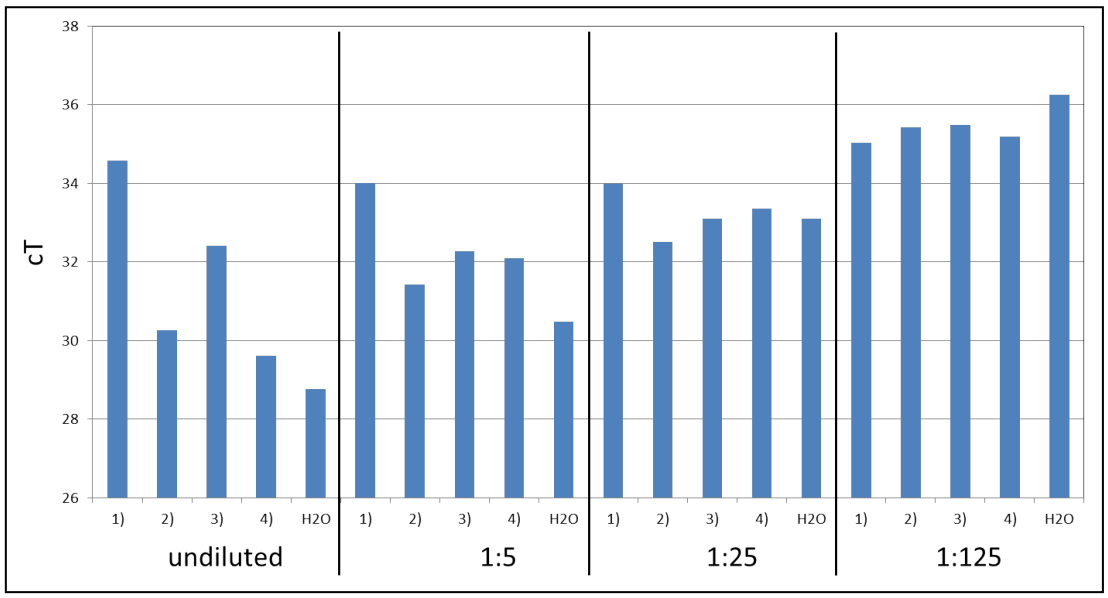

Fig. 39: Quantitative results of the same amount of CYV RNA (undiluted, 1:5, 1:25, 1:125 dilutions) from TaqMan real-time RT-PCR in different matrices using the Viral RNA Mini Kit for nucleic acid extraction (provided by Dr René Kallies)

The results of the two methods showed similar trends. Sample 1 showed a higher inhibitory effect than sample 2, which indicates inhibitory components were co-concentrated further during the CeUF step. Sample 3 showed higher inhibitory effects than sample 4, indicating that there were more inhibitory components distributed on the top disk. However, there could also be more viruses than on the second disk. Samples were diluted to reduce the inhibitory effect. Five-fold dilutions (1:5, 1:25, 1:125) were prepared for each sample (including control) and nucleic acids were extracted from 140 µl of each sample and dilution. The least inhibitory effect with a similar sensitivity for all samples was seen at a 1:25 dilution. As a result, 1:25 diluted samples were used for (RT)-PCR and Next Generation Sequencing (NGS).

(RT)-PCR reactions

Samples were analyzed for the presence of viral nucleic acids using 31 different (RT)-PCR protocols specific for detection of viruses belonging to 20 eukaryotic virus

families. None of the 31 (RT)-PCR assays showed a specific amplification product at the correct size after gel electrophoresis (most showed a smear or a multiple band pattern).

Next Generation Sequencing (NGS)

RNA was reverse transcribed using a random octamer oligonucleotide linked to a specific primer sequence. RNA was removed by treatment with RNase H and PCR was performed using a 1:9 mixture of the above primer and a primer targeting the specific sequence. 454 sequencing of random amplified nucleic acids resulted in a total of 62,872 reads. Reads were assembled and analyzed for virus-related sequences (as above). No virus-related sequence could be identified.

Bioinformatical analysis

Amplicons were deep sequenced using the Roche 454 GS Junior platform. In total, 96,130 sequencing reads were obtained. Reads were assembled in order to reduce redundancies and were then analyzed for the presence of virus-related sequences using different blast algorithms. Five sequences were identified with significant similarities to phage sequences from the final concentrates of $30m^3$ ground water after UF-MAF(Big)-CeUF.

3.3.4.3 Summary

In NGS, both runs resulted in an average number of reads. In addition, read assembly resulted in a high number of contigs as well as singletons (reads that were not assembled into a contig; one contig represents at least two reads that overlap by at least 40 nt). Approximately 100 sequences were virus positive when blasted against the virus database alone. All of these sequences were then blast-analyzed against the non-redundant database. Most of these sequences were of bacterial origin and from marine organisms. Five sequences related to phage genomes could be identified. All of

them were in samples from ground water. Metagenomic analyses would be performed later.

The large number of reads in sequencing indicates a high diversity of different sequences in the samples. Therefore, the water concentration method is suitable for cultivation independent amplification and sequencing approaches. On the other hand, it was shown that using the nucleic acid extraction method (Viral RNA Mini Kit Qiagen) resulted in similar inhibitory effect in PCR as using the ViralXpress nucleic acid extraction method, which was proven to be insufficient to remove inhibitors. Therefore, higher sensitivity and more information could be expected using more specific nucleic acid extraction methods, such as NucliSens Magnetic Extraction kit.

3.3.5 Summary

We proposed a three-step concentration method (UF-MAF-CeUF) for concentrating viruses from large volume of water (> 10 m^3). A concentration factor of 2.4×10^5 was obtained within 7 hours when concentrating bacteriophage MS2 from 10-m^3 tap water. In a comparative study, MAF-CeUF was proven to be the best choice to combine with UF due to its rapidness and effectiveness in purification. It was proven that in concentrating a wide range of viruses, including concentration techniques of different mechanisms in one combination system is important to compensate for the drawbacks of a single method. Accumulated particle loading and inhibitors for PCR detection were shown to be main obstacles when processing large volumes of water for UF-MAF-CeUF. However, we have proven that a more specific nucleic acid extraction method could facilitate solving inhibitory problems. With the UF-MAF-CeUF, 30m^3 of tap or ground water could be reduced to 1 mL in 20 hours. Various viruses and microorganisms could be simultaneously concentrated from the large volume matrix. Five sequences related to phage genomes could be identified by NGS in concentrates from ground water. Due to this high diversity maintained in the final eluate of this concentration system, it is suitable for combination with sequencing approaches and pathogenic virus identification.

Conclusions and Outlook

4 Conclusions and Outlook

Enteric viruses in water pose a great threat to human health. Several illnesses and water-associated outbreaks were caused by waterborne transmitted viruses. However, the concentrations of viruses are too low to be detected directly. The viruses need to be concentrated from large volume of water. Therefore, rapid and effective methods were developed based on MAF for the concentration and purification of waterborne viruses.

