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Enhanced detection of pathogenic enteric viruses in coastal marine environment by concentration using methacrylate monolithic chromatographic supports paired with quantitative PCR

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ABSTRACT

Currently, around 50% of the world's population lives in towns and cities within 100 km of the coast. Monitoring of viruses that are frequently present in contaminated coastal environments, such as rotavirus (RoV) and norovirus (NoV), which are also the major cause of human viral gastroenteritis, is essential to ensure the safe use of these water bodies. Since exposure to as few as 10–100 particles of RoV or NoV may induce gastrointestinal disease, there is a need to develop a rapid and sensitive diagnostic method for their detection in coastal water samples. In this study, we evaluate the application of methacrylate monolithic chromatographic columns, commercially available as convective interaction media (CIM®), to concentrate pathogenic enteric viruses from saline water samples prior to virus quantification by one-step reverse transcription quantitative PCR (RT-qPCR). Using RoV and NoV as model enteric viruses, we present our results on the most effective viral concentration conditions from saline water matrices using butyl (C4) hydrophobic interaction monolithic support (CIM® C4). C4 monolithic columns exhibit a good capacity to bind both RoV and NoV and both viruses can be eluted in a single step. Our protocol using a 1 ml C4 column enables processing of 400 ml saline water samples in less than 60 min and increases the sensitivity of RoV and NoV detection by approximately 50-fold and 10-fold respectively. The protocol was also scaled up using larger capacity 8 ml C4 columns to process 4000 ml of seawater samples with concentration factors of 300-fold for RoV and 40-fold for NoV, without any significant increase in processing time. Furthermore, C4 monolithic columns were adapted for field use in an on-site application of RoV concentration from seawater samples with performance equivalent to that of the reference laboratory setup. Overall, the results from successful deployment of CIM C4 columns for concentration of rotavirus and norovirus in seawater samples reiterate the utility of monolithic supports as efficient, scalable and modular preparative tools for processing environmental water samples to enhance viral detection using molecular methods.

1. Introduction

Currently, half the world's population resides within 100 km of the coast, an area that includes most of the megacities, with the trend of coastal inhabitation expected to continue through the next few decades (Brown et al., 2013; Small and Nicholls, 2003). Accompanying the enhanced anthropogenic coastal activities are the concurrent fecal contaminant sources of pathogenic bacteria and enteric viruses that threaten the safe use of these coastal water bodies for recreational use (Wyn-Jones et al., 2011), aquaculture (Lees, 2000) and in general cause unintended biogeochemical changes in the coastal marine environment (Paerl et al., 2003). The use of fecal indicator bacteria as the sole measure of the risk of fecal

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contamination has been shown repeatedly to inadequately reflect the risk posed by pathogenic enteric viruses (Updyke et al., 2015). Two of the viruses that are most frequently present in contaminated coastal environments, rotavirus (RoV) and norovirus (NoV), are also the major cause of human viral gastroenteritis (Bishop, 2009; Robilotti et al., 2015). They are responsible for the manifestation of gastrointestinal disease in nearly 20% of children under the age of 5 and inflict significant childhood mortality in developing countries (Nwachuku and Gerba, 2006). Hence, vaccines to control RoV-induced gastrointestinal disease have been introduced over the past decade with successful outcomes, but there are no such prophylactic vaccines to mitigate the threat of gastroenteritis caused by NoV infection (Glass et al., 2014). Moreover, as children in the poorest countries account for a majority of enteric virus infection related deaths where vaccine adoption remains limited, a balanced approach that combines vaccination with sanitary management and rapid clinical and environmental monitoring of pathogenic enteric virus levels is essential in these vulnerable hotspots (Monroe, 2011).

Molecular techniques like quantitative PCR (qPCR) are the preferred choice for detection of most fecal pathogens due to their specificity and speed over traditional culture-based techniques (Girones et al., 2010; Schoen et al., 2011), especially for RoV and NoV which are reliably detected at concentrations of 1×10^3 particles per ml (Gutiérrez-Aguirre et al., 2008; Kageyama et al., 2003; Updyke et al., 2015). However, as gastroenteritis may manifest through exposure to as few as 10 particles of enteric virus (Haas et al., 1993), and since environmental water samples often contain inhibitors of qPCR (Gentry-Shields et al., 2013), a concentration procedure is usually employed as a preceding preparative step. Some widely adopted primary methods to concentrate enteric viruses use an adsorption elution principle using electropositive or electronegative filters, sedimentation by flocculation, or other techniques like size exclusion by ultrafiltration, or ultracentrifugation (Calgua et al., 2008; Fong and Lipp, 2005). Poor recovery rates and slow processing times are some limitations of these concentration methods in saline water matrices that often necessitate a secondary step to concentrate environmental samples starting with several liters of water down to as few as 1–10 ml to enable enteric virus detection (Ikner et al., 2012). Moreover, in many of the above filter based methods, the solutions used to elute the concentrated enteric virus particles often contain inhibitors that affect downstream detection by PCR (Ahmed et al., 2015).

Methacrylate monolithic columns, commercially available as convective interaction media (CIM®), are chromatographic supports based on fast convective flow, with average pore sizes ranging from 1.5 to 6 μm, and flexible ion exchange or hydrophobic interaction based active chemistries that are optimized for interaction and concentration of biomolecules like plasmids and viruses (Barut et al., 2005). CIM columns have been successfully utilized previously for the concentration of waterborne enteric viruses from various matrices including tap water (Gutiérrez-Aguirre et al., 2009), bottled mineral water (Kovač et al., 2009) and wastewater treatment plant effluents (Steyer et al., 2015). The high binding capacity of the CIM columns was recently utilized as a strategy to remove enteric viruses from wastewater treatment plant effluents (Rački et al., 2015).