The monolithic columns consist of a hydrolyzed macroporous epoxy-based polymer, which was prepared in different sizes, e. g. with diameters of 4.5 mm and 35.5 mm. High recoveries were achieved by MAF(Small) for the bacteriophage MS2 110 (±19)% (MS2 concentration = 23 - 1100 PFU mL^{-1}), 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 CF-UF(Small). MS2 spiked in 10 L of tap water was concentrated by the CF-UF(Small)-MAF(Small) method with a volumetric factor of 10^4 in 33 min. Because of the adsorption of the examined viruses to the macroporous surface of the MAF column at pH 3, concentrated matrix components by CF-UF can be removed. Furthermore, the detection limit for quantification of bacteriophage MS2 by RT-qPCR could be improved from 79.47 to 0.0056 GU mL^{-1} by a factor of 1.4×10^4.

Upscaled monolithic columns (diameter: 35.5 mm) were prepared in a cost-effective way (<2 Euro/disk) and successfully achieved the expected high flow rate (> 1 L/min) and binding capacities (>10^8 PFU). Even larger binding capacity could be obtained by stacking of monolithic disks. Almost all seeded bacteriophage MS2 in 10-L tap water could be recovered by MAF(Big) in 15 min resulting in a concentration factor of 500. By a two-step concentration method MAF(Big)-CeUF, a volumetric concentration factor of 10^4 could be achieved.

UF-MAF(Big)-CeUF was proposed for concentrating viruses from large volume of

water (> 10 m^3). A concentration factor of 2.4×10^5 was obtained within 7 hours when concentrating bacteriophage MS2 from 10 m^3 of tap water. In a comparative study, MAF-CeUF was proven to be the best choice to combine with UF due to its rapidity and effectiveness in purification. Although accumulated particle loading was shown to be a limiting factor when processing large volume of water with high turbidity, 30 m^3 of tap or ground water could be reduced to 1 mL in 20 hours by the designed three-step concentration method (UF-MAF-CeUF). Various viruses could be simultaneously concentrated from the large volume matrix. Five sequences related to phage genomes could be identified by NGS in concentrates from ground water.

High efficiencies were achieved by these MAF involved concentration methods from spiked samples while during analysis of viruses in concentrates from environmental samples or large volumes of water, the main obstacle was found to be the inhibitory effect in PCR. However, we proved that the inhibitors could be removed by an effective nucleic acid extraction method, i.e. Nuclisens Magnetic Extraction kit. Therefore, such a nucleic acid extraction method in combination with upstreamed concentration methods could be used for virus quantification in environmental samples, e.g. surface water, ground and surface water with large volumes. Higher recovery rates could be expected in the future by using the appropriate nucleic acid extraction method in combination of multi-step concentration methods. .

MAF is effective, fast, inexpensive and easy to apply. During such an adsorption-elution procedure, viruses could be concentrated and separated from the matrix of environmental water, which is important for rapid bioanalytical methods like PCR. With UF, microorganisms larger than the pore size of the multibore ultrafiltration membrane can be enriched. By the 2-step/3-step combination methods consisting of MAF and UF (CeUF), a wide range of viruses could be simultaneously concentrated and high concentration factors were obtained. It was shown that in concentrating a wide range of viruses, including concentration techniques of different mechanisms in one combination

system is important to compensate for the drawbacks of a single method. Combining with PCR, the rapid and effective analytical method could be used to monitor microbial levels in raw water and applied for risk assessment of drinking water. The water safety plan of the WHO would clearly profit from such analysis methods for viruses. It could facilitate carrying out corresponding action at the early stage of water treatment, which is considered to be the most effective way to minimize microbial risk[48].

We have proven that the diversity of viruses in the original sample can be maintained during compression of the sample volume. Besides qPCR and RT-qPCR, DNA or antibody microarrays[171] are possible detection platforms for multiplexed analyses. If various viruses in environmental or large volume samples could be simultaneously concentrated and detected, it would be a very powerful analysis method.

Experimental Section

5 Experimental section

5.1 Instruments and materials

5.1.1 Instruments

Analytical balance Mettler PB3000 Delta Range (Mettler-Toledo, Giessen, Germany)

Analytical balance Sartorius (Sartorius, Göttingen, Germany)

Autoclave, Laboklav 55MV-FA (SHP Steriltechnik, Magdeburg)

Centrifuge Universal 320R (Hettich, Tuttlingen, Germany)

Cytometer Cell LabQuantaTM SC Flow Cytometer (Beckman Coulter, Krefeld, Germany)

Deep well pump (Sumoto ONK 100, Nowax, Germany)

LightCycler 480 (Roche diagnostics, Mannheim, Germany)

Incubator (Binder, Tuttlingen, Germany)

Incubator B 290 (Heraeus, Hanau, Germany)

Microplate shaker EAS 2/4 (SLT Labinstruments, Crailsheim, Germany)

Millipore water purification system Milli-Q plus 185 (Millipore, Schwalbach, Germany)

pH meter SG2 (Mettler-Toledo, Giessen, Germany)

Peristaltic pump (VP-Antrieb, Vario-Pumpsystem, Glattbrugg, Switzerland)

Peristaltic pump (PD 5206, Heidolph Instruments, Schwabach, Germany)

Photometer Specord 250 plus (Analytik Jena, Jena, Germany)

Pipettes Eppendorf Research Plus (100 - 1000 μL, 10 - 100 μL, 0.5 - 10 μL) and Multipette (2 - 5 mL) (Eppendorf, Hamburg, Germany)

Turbidometer, Turb 430IR (WTW, Weilheim)

Ultrasonic bath Sonorex RK 102 (Bandelin, Berlin, Germany)

Vortexer, Top Mix FB15024 (Fisher Scientific, Pittsburg, USA)

5.1.2 Materials

Amicon Ultra-15 Centrifugal Filters (PLQK Ultracel-PL membrane, 50 kDa, UFC905024, Millipore, Germany)

Amicon Ultra-4 Centrifugal Filters (PLQK Ultracel-PL membrane, 50 kDa, UFC805008, Millipore, Germany)

Glass column (Diameter 4.5 mm/26.5 mm) (7328-01, J. T. Baker, Philippsburg, NJ, USA)

LightCycler Multiwell plate 96 (0472969001, Roche, Mannheim)

Petri dish (632181, Greiner Bio One, Frickenhausen, Germany)

Plastic PD-tip (50mL, Carl Roth GmbH, Karlsruhe, Germany)

Pre-filter housing Big Blue (15-8055, Apic Filter GmbH, Merklingen, Germany)

Pre-filter element (FPO-20-BB20, Apic Filter GmbH, Merklingen, Germany)

Marprene tubing, I.D. 6.4 mm, O.D. 9.6 mm (9020064016, Watson Marlow, Cornwall, UK)

Tygon tubing, I.D. 1.3 mm, O.D. 3.02 mm (T3608-23, Saint-Gobain Performance Plastics, Charny, France)

Sample container 10 L Nalgene®, (216-8506, VWR, Darmstadt, Germany)

5.2 Chemicals and Reagents

5.2.1 Chemicals

1,4-Dioxane (42510, Sigma-Aldrich, Steinheim, Germany)

(3-Glycidyloxypropyl)trimethoxysilane, Purity: > 98% (440167, Sigma-Aldrich, Taufkirchen)

Agar-Agar, bacteriological (2266.3, Carl Roth GmbH, Karlsruhe, Germany)

Beef extract (6193160500, Merck, Darmstadt, Germany)

Beef extract (B4888, Sigma-Aldrich, Steinheim, Germany)

Boron trifluoride diethyl etherate purum (15719, Fluka, Buchs, CH)

DyNAmo™ cDNA Synthesis Kit (241, Thermo Scientific, München, Germany)