The objective in the present study was the development of a protocol using CIM columns to concentrate RoV and NoV from saline water matrices and allow for downstream detection by one-step reverse transcription qPCR (RT-qPCR). The evaluation approach included screening of the CIM columns with various active chemistries to identify the most suitable chromatographic support, the optimization of chromatographic conditions for concentration of both RoV and NoV in a single step, testing the protocol with environmental water samples, and using CIM columns in an on-site experiment to test performance in portable applications.

2. Materials and methods

2.1. Stool samples positive for enteric virus

Clarified suspensions derived from clinical stool samples obtained from children hospitalized with acute gastroenteritis that were characterized to be positive for either RoV or NoV GII (Steyer et al., 2011) were provided by Dr. Andrej Steyer (Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, Slovenia). Where mentioned throughout this article, these stool suspensions were used to spike the water samples at the indicated dilutions to evaluate the performance of the CIM chromatographic supports in binding and concentrating RoV and/or NoV particles.

2.2. Water samples

To optimize the binding and concentration of RoV particles with CIM chromatographic supports (BIA Separations, Ajdovščina, Slovenia), salinity of either 0.3 M or 0.6 M (equivalent to 18 g/L or 35 g/L) NaCl was simulated by addition of either NaCl (Sigma Aldrich, St. Louis, MO, U.S.A.) or sea salt (Piranske soline, Portorož, Slovenia) to Type 1 ultrapure water (Merck Millipore, Billerica, MA, U.S.A.). The samples were buffered to pH 7.0 using 50 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES) and pH confirmed using a SevenMulti pH meter (Mettler Toledo, Schwerzenbach, Switzerland). Prior to virus spiking, buffered solutions were filtered through a 0.22 μm cut-off nitrocellulose filter (Sartorius, Gottingen, Germany). These simulated saline water samples were spiked with RoV and/or NoV positive stool suspensions at the dilutions indicated in the individual experiments.

Seawater and brackish water samples were collected in front of the Marine Biology Station in Piran, Slovenia and in Drnica River in Portorož, Slovenia respectively. 5000 ml of water was collected at the sub-surface level (≈0.3 m depth) using sterile Duran glass bottles (Duran Group, Mainz, Germany) at each station. The collected samples were immediately filtered through cellulose acetate membranes with a pore size of 0.8 μm (Sartorius, Gottingen, Germany) to remove bigger particles and organisms. These environmental water samples were spiked with RoV and/or NoV positive stool suspensions at the dilutions indicated in the individual experiments.

2.3. Concentration of enteric virus in saline water using CIM methacrylate monolithic supports

Chromatographic concentration of virus particles were performed on CIM monolithic columns with quaternary ammonium (QA), diethylaminoethyl (DEAE), sulphate (SO_3) and hydroxyl (OH) active chemistries, with a bed volume of 0.34 ml placed in a specially designed housing (BIA Separations, Ajdovščina, Slovenia). The CIMmultus 1 ml and 8 ml CIM high density butyl (C4) columns were provided in ready to use enclosure by the manufacturer (BIA Separations, Ajdovščina, Slovenia). Water samples (virus-spiked and/or environmental) were loaded through the CIM supports placed in an ÄKTApurifier 100 fast performance liquid chromatography (FPLC) system (GE Healthcare, Uppsala, Sweden) comprising a binary gradient P-900 pump, a combined detector UPC-900 for measuring UV absorbance and conductivity, and an automated fraction collector Frac-920. For the respective runs, the CIM-chemistry, column volume, buffer composition, sample volumes and flow rates for the load and elution steps are indicated in

Table 1. A wash step using an equilibration buffer (same composition as load buffer, but without added virus) was performed prior to elution. An aliquot of the load (L) sample, flow-through (FT), wash (W) and elution (E1, E2, E3 or E) chromatographic fractions was immediately stored at −20 °C for further processing. Where indicated, fresh aliquots of the elution fraction were immediately processed for negative staining Electron Microscopic visualization to evaluate the integrity of the viruses eluted from the CIM monolithic columns. See Supplementary Method S1 for a detailed protocol of the negative staining Electron Microscopy procedure. Between experiments, CIM columns were regenerated using contact with 1 M NaOH for 2 h and stored according to manufacturer's instructions (BIA Separations, Ajdovščina, Slovenia).

2.4. On-site concentration of rotavirus in saline water using CIM C4 columns

For the on-site concentration experiments, a standard gasoline power generator (Honda, AS Domžale Moto center Ltd., Trzin, Slovenia) was used as power supply. CIM C4 1 ml columns were transported to the planned location already equilibrated with the loading buffer (0.6 M NaCl, 50 mM HEPES, pH 7). Elution buffer (50 mM HEPES, pH 7, 10% Ethanol) and column regeneration buffer (1 M NaOH) were also previously prepared in the laboratory. Solutions were pumped using a Milton Roy LMI B71 dosing pump (Milton Roy Europe, Pont-Saint-Pierre, France) and a modular Smartline preparative UV Detector 200 (Knauer, Berlin, Germany) was used for detecting the elution of the concentrated fraction.

2.5. Quantification of rotavirus and norovirus in chromatographic fractions by RT-qPCR

RNA was extracted from a 140 μl aliquot of the CIM chromatographic fractions using a QIAamp viral RNA mini kit according to the manufacturer's instructions (QIAGEN, Chatsworth, CA, USA) to obtain 45 μl of isolated total RNA. For all samples, a known concentration (2 ng) of luciferase (luc) RNA (Promega, Madison, WI, USA) was added as an external control for the extraction procedure and to report potential PCR inhibitory effects inherent to the samples (Toplak et al., 2004). Additionally, a negative control (NCI) for each RNA isolation procedure, consisting of buffer only, was always included. RT-qPCR was performed as described in (Gutiérrez-Aguirre et al., 2008). Briefly, for each sample, a 10 μl reaction in triplicate using 2 μl total RNA was subjected to RT-qPCR assays for RoV (Gutiérrez-Aguirre et al., 2008) and/or NoV GII (Kageyama et al., 2003; Steyer et al., 2011), along with luc control (Toplak et al., 2004) using a commercial kit, AgPath-ID™ One-Step RT-PCR Kit (Life Technologies, CA, USA) on a 7900HT Fast Real-Time PCR System (Applied Biosystems, CA, USA). The primers and probes used for the RT-qPCR assays for RoV and NoV are indicated in Supplementary Table S1. The cycling conditions for the RT-qPCR assay were as follows: reverse transcription 48 °C, 10 min; denaturation 95 °C, 10 min; 45 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 60 s. The RT-qPCR reactions were planned in the GENEIO qPCR workflow application (BioSistemika LLC, Ljubljana, Slovenia) and the microplates for the qPCR reactions were prepared with assistance from the PLATR smart pipetting assistant (BioSistemika LLC, Ljubljana, Slovenia). The quantification cycle (Cq) for each individual amplification was obtained using SDS 2.4 software (Applied Biosystems, CA, USA). For all calculations, the baseline was set automatically and the fluorescence threshold was set manually at 0.065 for RoV and NoV, and at 0.4 for luc (a level that was above the baseline and sufficiently low to be within the exponential increase region of the amplification curve) in accordance to suggested practices of the MIQE qPCR guidelines (Shipley, 2011). Non-template controls were used to monitor for potential contamination within the qPCR reagents.

2.6. Calculation of rotavirus and norovirus recovery efficiency in chromatographic fractions

Total viral RNA was extracted from RoV and NoV positive stool suspension. Isolated RNA was 10-fold serially diluted from neat to 1×10^8 and assayed for RoV or NoV specific RT-qPCR in triplicate. A standard curve was obtained by plotting Cq values for each dilution against the Log_{10} dilution for RoV or NoV RNA (Supplementary Fig. S1 and S2). The Cq values obtained in RT-qPCR analysis for RoV and NoV on viral RNA isolated from CIM chromatographic fractions was normalized to the external luc control (Supplementary Tables S3 – S7 and Figs. S3 – S7). See the Supplementary Method S2 for further details on the computation of recovery efficiency and concentration factor during chromatographic concentration of RoV and NoV by CIM monolithic columns.

3. Results

3.1. Preliminary screening of CIM columns with different active side chains to optimize rotavirus binding in high saline water matrices

Since most enteric viruses are considered to exist as negatively charged particles in fresh water, CIM columns with electropositive quaternary ammonium (QA) active chemistry have been utilized to successfully concentrate RoV from tap water, stream water and bottled water. Using the CIM QA columns, RoV and other important enteric viruses are bound in the absence of salt, and the bound viruses are eluted using buffers with 1 M NaCl (Gutiérrez-Aguirre et al., 2009; Kovač et al., 2009; Steyer et al., 2015). As variations in ionic concentrations and electrolytic conditions are known to

Table 1
CIM concentration run parameters.

Run	CIM chemistry	Column volume	Sample loading conditions			Elution conditions		
			Load Buffer	Volume	Flow rate	Buffer	Volume	Flow rate
1	Quaternary ammonium (QA)	0.34 ml	Buffer A1[a]	120 ml	3 ml/min	Buffer B1[c]	5 ml	1 ml/min
2	Di ethyl amino ethyl (DEAE)	0.34 ml	Buffer A1[a]	120 ml	3 ml/min	Buffer B1[c]	5 ml	1 ml/min
3	Sulphate (SO_3)	0.34 ml	Buffer A1[a]	120 ml	3 ml/min	Buffer B1[c]	5 ml	1 ml/min
4	Hydroxyl (OH)	0.34 ml	Buffer A2[b]	120 ml	3 ml/min	Buffer B2[d]	1.5 ml	1 ml/min
5	High density butyl (C4)	1.0 ml	Buffer A2[b]	400 ml	10 ml/min	Buffer B2[d]	5 ml	3 ml/min
6	High density butyl (C4)	1.0 ml	Seawater	400 ml	10 ml/min	Buffer B2[d]	5 ml	3 ml/min

[a] Buffer A1: 0.3 M NaCl, 50 mM HEPES, pH 7.0.
[b] Buffer A2: 0.6 M NaCl, 50 mM HEPES, pH 7.0.
[c] Buffer B1: 1 M NaCl, 50 mM HEPES, pH 7.0.
[d] Buffer B2: 50 mM HEPES, pH 7.0.