Ethanol (24194, Sigma-Aldrich, Steinheim, Germany)

Gelatin from cold water fish skin (G7041, Sigma-Aldrich, Steinheim, Germany)

Glycine (33226, Sigma-Aldrich, Steinheim, Germany)

Hellmanex solution (320.000, Hellma GmbH, Mühlheim, Germany)

Hydrochloric acid 37% (84422, Sigma-Aldrich, Steinheim, Germany)

LightCycler 480 SYBR Master I (04707516001, Roche, Mannheim)

Magnesium chloride hexahydrate (1.05832, Merck, Darmstadt, Germany)

Methanol (65548, Sigma-Aldrich, Steinheim, Germany)

mTBE - *tert*-butylmethyl ether (20249, Sigma-Aldrich, Steinheim, Germany)

Nitrogen 5.0 (Air Liquide, München, Germany)

NZCYM (X974.1, Carl Roth GmbH, Karlsruhe, Germany)

NucliSens Magnetic Extraction reagent (200293, Biomerieux, France)

NucliSens Lysis buffer (200292, Biomerieux, France)

Polypox R9, Polyglycerol-3-glycidyl ether (kindly provided as a gift by UPPC, Mietlingen, Germany)

Sodium carbonate (71351, Sigma-Aldrich, Steinheim, Germany)

Sodium chloride (13565, Sigma-Aldrich, Steinheim, Germany)

Sodium bicarbonate (S5761, Sigma, Germany)

Sodium hydroxide (S5881, Sigma-Aldrich, Steinheim, Germany)

Sodium hypochlorite solution, 10% (RT) (71696, Sigma-Aldrich, Taufkirchen, Germany)

Sulfuric acid (84720, Sigma-Aldrich, Steinheim, Germany)

Toluene (89677, Sigma-Aldrich, Steinheim, Germany)

TRIS, 2-Amino-2-(hydroxymethyl)-propan-1,3-diol, > 99% (T1378, Sigma-Aldrich, Steinheim, Germany)

Sodium bisulphate (13438, Sigma-Aldrich, Steinheim, Germany)

ViralXpress Nucleic Acid Extraction kit (5540, Millipore, Germany)

Buffers

Beef extract glycine buffer (BEG buffer)

1.5 g beef extract (3% w/v)

1.875 g glycine (0.5 M)

fill to 50 mL with ultrapure water

autoclave at 121°C

Carbonate buffer (pH 8.2)

31.8 mg Na_2CO_3

58.8 mg $NaHCO_3$

fill to 1 L with ultrapure water

SM buffer, (pH 7.5)

50 mL 1 M TRIS-HCl buffer, pH = 7.5

5 mL gelatine in H_2O (2% w/v)

5.8 g NaCl

2 g $MgSO_4 \cdot 6\ H_2O$

fill to 1 L with ultrapure water

autoclave at 121°C

TRIS-HCl buffer, (pH 7.5)

24.23 g TRIS

13.7 mL 37% HCl

fill to 200 mL with ultrapure water

5.2.2 Bacteria, Viruses and Primers

Bacteria:

Escherichia coli, DSM 5695 (Leibniz-Institut DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig).

Viruses:

Bacteriophage MS2, ATCC 15597, DSM 13767 (Umweltbundesamt, Berlin),

Human adenoviruses serotype 2 (hAdV2) were grown in human embryonal kidneycells (HEK 293T, DSMZ ACC635) in the biosafety level 2 (BSL-2) facility of the UBA,

Murine noroviruses type 1 (MNV-1) were grown in mouse leukaemic monocyte macrophage cells (RAW 264.7, ATCC TIB-71) in the BSL-2 facility of the UBA.

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 qPCR and calculated as genomic units (GU)/mL.

Primers:

REV-MS2-3-BIO (5'-CGT GGA TCT GAC ATA C-3', H 1865336-5076-1/1, Eurofins MWG Operon, Ebersberg),

FWD-MS2-3-DIG (5'-CGT GGC AAT AGT CAA A-3', 41-149-1/3, Eurofins MWG Operon, Ebersberg).

5.3 Procedures

5.3.1 Detection methods

5.3.1.1 Cultivation and quantification of bacteriophages

The quantification and preparation of fresh bacteriophage MS2 (DSM 13767) stock solution with cell culture was done using a double agar layer plaque technique [190]. For the preparation of the bottom agar layer, 30 mL of molten NZCYM agar (1.5% w/v agar) was filled in petri dishes (d = 96 mm). From each bacteriophage sample, a ten-fold dilution series was prepared using SM buffer. 100 µL of each dilution was preincubated with the same volume of bacterial host suspension (*E. coli*, DSM 5695, c = 1 x 10^8 cells/mL) for 20 min at 37 °C. Afterwards, 3 mL of molten NZCYM agar (0.7% w/v agar) were mixed with the preincubated suspension of bacteria and phage, then plated on the bottom agar layer. The petri dish was shaken to ensure total coverage. Plaques were counted after an incubation time of 16 h at 37 °C. The calculation of phage concentration in PFU/mL was performed as described in DIN EN ISO 10705-1:2001.

For the preparation of a fresh bacteriophage stock solution, a confluent lysed plate was covered with 5 mL of sterilized SM buffer and was shaken for 3 h at RT. The supernatant was transferred to a tube and centrifuged for 10 min at 4500 rpm and 4 °C. The supernatant was filtered through a sterile syringe filter (0.22 µm) to remove the bacteria. Typically the filtered supernatant had a concentration of 10^{10} PFU/mL. This stock solution was aliquoted and frozen.

5.3.1.2 PCR assay

Nucleic acid extractions

Unless stated elsewhere the RNA of bacteriophage MS2 was mainly extracted using the ViralXpress Nucleic Acid Extraction kit from Millipore. 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 was used. The washing step of the pellet was performed with 400 µL of ethanol. Finally, the pellet was resuspended in 20 µL of RNase-free water.

Nucleic acids from vertebrate viruses hAdV2 and MNV-1 were extracted at the German Federal Environment Agency (UBA, Berlin) using the NucliSens Magnetic Extraction kit according to the manufacturer's manual. Sample was added into 2 mL lysis buffer. Nucleic acid was extracted by adding 50 µL silica beads. After washed 4 times by special washing buffers, nucleic acids were eluted from silica beads 2 times by 50 µL elution buffer, respectively. Nucleic acids were eluted in 100 µl elution buffer and 10 µL volumes were subjected to qPCR.

cDNA synthesis for reverse transcription

For this step the DyNAmo cDNA Synthesis kit from Thermo Fisher Scientifics was used. The reaction was performed according to the manufacturer`s recommendations by adding 4 µL of extracted template RNA.

SYBR GreenPCR amplification of bacteriophage MS2

A 314 bp long cDNA fragment was amplified by qPCR coding for the replicase gene of bacteriophage MS2. The used oligonucleotides were synthesized by Eurofins MWG, as reported elsewhere[191]. 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. 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 0.4 µM of each primer were used each. The reaction volume was adjusted by using PCR-grade ultrapurified

water to achieve a final volume of 20 µL. Unless stated otherwise the following program consisted of 55 cycles and an initial denaturation step at 95 °C for 15 min and a three-step cycling at 94 °C for 15 sec, 50 °C for 30 sec, and 72 °C for 30 sec. For quantification of MS2 a RNA standard (Roche Diagnostics) was used. The concentration was given to 0.8 µg/µL, which corresponds to 4×10^{14} GU/mL.