effect the isoelectric point of virus particles (Michen and Graule, 2010), they may have a different binding preference to CIM QA columns at higher salinity of seawater compared to fresh water matrices with negligible salinities used in the previous studies. Thus, CIM columns with a wide range of ion exchange or hydrophobic interaction chemistries were screened to determine optimized RoV binding in saline water matrices with chromatographic run conditions as summarized in Table 1. Notably, considering the eventual applicability of the method for environmental monitoring, for all the CIM concentration runs, the entire procedure was completed in approximately 60 min per sample. For the CIM columns with ion exchange chemistries; QA, DEAE; and SO_3 columns; concentration was performed on 120 ml buffered water samples (50 mM HEPES pH 7) spiked with stool samples positive for RoV diluted by a factor of 1×10^4. The salinity was maintained at 0.3 M NaCl which is equivalent to approximately half that of seawater in Piran bay, Slovenia, as higher salinity levels were not conducive to RoV binding (data not shown). Bound RoV was eluted from the CIM ion exchange columns using 5 ml of buffer as above (50 mM HEPES pH 7) but with higher salt concentration of 1 M NaCl to facilitate RoV elution (Run 1–3, Table 1). The CIM chromatographic fractions collected were: the water sample containing RoV to be pumped through the CIM column (load), the flow through (FT) from the CIM column, a fraction from a wash step using a buffer of the same pH and salinity as load (wash), and the fraction containing eluted RoV (eluate). Aliquots of these fractions were used for determination of RoV concentration using RT-qPCR (section 2.5) and standard curve quantitation as described in section 2.6 and Supplementary Method S2. The ideal concentration factor assuming 100% recovery efficiency with 120 ml load and 5 ml eluate is 24-fold. For the CIM QA, DEAE, and SO_3 columns the concentration factors and recovery efficiencies obtained for these CIM columns were 5-fold (17%), 8-fold (35%) and 5-fold (21%) respectively (Run 1–3, Table 2). The low concentration factor and recovery efficiencies obtained may be due to weak or incomplete RoV binding as evidenced by the relatively high amount of RoV detected in the wash and FT fractions for these CIM column runs (Run 1–3, Table 2). Since short range hydrophobic interactions are relatively less affected by higher salinity when compared to electrostatic interactions (Thomas and Elcock, 2006), we decided to evaluate CIM hydroxyl (OH) and butyl (C4) columns with hydrophobic interaction chemistries to concentrate RoV in saline water. For the CIM OH and C4 columns, the load sample was buffered water (50 mM HEPES pH 7), spiked with stool samples positive for RoV diluted by a factor of 1×10^4, and salinity was maintained at 0.6 M NaCl, which is approximately that of seawater in Piran bay, Slovenia at 35 g/L. Bound RoV was eluted using just buffer (50 mM HEPES pH 7) with no salt added. RoV particles bound very poorly to the CIM OH column, but RoV binding to the CIM C4 column was effective with 70% recovery efficiency and 60-fold concentration factor (Runs 4–5, Table 2). Moreover, no RoV was detected by qPCR in the wash and FT fractions indicating that RoV binding was almost complete using the CIM C4 column (Run 5, Table 2). When RoV concentration using CIM C4 column was repeated using a seawater sample collected from the Adriatic coast of Slovenia with no pH conditioning or any other chemical modification, an effective 63% recovery efficiency with 50-fold concentration factor was obtained (Run 6, Table 2), indicating the choice of CIM C4 column as the most effective for RoV concentration from high salinity water samples.

3.2. Optimizing simultaneous concentration of rotavirus and norovirus with CIM C4 column

Given the continued lack of prophylactic vaccines against NoV, any monitoring protocols for assessing the risk of viral gastroenteritis should include both RoV and NoV detection. Thus, we tested the ability of CIM C4 column to concentrate a saline water sample spiked with both RoV and NoV (Table 3). Similarly, as for RoV, NoV levels in the CIM C4 fractions were determined using RT-qPCR (section 2.5) and standard curve quantitation as described in section 2.6 and Supplementary Method S2. The CIM C4 column appears to bind both RoV and NoV very well as evidenced by the relatively low amount of virus detected by RT-qPCR in the wash and FT fractions (Table 3). The ideal concentration factor assuming 100% recovery efficiency with 400 ml load and 5 ml eluate is 80-fold. However, the buffer employed in the chromatographic run appears to be unable to efficiently elute the bound NoV from the CIM C4 column resulting in no net concentration (1-fold concentration factor). Additionally, the presence of NoV appeared to affect the elution of bound RoV as the concentration factor of RoV was reduced to 19-fold in presence of NoV (Table 3), versus 50-fold–60-fold when RoV was concentrated alone through CIM C4 column (Table 2), despite using identical elution conditions. To improve the elution of bound RoV and NoV from the CIM C4 columns, we investigated the effect of lowering the polarity of the elution buffer (50 mM HEPES pH 7) by addition of 5%, 10% or 15% isopropanol on

Table 2
Preliminary screening with CIM columns for rotavirus concentration from saline water.

Run	CIM chemistry	Load salinity	Virus recovery (Cq, RoV)				Concentration factor	Recovery efficiency
			Load	FT	Wash	Eluate		
1	QA	0.3 M NaCl	29.76	29.55	28.56	27.53	5-fold	21%
2	DEAE	0.3 M NaCl	29.87	30.57	29.11	27.50	8-fold	35%
3	SO_3	0.3 M NaCl	29.24	30.07	30.82	28.02	4-fold	17%
4	OH	0.6 M NaCl	31.52	31.72	30.42	31.33	0.3-fold	1%
5	C4	0.6 M NaCl	31.54	nd	nd	25.86	60-fold	75%
6	C4	seawater	29.87	36.35	36.44	24.43	50-fold	63%

nd: not detectable.

Table 3
Simultaneous concentration of rotavirus and norovirus with CIM C4 column.

Virus recovery	Virus recovery (Cq)						Concentration factor
	Load (400 ml)	FT (400 ml)	Wash (15 ml)	Eluate 1 (5 ml)	Eluate 2 (5 ml)	Eluate 3 (5 ml)	
Rotavirus	25.09	33.56	33.24	20.95	23.52	25.08	19-fold
Norovirus	26.78	32.51	31.32	26.77	27.70	28.45	1-fold

Table 4
Optimizing recovery of rotavirus and norovirus during simultaneous concentration with CIM C4 column.

Virus Recovery	Elution buffer	Virus recovery (Cq)						Concentration factor
		Load (400 ml)	FT (400 ml)	Wash (15 ml)	Eluate 1 (5 ml)	Eluate 2 (5 ml)	Eluate 3 (5 ml)	
Rotavirus	50 mM HEPES	27.31	33.32	34.18	23.75	26.16	28.09	19-fold
	5% Isopropanol	29.72	34.36	36.61	24.48	28.89	29.96	64-fold
	10% Isopropanol	32.49	nd	nd	26.95	29.48	29.74	56-fold
	15% Isopropanol	28.89	34.01	nd	23.79	28.83	29.85	41-fold
Norovirus	50 mM HEPES	29.50	nd	nd	29.87	29.74	31.14	1-fold
	5% Isopropanol	28.81	nd	nd	27.05	29.01	29.71	5-fold
	10% Isopropanol	29.75	nd	nd	25.89	29.21	29.56	13-fold
	15% Isopropanol	28.91	nd	nd	26.57	28.74	29.71	5-fold

nd: not detectable.

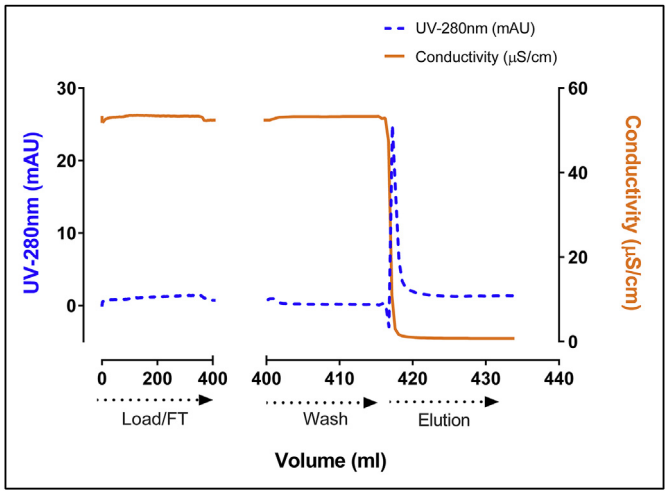

Fig. 1. Chromatographic concentration of rotavirus and norovirus in a saline water sample by 1 ml CIM C4 monolithic column. Chromatogram showing a concentration run where 400 ml of saline buffer (50 mM HEPES, 0.6 M NaCl, pH 7) spiked with stool samples positive for rotavirus and norovirus were loaded through a CIM C4 1 ml column at a flow rate of 10 ml/min. After loading the 400 ml sample, CIM C4 column was washed with 15 ml of the saline buffer following which viruses were eluted with 5 ml elution buffer (50 mM HEPES, 10 % Isopropanol, pH 7) in three consecutive steps. Throughout the concentration run, absorbance (UV-280 nm, broken line, left y-axis) and conductivity (μS/cm, solid line, right y-axis) were monitored and are plotted against progressing volume (x-axis). The volume (x-axis) is split in two parts (0–400 ml; 400–440 ml) for better visualization of the elution step. Broken arrows below the x-axis indicate the corresponding chromatographic fractions of Load/FT, wash and elution steps respectively.

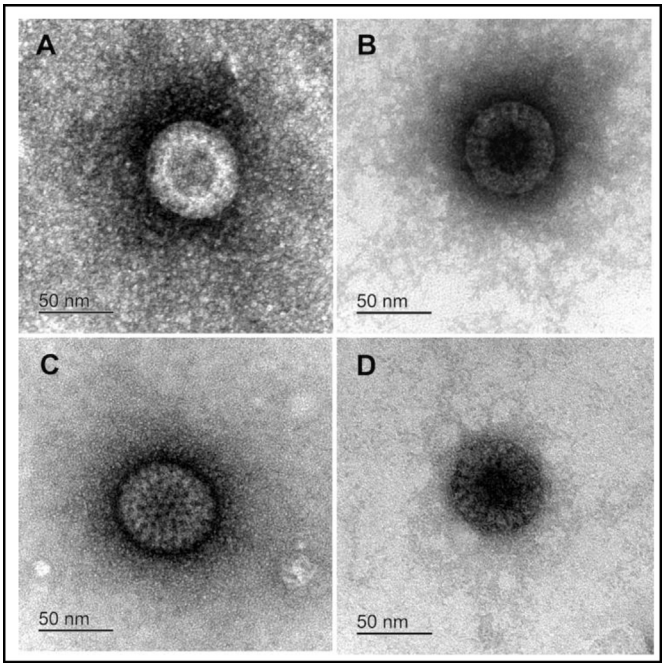

Fig. 2. Electron micrograph of rotavirus particles before and after elution from CIM C4 monolithic column. Panels A and B show electron microscope micrographs corresponding to the original RoV clarified stool suspension used as spike. Panels C and D show micrographs of the fraction corresponding to the elution step in Fig. 1. All micrographs were taken at the same magnification.

the simultaneous concentration of RoV and NoV by CIM C4 columns (Table 4). The concentration factor of RoV/NoV improved from 19-fold/1-fold to 64-fold/5-fold, 56-fold/13-fold and 41-fold/5-fold with addition of 5%, 10% or 15% isopropanol in the elution buffer, respectively. Observing the chromatogram of the concentration run using the 10% isopropanol elution buffer, the peak of elution, as indicated by a sharp change in UV absorbance, corresponds to the addition of the elution buffer with 10% isopropanol to the CIM C4 column (Fig. 1). Taking into account both the chromatogram and the RT-qPCR results (Table 4), confirms the elution buffer (10% isopropanol, 50 mM HEPES pH 7) to be the most efficient for eluting column bound of RoV and NoV and was therefore used as the default elution buffer in all subsequent concentrations of RoV and NoV by CIM C4 columns. Furthermore, when the RoV positive stool suspension and the eluate fraction E1 from the CIM C4 concentration run using this elution buffer (10% isopropanol, 50 mM HEPES pH 7) were characterized through a transmission electron microscope (TEM), no alterations in the appearance of the RoV particles induced by the passage through the CIM C4 monolithic column were noticed (Fig. 2). Due to the lower efficiency of NoV elution compared to that of RoV, NoV particles were not observed, as they were most probably at concentrations lower than that needed for detection through the TEM.