TaqMan 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[192], using a TaqMan probe Ad:ACDEF (5′-(6FAM)-CCG GGC TCA GGT ACT CCG AGG CGT CCT-TAMRA-3′) and degenerated primers AdHexup 5′-CWT ACA TGC ACA TCK CSG G-3′ (forward), AdHexdo 5′-CRC GGG CRA AYT GCA CCA G-3′. Murine norovirus (MNV-1) RNA was amplified using a single-step RT-qPCR as described elsewehere[193]. MNV-1-specific RT-PCR primers (MNV Polymerase 5 Forward, 5′-TCTTCGCAAGACACGCCAATTTCAG-3′ and MNV Polymerase 5 Reverse, 5′-GCATCACAATGTCAGGGTCAACTC-3′) targeting the polymerase gene of MNV-1 (GenBank accession no. AY228235) were used.

5.3.2 Preparation of MAF(Small) and optimization of conditions

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[141]. First of all contaminants of the glass column especially glass dust must be removed.

For this purpose glass columns were cleaned by sonication in 2% Hellmanex for 1 h,

shaken overnight, and sonicated for 1 h afterwards. The columns were washed with purified water and treated with a mixture of 37% HCl and methanol (1:1, v/v) for 1 h by shaking. Before glass columns were shaken for 1 h in 99 – 100% H_2SO_4, they were washed with purified water. Then the glass columns were cleaned with purified water and dried under a N_2 stream. The dry columns were directly silanized by filling with 150 µL of GOPTS and incubation for 1 h at room temperature. Afterwards the columns were cleaned successively by sonication in ethanol, methanol and ethanol for 15 min and finally dried under a N_2 stream.

For the preparation of the monolithic column a 60:40 (v/v) mixture of toluene and tert-butyl methyl ether (mTBE) was used as porogen. To prepare one unit of polymerization mixture (for four monolithic columns with volume of 100 µL), 1413 µL toluene and 942 µL mTBE were mixed together firstly and incubated at 28 °C. A short time before starting the polymerization reaction 100 µL of boron trifluoride diethyl etherate ($BF_3 \cdot Et_2O$) were mixed with 900 µL of iced dioxane (1:10, v/v). Then 37.5 µL of a mixture of $BF_3 \cdot Et_2O$ and dioxane were added to a mixture of toluene and mTBE and mixed for 20 s. After addition of 600 µL polyglycerol-3-glycidyl ether (R9, 20:80, v/v, monomer/porogen ratio), the solution was mixed intensively for 1 min. 100 µL of the reaction mixture was poured into the silanized glass column with sealed outlet. The glass column was closed with the column fittings and the polymerization was allowed to proceed at room temperature for 1 h. After this, the column fitting was attached to a peristaltic pump. More than 100 mL methanol was pumped through the column at a flow rate of 0.5 mL/min to remove the porogenic solvents and other unreacted soluble compounds. The monolithic column was kept in methanol before surface activation.

The monolith has macropores with a size of 21 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 was pumped through the column at a flow rate of

0.3 mL/min under recirculation by using a peristaltic pump. The reaction was allowed to proceed for 3 h at 60 °C . The monolithic 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 1M HCl.

Glass wool of the same dry weight as monolithic columns (0.017 g) was packed into the same glass columns (ID 4.5 mm). 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.

Description of the MAF process

The MAF process was based on an adsorption-elution procedure by monolithic affinity columns. MS2 adsorption at different water pH levels, flow rates and binding capacity of the monolithic column were evaluated. First, the water sample was acidified with 1M HCl to pH 3 and spiked with MS2 stock solution mixed intensively by vortexing. Then the sample was pumped through the column at a flow rate of 10 mL/min using a peristaltic pump (MS-Reglo, a peristaltic pump) and a tubing with an inner diameter of 1.85 mm. Afterwards, captured viruses were eluted by pumping 1 mL of BEG buffer though the column at a flow rate of 1 mL/min. The MAF process for 100 mL of water sample took 11 min. Adsorption rate was calculated from concentrations of bacteriophages MS2 present in the input and the filtrate. Recovery efficiency was calculated based on concentrations of MS2 present in the input and the eluate.

$$\text{Adsorption} = \left(1 - \frac{C(MS2)_{\text{Filtrate}}}{C(MS2)_{\text{Input}}}\right) \times 100\%$$

$$\text{Recovery} = \frac{C(MS2)_{\text{Eluate}}}{C(MS2)_{\text{Input}}} \times 100\%$$

Concentrations of MS2 in the input, the filtrate and the eluate were determined by double layer plaque assays. An image of the monolithic column and a schematic diagram

are shown in Fig. 17.

Evaluation of MS2 adsorption at different water pH levels

100 mL of tap water was adjusted to pH 2, 3, 4, or 5 using 1 M HCl. The samples were spiked with 5 x 10^4 PFU of bacteriophage MS2 and vortexed intensively to disrupt aggregates. The water samples were subsequently pumped through activated monolithic and glass wool columns at a flow rate of 10 mL/min. Captured viruses were eluted using 1 mL glycine-beef extract buffer of pH 9.5.

Comparison of MS2 recoveries at different flow rates

100 mL of tap water (pH adjusted to 3) was seeded with MS2 to get a final concentration of 300 PFU/mL. Then, virus-seeded samples were passed through the activated monolithic column at velocities ranging from 5, 10, 20, 22 to 40 mL/min. Captured viruses were eluted using 1 mL BEG buffer.

Capacity determination of the monolithic affinity column

Bacteriophage MS2 stock solution was spiked in 1000 mL tap water to get a final concentration of 300 PFU/mL. The experimental procedure was the same as described for MAF process in the method section. To get the profile of the binding procedure, aliquots of input and filtrate solutions were collected at certain time points and enumerated by plaque assay.

Concentration of human adenoviruses and murine noroviruses by monolithic columns

Samples of 300 mL tap water were spiked with 3.2 x 10^2 GU/mL for each virus and adjusted to pH 3. After concentration, viruses were recovered in 1 mL elution buffer by pure gravity elution. Concentrations of hAdV2 and MNV-1 were measured by TaqMan PCR.

5.3.3 Characterization of CF-UF-MAF(Small) - PCR

The instrumentation of CF-UF was built up by by Dr. Martin Rieger in cooperation with the workshop[183, 187]. The CF-UF unit was adapted from a first crossflow microfiltration system as described elsewhere[64]. A transmembrane pressure is generated by a peristaltic pump circulating water over a hollow-fibre ultrafiltration module with a maximum flow rate of 3.65 L/min. Marprene tubings are used with an inner diameter of 6.4 mm. The CF-UF module (Inge AG, Greifenberg) contained 24 multibore fibres with a length of 0.5 m and a pore size of 20 nm. Filtration was performed at a recirculation flow rate of 3.65 L/min. A TMP of 0.2 bar was achieved without restriction. A permeate rate of 504 ± 21 mL/min was gained. Afterwards, the system was flushed with a recirculation rate of 3.65 L/min for 1 min in the forward flushing mode and afterwards 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. In total, a sample volume of 10 L was concentrated in 22 min. The secondary concentration process was based on MAF. After a primary concentration with CF-UF, the eluate was acidified with 1M HCl to pH 3 and mixed intensively by vortexing. The MAF process was carried out as described in section 5.3.2.

10-L tap water samples were spiked with MS2 to a final concentration between 0.53 and 1120 GU/mL, which were calculated from MS2 concentration in the stock solution and dilution factors. The water sample was prepared in autoclavable Nalgene bottles (VWR International GmbH, Ismaning, Germany) and shaken by hand for some minutes. 10 L of the surface water (kept overnight in a cool box and tested within 24 h after sampling) were taken from an urban (Teltow-Kanal, Berlin) as well as an alpine river (Mangfall, Munich). The samples were processed by the combined CF-UF-MAF concentration method on the next day. The RT-qPCR was performed by Sandra Lengger[183].

5.3.4 Preparation of MAF(Big) and optimization of conditions

Preparation of monolithic disk

The key part, monolithic disk is prepared in a separate mold. Due to the material property, the monolithic polymer tends to attach to the inner wall of the mold. Molds of different materials were tested, e.g. fine polished stainless, PP and PTFE *etc*. Mold made of PTFE is selected.

The protocol for polymerization of was up-scaled from the small one[141]. For the preparation of the monolithic column a 60:40 (v/v) mixture of toluene and tert-butyl methyl ether (mTBE) was used as porogen. For one monolithic disk (column volume of 11.97 mL), first, 5.65 mL toluene and 3.77 mL mTBE were mixed together first and incubated at 28 °C. A short time before starting the polymerization reaction 100 µL of boron trifluoride diethyl etherate ($BF_3 \cdot Et_2O$) were mixed with 900 µL of iced dioxane (1:10, v/v). Then 150 mL of the mixture of $BF_3 \cdot Et_2O$ in dioxane were added to the mixture of toluene and mTBE and mixed for 20 s. After addition of 2.40 mL polyglycerol-3-glycidyl ether (R9, 20:80, v/v, monomer/porogen ratio), the solution was mixed intensively for 1 min. All of the reaction mixture was poured into the PTFE mold (inner diameter: 3.86 cm) and covered with glass lid. The polymerization was allowed to proceed for 1 h. After this, the monolithic disk was taken out of the mold. A small thin spatula could be used to separate the polymer from the inner wall of the mold. Then the monolithic disk was put into a PP housing with PTFE holder. 50 mL methanol was added in to remove the porogenic solvents and other unreacted soluble compounds. The monolithic disk was kept in methanol before surface activation. The monolithic disk was hydrolyzed by 0.5 M sulfuric acid recirculating with a peristaltic pump for 3 h at 60 °C. 3 disks could be stacked in the PTFE cylinder. Then the cylinder was filled with water and sealed with the fitting. Afterwards ultrapure water was pumped through the column used to remove the acid. And the hydrolyzed monolithic disks were kept in ultrapure water at 4 °C.

To have a cost-effective method, a disposable plastic tip made of polypropylene (PP) was selected as the housing of the monolithic column. To reduce the resistance at a high flow rate, the narrow end of the outlet was cut. A disk-shape monolith was prepared in a PTFE mold. The desired dimension can be achieved by using a mold of defined size. The proposed column consists of 5 parts: the commercial PP housing, the PTFE holder with bore holes (2 mm in diameter), the monolithic disk, the fitting and the blocker (Fig. 40)

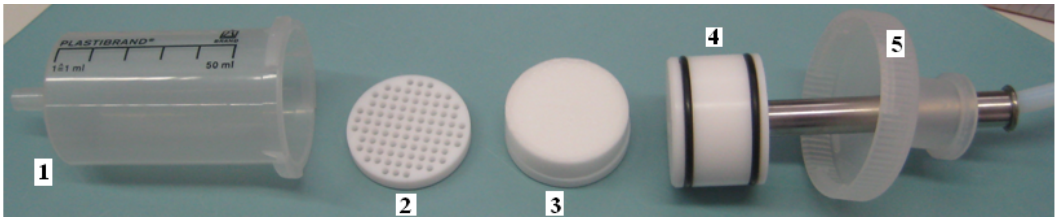

Fig. 40: Components of MAF(Big): 1) Column PP housing (disposable syringe, outlet cut to an inner diameter of 4 mm); 2) PTFE holder with bore holes (2 mm in diameter); 3) monolithic disk; 4) column fitting; 5) blocker.

Description of the MAF(Big) process

The MAF(Big) is partly adapted from MAF(Small) with some modification. The dimension of monolithic disk, flow rates and binding capacity of the monolithic disk were evaluated. Before starting the concentration procedure, the column was equilibrated with tap water of pH 3. Water sample was acidified with 1M HCl to pH 3 and mixed by magnetic stirring bar during filtration. Then the sample was pumped through the column at a flow rate of 1 L/min using a peristaltic pump (Pumpdrive 5206, Heidolph, Kelheim, Germany) and a Marprene tubing with an inner diameter of 6.4 mm (Watson Marlow, Rommerskirchen, Germany). Afterwards, adsorbed MS2 were eluted with BEG buffer. After disconnection of the Marprene tubing, 20 mL of BEG buffer was injected into the hosing by syringe. Then the Marprene tubing was reconnected with the column again. After an equilibration time of 2 min, the pump was restarted to pump air into the housing to press 1/3 of the liquid out (Fig. 41). It was repeated for 3 times to pump out all the

eluate. If more than one monolithic disk were used, the elution for each disk was performed separately. A schematic diagram is shown in Fig. 28. After using, the fitting and rubber tube are cleaned with 70% ethanol and ultrapure water. Housing together with PTFE holder and monolithic disks were disinfected by autoclave. After autoclaving, the PTFE holder can be reused.

Adsorption rates were calculated from concentrations of bacteriophages MS2 present in the inputs and filtrates. Recovery efficiency was calculated based on concentrations of MS2 present in the input and eluate. The numbers of MS2 were determined by double layer plaque assays or PCR.

Fig. 41: Image of setup of MAF(Big)(left) and elution step (right)

Optimization of dimensions

Monolithic disks with different diameters (3.56, 3.66, 3.76 and 3.86 cm) were prepared in PTFE molds having the same inner diameter. The volume of mixture for polymerization was kept at 11.97 mL. Afterwards, monolithic disks with a diameter of 3.86 cm and length of 0.8, 1.0 and 1.2 cm were prepared. Corresponding volume of

mixture for polymerization were 9.34, 11.97 and 14.01 mL.

10.5 L of tap water was acidified to pH 3 by 1 M HCl. Samples were spiked with bacteriophage MS2 stock solution to get final concentrations from 6.4 x 10^2 to 1.5 x 10^3 PFU/mL. A magnetic stirring bar was used for mixing during filtration. 10-L water samples were pumped through activated monolithic column at a flow rate of 1 L/min. Captured viruses were eluted by 20 mL BEG buffer.

Comparison of MS2 recoveries at different flow rates

10.5 L of tap water (pH adjusted to 3.0) were spiked with MS2 stock solution to get concentrations between 900 and 1600 PFU/mL. 10-L virus-seeded samples were concentrated by monolithic columns at velocities varied from 1060 mL/min to 1690 mL/min. Captured viruses were eluted using 20 mL of BEG buffer (pH 9.5).