3.3. *Dynamic range concentration of rotavirus and norovirus with CIM C4 column*

Any robust protocol for environmental viral monitoring must be able to process and detect viral loads across a wide dynamic range. To test the performance of CIM C4 columns in concentrating RoV and NoV at different load levels, we added virus-positive stool samples at dilution factor of either 10^4 RoV and 10^4 NoV (high viral load) or 10^7 RoV and 10^5 NoV (low viral load) respectively to the input saline water samples (Table 5). For each of the high and low viral load samples, duplicate concentration runs through a CIM C4 column were performed and amount of RoV and NoV in various fraction determined by RT-qPCR (Table 5). For the high viral load sample runs, CIM C4 concentration enhanced RoV detection by RT-qPCR from Cq ~29.0 in the load to Cq ~23.6 in the eluate fraction

Table 5
Dynamic range of concentration of rotavirus and norovirus with CIM C4 column at high and low viral loads levels.

Viral load	Virus recovery	Run	Virus recovery (Cq)						Concentration factor
			Load (400 ml)	FT (400 ml)	Wash (15 ml)	Eluate 1 (5 ml)	Eluate 2 (5 ml)	Eluate 3 (5 ml)	
High	Rotavirus	Run 1	29.11	32.65	34.48	23.46	27.41	27.21	63-fold
		Run 2	29.04	31.73	34.39	23.73	28.18	29.27	48-fold
	Norovirus	Run 1	29.77	nd	nd	26.90	29.41	32.59	7-fold
		Run 2	29.66	nd	nd	26.73	30.08	31.36	7-fold
Low	Rotavirus	Run 3	nd	nd	nd	32.33	37.72	41.18	nm
		Run 4	nd	nd	nd	32.91	nd	nd	nm
	Norovirus	Run 3	nd	nd	nd	31.45	44.15	nd	nm
		Run 4	nd	nd	nd	31.72	33.98	nd	nm

nd: not detectable.
nm: not measurable.

which represents a concentration factor of 50-fold–60-fold. In parallel, NoV detection by RT-qPCR in the high viral load samples were enhanced from Cq ~29.7 in the load to Cq ~26.8 in the eluate fraction which represents a concentration factor of 7-fold (Runs 1–2, Table 5). Crucially, in the low viral load samples where both RoV and NoV were beyond detection limits of RT-qPCR in the load fractions (Cq nd), concentration with CIM C4 column enabled detection of both RoV and NoV in the eluate fractions (Runs 3–4, Table 5). This shows that CIM C4 column concentration method may be successfully employed for enhancing the sensitivity of detection of enteric viruses in environmental saline water samples where RoV and NoV are often present at concentration levels undetectable solely by qPCR.

3.4. Concentration of rotavirus and norovirus from spiked river and seawater with CIM C4 column

To determine the ability of CIM C4 columns to concentrate RoV and NoV present across a range of salinities, we processed 4000 ml of brackish river water and seawater collected along the Adriatic coast in Piran, Slovenia, each spiked with RoV and NoV positive stool samples at dilution of 1×10^4. To process the larger volume of water in approximately the same time as for the previous experiments, we used a larger CIM C4 column with 8 ml column volume and a flow rate of 60 ml/min that enables the concentration of 4000 ml of environmental saline water samples in about 70 min. Testing the brackish river water sample for concentration of RoV and NoV using the 8 ml CIM C4 column, we observed a concentration factor of 227-fold for RoV and 40-fold for NoV (Table 6). For the seawater sample, a concentration of 307-fold for RoV and 43-fold for NoV was obtained. Thus, in addition to enabling processing of the large volume water samples of different salinities in shorter time, the 8 ml CIM C4 column allows for volume reduction from 4000 ml load to 12 ml eluate with theoretical concentration factor of 333-fold at 100% recovery efficiency (Fig. 3). This is true scaling up from the 80-fold concentration factor achievable using the 1 ml CIM C4 column used in the preceding experiments (Fig. 1).

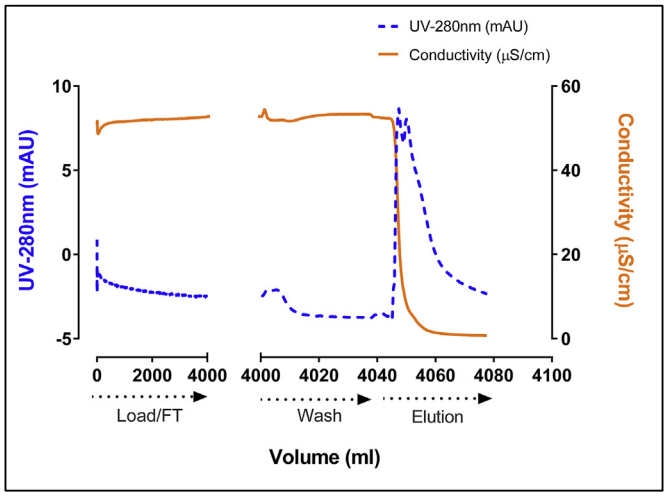

Fig. 3. Chromatographic concentration of rotavirus and norovirus in a seawater sample by 8 ml CIM C4 monolithic column. Chromatogram showing a concentration run where 4000 ml of seawater sample spiked with stool samples positive for rotavirus and norovirus were loaded through a CIM C4 8 ml column at a flow rate of 60 ml/min. After loading the 4000 ml sample, CIM C4 column was washed with 40 ml of saline buffer (50 mM HEPES, 0.6 M NaCl, pH 7) following which viruses were eluted with 12 ml elution buffer (50 mM HEPES, 10 % Isopropanol, pH 7) in three consecutive steps. Throughout the concentration run, absorbance (UV-280 nm, broken line, left y-axis) and conductivity (µS/cm, solid line, right y-axis) were monitored and are plotted against progressing volume (x-axis). The volume (x-axis) is split in two parts (0–4000 ml; 4000–4100 ml) for better visualization of the elution step. Broken arrows below the x-axis indicate the corresponding chromatographic fractions of Load/FT, wash and elution steps respectively.

3.5. On-site concentration of rotavirus from spiked seawater with CIM C4 column

In addition to precision and adaptability, portability is also a desirable characteristic of any enteric virus concentration method for its eventual adoption for use in the field. Therefore, the CIM C4 column was deployed in a portable setup to concentrate seawater

Table 6
Concentration of rotavirus and norovirus in environmental water samples with CIM C4 column.

Environmental sample	Virus recovery	Virus recovery (Cq)						Concentration factor
		Load (4000 ml)	FT (4000 ml)	Wash (40 ml)	Eluate 1 (12 ml)	Eluate 2 (12 ml)	Eluate 3 (12 ml)	
River	Rotavirus	31.13	33.89	34.12	24.00	27.72	29.25	227-fold
	Norovirus	30.06	nd	31.09	24.82	26.93	27.98	40-fold
Sea	Rotavirus	31.56	34.27	33.82	23.88	27.66	29.66	307-fold
	Norovirus	29.71	nd	31.30	24.18	26.61	28.08	43-fold

nd: not detectable.

Table 7
On-site concentration of rotavirus in seawater samples with CIM C4 column.

Seawater	RoV (Cq) load	RoV (Cq) concentrate	Concentration factor	Recovery efficiency
Non-spiked	nd	nd	nm	nm
spiked	31.05	24.82	98-fold	65%

nd: not detectable.
nm: not measurable.

samples, similar to a previous field setup using CIM QA columns on stream water samples (Gutiérrez-Aguirre et al., 2011). 450 ml of seawater samples (load), either non-spiked, or spiked with stool samples positive for RoV diluted by a factor of 1×10^4, were concentrated through a 1 ml CIM C4 column using a transportable dosing pump for pumping the seawater samples. Bound RoV was eluted (concentrate) using 3 ml elution buffer (50 mM HEPES pH 7 with 10% isopropanol) and elution step was monitored with a modular preparative UV detector. The amount of RoV in the load and concentrate were quantified by RT-qPCR. No RoV was detected (Cq nd) in the non-spiked seawater sample in the load or concentrated fractions. However, in the spiked seawater samples, on-site CIM C4 concentration enhanced RoV detection by RT-qPCR from Cq ~31.0 in the load to Cq ~24.8 in the concentrate (Table 7). Moreover, concentration factor of 98-fold and 65% recovery efficiency of the on-site method is comparable to the performance of the method using the reference FPLC system (Table 5), which indicates that the CIM C4 method is a flexible protocol capable of being adapted to on-site environmental monitoring of RoV and NoV in seawater or coastal brackish water samples.

4. Discussion

4.1. Viral concentration methods for enteric viruses in saline water matrices

The challenge of developing sensitive detection methods for enteric viruses in environmental water samples has garnered considerable attempts with varied levels of success. Many of the commonly used techniques for viral concentration rely on the principle of viral adsorption from larger volumes of environmental water and subsequent elution in more concentrated form (Fong and Lipp, 2005). The rationale for the wide adoption of electropositive filters as supports used for viral adsorption is their electrostatic interaction with most of viruses that are considered to be negatively charged particles in ground and fresh water matrices (Ikner et al., 2012). These electropositive filters display poor effectiveness in saline water samples due to presence of higher concentration of salts in the saline water matrices which interferes with electrostatic interactions and instead promotes hydrophobic interactions (Lukasik et al., 2000). As an alternative to electropositive filters, few research groups have used electronegative filters to concentrate enteric viruses. Katayama et al., using an electronegative HA membrane filter, obtained efficient recovery of poliovirus from seawater samples, but more recent attempts to concentrate NoV and RoV with a similar approach have resulted in variable and inefficient performance in environmental water matrices (De Keuckelaere et al., 2013; Katayama et al., 2002). Additionally, this technique is effective only with acidification of water samples to a low pH, complicating the adoption for routine environmental monitoring when processing larger numbers or volumes of saline water samples (Ikner et al., 2012).

Calgua et al. developed a skimmed milk flocculation method for concentration of adenoviruses (AdV) from seawater matrices but this method takes more than 10 h processing time per sample (Calgua et al., 2008). Another method combining filtration with glass wool filter and nitrocellulose membrane in addition to skim milk flocculation was successfully used for environmental monitoring of AdV and NoV in saline water samples from European recreational areas with processing time of around 2 h (Wyn-Jones et al., 2011). However, this method also involves pre-acidification of the seawater samples prior to concentration which adds additional time and cost constraints for routine use in the field. Gibbons et al. employed electropositive, alumina nanofiber (NanoCeram) cartridge filters for concentration of enteric viruses from seawater samples and obtained recovery efficiencies of 80–90% for NoV but less than 5% for AdV (Gibbons et al., 2010). NanoCeram filters have also been recommended in the United States Environment Protection Agency (U.S. EPA) method 1615 for monitoring of enteric viruses in environmental water samples. However, the testing of this method for NoV concentration and detection revealed that recovery efficiency was 30% in groundwater samples but only 10% in surface water samples (Cashdollar et al., 2013). Moreover, the elution step of the NanoCeram method involves use of beef extract, which has been reported to contain inhibitors that potentially affect virus detection using molecular methods such as qPCR (Ahmed et al., 2015; Ikner et al., 2012). Ultrafiltration that uses a size exclusion principle and ultracentrifugation are some other techniques for virus concentration from water samples, but are employed more as finishing secondary steps and usually preceded by a filter based concentration step for clarification of water samples. Major limitations of ultrafiltration and ultracentrifugation include very high cost of equipment and consumables, longer processing time, and an inability of scaling up to handle larger water volumes.