Test with samples with various amounts of MS2

10.5 L of tap water (pH adjusted to 3.0) were spiked with MS2 stock solution to get concentrations between 10^3 and 10^8 PFU/mL. 10-L virus-seeded samples were concentrated by monolithic columns at velocity of 1 L/min. Captured viruses were eluted using 20 mL of BEG buffer (pH 9.5).

Simultaneous concentation of MS2, ΦX174, human adenoviruses and murine noroviruses and by monolithic columns

MAF(Big) was tested with bacteriophage MS2, ΦX174, human adenoviruses and murine noroviruses (as surrogate for human noroviruses) by our project partners (Dr. Hans-Christoph Selinka and Dr. Nils Marten Hartmann from UBA). 10 L of acidified tap water (pH 3) was spiked with a mixture of MS2 (4.0× 10^2 PFU/mL), ΦX174 (5.7× 10^2 PFU/mL), human adenoviruses (2.5× 10^3 CP/mL) and murine noroviruses (7.5× 10^2 CP/mL). 10-L virus-seeded samples were concentrated by monolithic columns at velocity of 0.5 L/min. Captured viruses were eluted using 20 mL of BEG buffer (pH 9.5).

Bacteriophage MS2 and ΦX174 were tested by plaque assay. Human adenoviruses and murine noroviruses were detected by TaqMan qPCR.

5.3.5 MAF(Big) - CeUF for environmental samples

MAF(Big) tested with urban river water

10-L samples of Havel river water were taken on 18.04.2012 at Freibrücke, Berlin-Spandau, between 9 to 10 a.m. They were stored at 6 ± 2 ℃. 10 L of acidified river water (pH 3) was spiked with a mixture of MS2 (4.0×10^6 PFU/mL), ΦX174 (4.9×10^5 PFU/mL), human adenoviruses (2.49×10^6 CP/mL) and murine noroviruses (6.03×10^5 CP/mL). Virus-seeded samples were concentrated by MAF(Big) at velocity of 0.5 L/min. Captured viruses were eluted using 20 mL BEG buffer (pH 9.5). Bacteriophage MS2 and ΦX174 were tested by plaque assay. Human adenoviruses and murine noroviruses were detected by qPCR. The experiments were done by our project partners Dr. Hans-Christoph Selinka and Dr. Nils Marten Hartmann from UBA. The setup of MAF(Big) they used and monolithic disk are shown in Fig. 42.

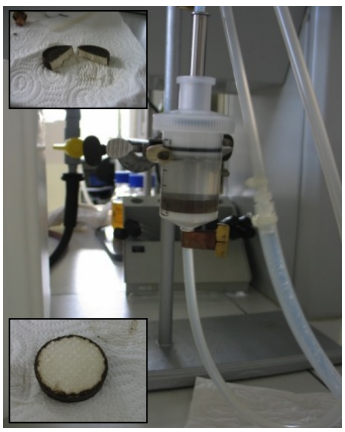

Fig. 42: Setup of MAF(Big) used in UBA for river water

Test with lake water

10-L samples were taken from Starnberg lake on 24.07.2012 between 9 to 10 a.m (Fig. 43). 10-L samples were acidified and concentrated by monolithic columns at velocity of 1 L/min. The setup of MAF was modified in following aspects: 1) a bouncy O-ring was inserted between the polymer and housing to block the gap; 2) two monolithic disks were stacked to increase column length. The setup used is shown in Fig. E4. The stacked disks were eluted separately in order to maintain the high elution efficiency achieved by 20 mL elution buffer. The eluate of MAF was further concentrated by CeUF. The sample was added in a CeUF unit and centrifuged at 1500 g to a final volume of ~ 1 mL. Aliqots of input, eluate from top and bottom disks and final concentrate 1 and 2 were collected. Naturally occurring bacteriophage MS2 were quantified by RT-qPCR. In addition, to improve the specificity, the annealing temperature in PCR program was increased to 60 °C quantification of MS2 in environmental samples.

Fig. 43: Setup of improved MAF(Big) with O-ring and stacked-disk

Test with wastewater

Wastewater samples were taken from water treatment plant in Garching on 2.07.2012. In the first experiment, 4-L acidified samples were spiked with MS2 stock (6.1×10^5 GU/mL) and filtrated by MAF(Big). The procedure by MAF(Big)-CeUF was done as the same as described above for lake water.

In the control experiment, 4-L acidified samples were filtrated by MAF(Big). The MAF(Big)-CeUF was carried out like described before. Aliqots of input, eluate from top and bottom disks and final concentrate 1 and 2 were collected and spiked with MS2 stock (7.8×10^9 GU/mL). Bacteriophage MS2 were quantified by RT-qPCR.

5.3.6 UF-MAF(Big) - CeUF for large volumes of water

5.3.6.1 The ultrafiltration system for large-volume water

The ultrafiltration system used for large-volume water is adapted from Dr. Rieger's work[187]. It is an up-scaled version of CF-UF used in former study[64] and was built up at IWC, TUM. The instrument can be run by local electricity supply or a petrol power generator on board. The ultrafiltration is driven by a rotary pump producing a maximum flow rate of 10 m^3/h at a delivery height of 16 m (1.6 bar). The transmembrane pressure can be applied on both sides of the ultrafiltration module for filtration and elution, which is controlled by a pressure gauge. The ultrafiltration module (dizzer S 0.9 MB 6.0) consists of polyethersulfone multibore hollow fibers with a nominal pore size of 20 nm and a membrane area of 6 m^2. The ultrafiltration process consisted of four steps (conditioning, filtration, elution and disinfection). Changes of statuses can be controlled by four valves and three buttons, which are described in Table 29. In a concentration procedure, the first step was the conditioning of the CF-UF system. Therefore, the input and conditioning tubes were put in the 1-m^3 buffering container with clean water. SV and

CV were opened whereas DV was switched between open and closed to get the air out of the system. When there was no air bubble came out of the conditioning tube, CV was switched off. When the white button is off, the water fills in the back-flushing tank directly. When the tank is full, water would spray from the hole on the top of the back-flushing tank. Then press the white and red buttons at the same time to change the pathway of water to the outlet tube. When there was no air bubble in the system and the backflushing tank is full, the conditioning step is finished. For cross-flow filtration DV was opened, while it was closed for dead-end filtration. The syringe pump together with stock solution of viruses can be fixed by a PTFE adapter for model virus addition. Filtration started after switching on the rotary pump (it can be switched on by press the green button outside or the button under the red number). It runs at full power (60Hz), corresponding to 1724 L/h in deadend mode and 984 L/h in crossflow mode. Record the initial values on volume meter at the outlet and pressure gauges (P1, P2 and P3). Transmembrane pressure is calculated from the average of P1 and P2 minus P3. TMP was 0.65 bar in deadend mode and 0.45 bar in crossflow mode. When the filtration was done, the rotary pump was switched off and SV was closed. In elution step, EV was open and elution started when the green button was pressed. Then 20-L of concentrate (the dead volume of the system is 14 L) was eluted by backflushing the system with 2.5 bar pressure from a compressor. When elution is finished, V4 was closed and the red and the white buttons were switched off. Afterwards, the system was disinfected by 200 mg/L chlorine in recycling. All of input, outlet and conditioning tubes were put in 10-L bottle containing chlorine ($c(Cl_2)$ = 200 mg/L). The machine ran in a conditioning mode. But to avoid chlorine going into the backflushing tank, the white and red buttons should be turned on to make sure chlorine solution going out from outlet tube. After disinfection, the system was washed with 100 L clean water. To maintain the membrane in a good status, the system was filled with 0.75% (w/v) sodium bisulphate in the end according to the manufacturers' advice.

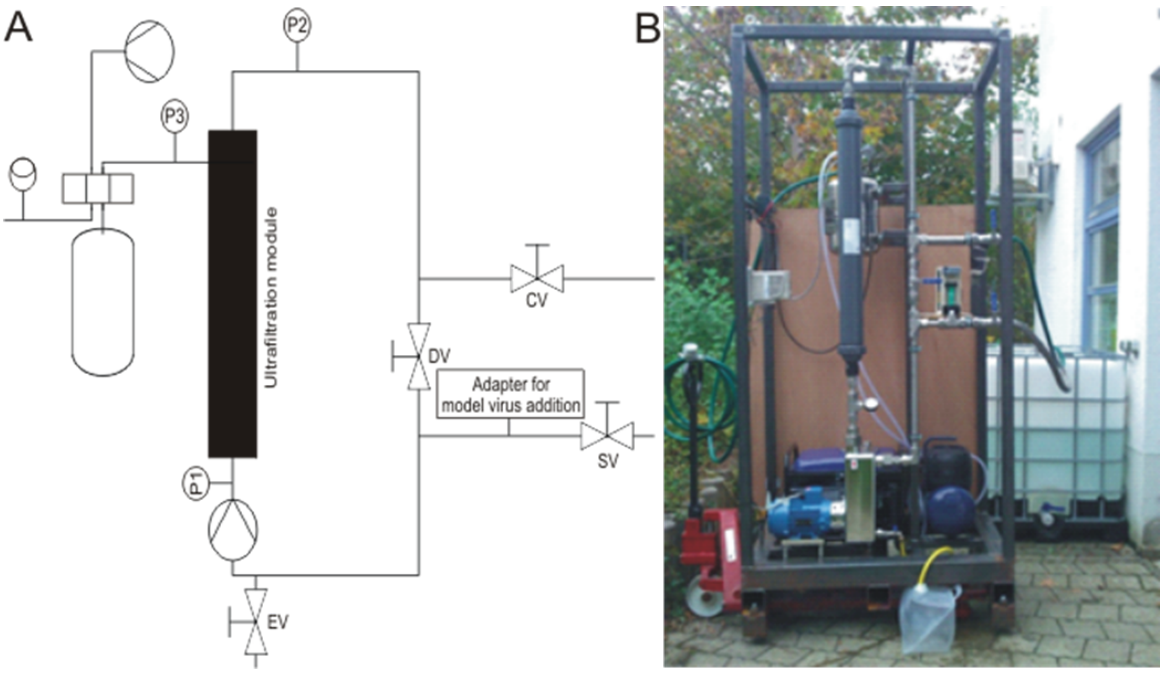

Fig. 44: Picture of the ultrafiltration setup. All process steps are manually controlled (sample valve, SV; conditioning valve, CV; dead-end filtration valve, DV and elution valve, EV). The filtrated volume and the transmembrane pressure can be observed by a volume flow meter and three manometers (P 1-3)

Table 29: Setting of valves and buttons during filtration procedure

	SV	**DV**	**CV**	**EV**	**Button Red + white**	**Button green**
Conditioning	Open	Open/Close	Open	Close/Open	When backflushing tank full, switch it on	-
Filtration	Open	CF-UF (open) DEUF (close)	Close	Close	On	-
Elution	Close	Open	Close	Open	When elution is finished, switch it off	On
Disinfection	Open	Open/Close	Close	Close/Open	On	-

5.3.6.2 UF-MAF(Big)-CeUF for 10 m^3 tap water

Tap water was taken from IWC's pipeline, which was provided by Stadtwerke München, Germany. Physical and chemical parameters of tap water used: conductivity: 456 µs/cm; pH = 7.34; turbidity: < 0.01 NTU.

During ultrafiltration process, 10^{10} GU MS2 were continuously injected into 10 m^3 of tap water by a syringe pump. To have a rapid analysis method, the ultrafiltration was operated in DE-UF mode at a flow rate of about 1700 L/h. After 5.9 hours, filtration was finished. The 20-L retentate was further compressed by MAF(Big). For MAF step, one disk column was used. MAF procedure was done as described before. After filtration, the monolithic disk was eluted from forward direction and also in backward direction (in case only forward elution is not enough). 26.83 mL eluate from forward direction and 12.39 mL eluate from backward direction was mixed together for next step. In CeUF step, 10 mL was added into centrifugal filter (Amicon Ultra-4 Centrifugal Filters) and centrifuged at 1500 g to a final volume of about 1 mL. Aliquots of input and eluates of each step were collected and analysis by RT-qPCR. The concentration of MS2 in eluate of MAF(Big) from backward direction is too low to be detected. Therefore, the recovery of MAF step is calculated from the concentration of MS2 in eluate of MAF(Big) from forward direction and corresponding volume.

5.3.6.3 Testing real samples in the fieldin

35 m^3 ground water was processed to eliminate manganese and iron, which was provided by German Federal Environment Agency (Umweltbundesamt, UBA). Physical and chemical parameters of used ground water: conductivity: 1178 µs/cm; pH = 7.64; turbidity:0.29 $\pm$ 0.07 NTU.

35 m^3 processed ground water (manganese and iron eliminated) floating in outdoor

channel was spiked with 0.01% (3 L) mechanically treated sewage, stock solutions of murine noroviruses and MS2 bacteriophages. Firstly water was prefiltrated by a prefilter equipped with nylon meshes of 25 µm to remove big particles and suspended matters. The ultrafiltration was operated in CF-UF mode. During the filtration procedure, the flow rate kept decreasing due to high turbidity of water (Table 26). At the beginning the flow rate was 1.15 m^3/h. When 7 m^3 water was filtrated, the flow rate decreased to 0.41 m^3/h (Fig. 37). In this case, backflushing (2.5 bar) was used to remove the concentrate and particles two times (eluate 1, 2). Afterwards, another 3 m^3 was filtrated, but due to high backpressure from the blocked membrane, the pump was overloaded. To compress the total volume of eluate from the primary step, eluate 2 was pumped into the ultrafiltration system again. Then the system was backflushed twice (eluate 3, 4). To avoid the forming of colloid during this time, samples were acidified.

To further reduce the volume, eluate 1 was pumped through 3-disk monolithic column. But when 2 L of the eluate 1 passed through, the column was blocked by particles. These three disks were eluted separately by 20 mL BEG buffer (pH 9.5). 10 mL of eluate was taken out for detection. The rests were further concentrated by centrifugal filter (Amicon Ultra-15). 3 final concentrates of 1 mL were collected for detection.

In the concentration system 2 (UF-GW-FL), the rest of eluate 1 (18 L) and 10 L out of eluate 3 was pumped through the first glass wool column. Then the glass wool column was also blocked. So a second glass wool column was used for the rest of Eluate 3. Elution and flocculation was done according to the protocol developed in UBA. 200 mL of BEG buffer was used for each column and eluate was flocculated at pH 3, pellet was resuspended in 20 mL PBS.

By the concentration system 3 (UF-(GW-)MAF-CeUF), the filtrate of the first glass wool column (28 L) was concentrated by a 2-disk monolithic column. These two monolithic disks were eluated separately by 20 mL of BEG buffer. 10 mL was taken out for following detection. The rest was further concentrated by CeUF. 2 final concentrates

of 1 mL were collected for detection. Nucleic acids from the vertebrate viruses hAdV2 and MNV-1 were extracted using the NucliSens Magnetic Extraction kit according to the manufacturer‘s manual. The presence of human adenoviruses was quantified by TaqMan qPCR.

5.3.6.4 UF-MAF(Big)-CeUF for 30 m^3 tap water and ground water

Process of 30-m^3 tap water samples

The experiment with tap water was carried out like before. Due to the low turbidity, UF was operated in DE-UF mode at flow rate about 1700 L/h, TMP = 0.8 bar. MAF step one-disk column was used. 30 m^3 water was reduced into 1 mL by UF-MAF-CeUF system within 20 hours. 1 mL out of 20 mL eluate of MAF and 1 mL final concentrate after three-step UF-MAF(Big)-CeUF were collected for pyrosequencing analysis.

Process of 30-m^3 ground water samples

For the experiment with ground water in IWC, TUM, the ground water level was monitored when it was pumped out at a flow rate of 1.4×10^4 L/h. The radius of influence was calculated according to

$$R_0 = 3000 \times (H\text{-}h) \times K^{1/2}$$

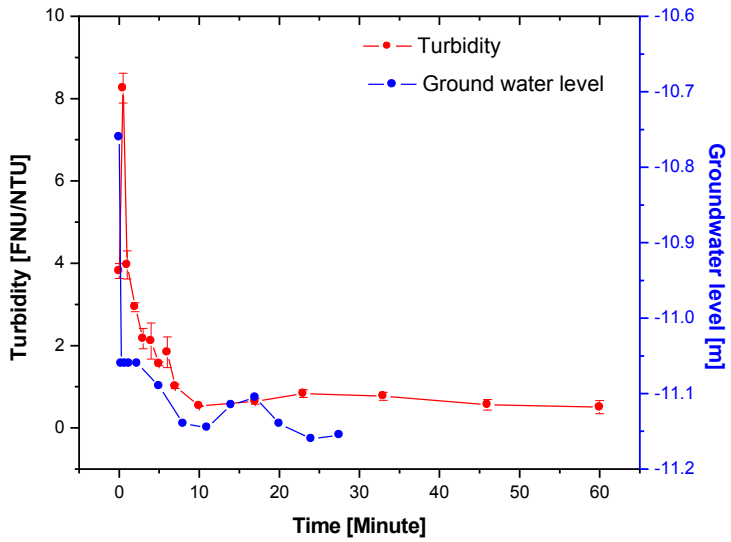

Fig. 45 Ground water level and turbidity of water when it was pumped out at a flow rate of 1.4×10^4 L/h

For hydraulic conductivity K = 10^{-3}, radius of influence is 37.95 m (maximum).

For hydraulic conductivity K = 10^{-4}, radius of influence is 12 m.

For hydraulic conductivity K = 10^{-5}, radius of influence is 3.79 m (minimum).

The bottom of the groundwater well was -12.8 m. The depth of the groundwater was 2 m. So only the top 0.4 m was used (Fig. 45). It would be no problem to keep the pump running at a flow rate of about 1.44×10^4 L/h during the whole experiment. But at such a high flow rate, the turbidity kept at a very high level (0.6 ± 0.2 NTU) even after 60 min. Therefore a deep well pump with a lower flow rate (2400 L/h) was installed. The turbidity was reduced to 0.12 ± 0.02 NTU (Fig. 46).

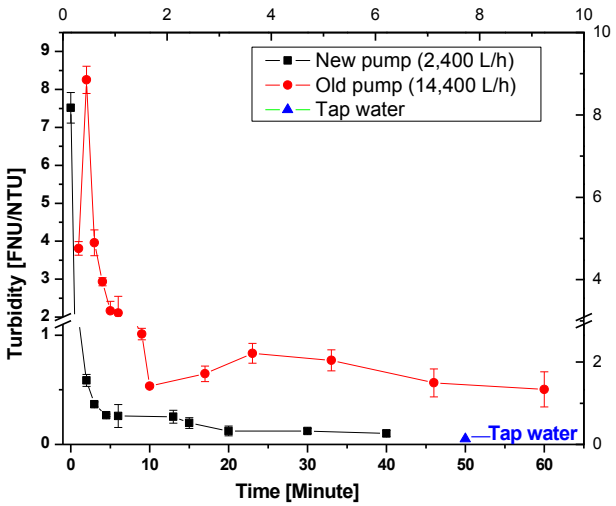

Fig. 46 Change of turbidity using different pumps

Due to the low turbidity, UF was operated in DE-UF mode at flow rate about 1700 L/h. Two monolithic disks were used in MAF step. On the first disk, the color of the disk turned from white to brown after filtration. Particles from matrix were kept on the first one. These two disks were eluted separately. The eluates were processed by CeUF separately. Two of 1-mL final concentrate were obtained and collected for following pyrosequencing analysis. The ground water, concentrate from UF and flow through of MAF were also collected to measure amounts of metallic elements by atomic absorption spectrometry.

Inhibitory study

In inhibitory study, nucleic acid extraction with ViralXpress Nucleic Acid extraction kit was done as described before. Using the Viral RNA Mini Kit (analyzed by Dr. Kallies and Prof. Drosten at University of Bonn), nucleic acids (both RNA and DNA) were extracted according to the manufacturer's instructions. From each column, nucleic acids were eluted in 2 x 50 µl buffer. For each of the four samples 195 µl were spiked with 5 µl (containing approx. 100,000 copies) of Culex-Y-virus (CYV) RNA. The same amount of CYV RNA was spiked into 195 µl of molecular grade water that was used as a control.

CYV RNA was detected using a one-step TaqMan real-time RT-PCR assay[189].

Appendix

6 Appendix

6.1 Abbreviations

BEG	Buffer containing 3% beef extract and 0.5 M glycine
CeUF	Centrifugal ultrafiltration
CF-UF	Crossflow ultrafiltration
conc.	Concentration
Cp	Crossing point
CM	Concentration method
CYV	Culex-Y-virus
Da	Dalton
DE-UF	Deadend ultrafiltration
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
GOPTS	3-Glycidoxypropylmethoxysilane
GU	Genomic unit
hAdV2	Human adenoviruses serotype 2
ID	Inner diameter
IWC	Institute of Hydrochemistry
LOD	Limit of detection
MAF	Monolithic adsorption filtration
MAF(Small)	Monolithic adsorption filtration based on monolithic column with diameter of 4.5 mm
MAF(Big)	Monolithic adsorption filtration based on monolithic column with diameter of 35.5 mm
MNV	Murine noroviruses type 1
mTBE	2-Methoxy-2-methylpropane
MWCO	Membrane molecular weight cutoff

NGS	Next generation sequencing
NTU	Nephelometric turbidity unit
PBS	Phosphate buffered saline
PFU	Plaque forming unit
PP	Polypropylene
PTFE	Polytetraflourethen
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
SEM	Scanning electron microscope
UBA	Umweltbundesamt, German Federal Environment Agency
UF	Ultrafiltration
USEPA	US Environmental Protection Agency
UV	Ultra violet
VIRADEL	Virus adsorption and elution
WHO	World Health Organization

6.2 List of Figures

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7 References

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