4.2. Methacrylate monolithic hydrophobic interaction chromatography as a tool to concentrate enteric viruses in saline water matrices

CIM methacrylate monolithic columns which are optimized for interaction and concentration of biomolecules like plasmids and viruses (Barut et al., 2005), address many of the limitation posed during the concentration of viruses from water matrices using many of the methods discussed above. This is due to some unique characteristics of the CIM monolithic columns such as their large average pore sizes ranging from 1.5 to 6 μm, availability of flexible ion exchange or hydrophobic interaction based active chemistries, and fast convective flow, allowing them to be used for effective concentration of enteric viruses from a wide range of water matrices including bottled water, streams, rivers and wastewater (Gutiérrez-Aguirre et al., 2009; Kovač et al., 2009; Rački et al., 2015; Steyer et al., 2015). The CIM QA columns used for most of the aforementioned concentration applications rely on ion exchange interactions with enteric viruses which may be diminished in saline water matrices (Lukasik et al., 2000). Our results show that the CIM columns with ion exchange chemistries such as QA, DEAE and SO_3 were inefficient in concentrating RoV in saline water samples (Table 2). Since hydrophobic interactions are relatively less affected by higher salinity (Thomas and Elcock, 2006), we screened the ability of CIM C4 and CIM OH hydrophobic interaction chemistry columns to concentrate RoV in saline water samples. The CIM C4

columns that utilize hydrophobic interactions were much more effective in binding and concentrating RoV in saline water samples (Table 2).

Since prophylactic vaccines against NoV infection are still lacking, monitoring protocols for risk assessment of enteric virus infection must include detection of NoV levels. When tested with saline water samples containing both RoV and NoV, the CIM C4 columns were able to effectively bind the majority of both RoV and NoV particles as there was a relatively low amount of either NoV or RoV detected in the wash of FT fractions (Table 3). However, a simple isocratic elution using buffer without salt was unable to elute the bound NoV particles from the CIM C4 columns (Table 3). One reason for this could be that NoV particles may aggregate to a greater extent than RoV at higher salinity (da Silva et al., 2011), which would affect the elution from the CIM C4 columns. We tried using non-ionic surfactants to disrupt NoV aggregation during the elution step (Mertens and Velev, 2015), but this did not yield any improvements in recovering NoV from the CIM C4 columns (data not shown). Another strategy for improving elution from hydrophobic columns is the addition of organic solvents to lower the polarity and disrupt the interaction between the adsorbent and viral particles (McCue, 2009). When we employed 5%, 10% and 15% isopropanol in the buffers during the elution step, we observed a clear enhancement of recovery efficiency for both RoV and NoV, with 10% isopropanol giving the best results for combined elution of RoV and NoV from the CIM C4 columns (Table 4). Despite the improvements in elution with 10% isopropanol, the recovery efficiency of NoV (~10%) was still consistently lower than RoV (~60%) over multiple runs, and with different flow rates, column sizes and viral loads (Tables 4–6). We chose stool samples containing NoV GII since this genogroup is responsible for the majority of human NoV infections (Riddle and Walker, 2016) and the most relevant in context of waterborne contamination in Slovenia (Steyer et al., 2015, 2011). In the future, additional NoV genotypes could be tested to evaluate the concentration efficiency of NoV using CIM C4 columns. Since most NoV genotype share well conserved hydrophobic amino acid residues in the capsid (Imai et al., 2011), there is likely to be consistency in hydrophobic interactions with CIM C4 columns amongst viral particles belonging to the different NoV genotypes.

The trend of lower concentration efficiency for NoV compared to other enteric viruses has been observed in many previous studies that have employed varying concentration tools. Cashdollar and Wymer conducting a meta-analysis of six different studies from 2007 to 2011 for NoV concentration from various water matrices observed that the average efficiency of NoV concentration was below 30%, a value lower than that obtained for other waterborne viruses (Cashdollar and Wymer, 2013). The method with one of the highest efficiency of NoV concentration from seawater is based on adsorption to a NanoCeram cartridge filter and elution using beef extracted recirculated with a peristaltic pump (Gibbons et al., 2010). The above method is able to concentrate 40 L of seawater into 500 ml at ~90% efficiency which computes to a concentration factor of 70-fold. Despite the lower concentration efficiency ~10%, the 8 ml CIM C4 columns yielded a 40-fold concentration in both brackish river water and seawater (Table 6). Moreover, the use of beef extract during elution may interfere with downstream molecular detection techniques like qPCR (Ahmed et al., 2015), which can lead to overestimation of virus recovery. Future improvements in NoV elution from CIM C4 columns could be attained by testing a wider range of organic solvents like glycols, acetonitrile and alcohols to disrupt the hydrophobic interactions between the CIM C4 columns and the adsorbed NoV particles (McCue, 2009). There are examples of CIM methacrylate monolithic columns of a particular interaction chemistry displaying concentration ability for enteric viruses other than RoV, such as for astrovirus and sapovirus with QA ion-exchange CIM columns in inland wastewaters (Steyer et al., 2015). At any rate, the differences in concentration efficiencies amongst the various waterborne enteric viruses highlights the need for contextual optimizations for individual enteric viruses in different water matrices (Cashdollar and Wymer, 2013).

As a tool for primary concentration waterborne enteric viruses, the performance of CIM C4 columns compare favorably with other available concentration methods. Using the 8 ml CIM C4 column, we achieved a flow rate of 60 ml/min that enables the concentration of 4000 ml of samples in approximately 70 min into a final eluted volume of 12 ml which represents a theoretical concentration factor of 333-fold at 100% recovery efficiency (Fig. 3). The protocol with the CIM C4 columns does not require any pre-treatment of the saline water samples with ions, salts or buffers to alter the pH which makes for simple processing for enteric virus monitoring applications. The pre-filter step with 0.8 μm filter in our protocol serves two purposes: as it is not generally advisable to store raw seawater for any considerable duration, a quick filtration through 0.8 μm filters allows for overnight storage and transport of seawater from various sampling sites. And although not strictly necessary, using a pre-filtration step with 0.8 μm filters improves the flow-rate and life-cycle of the CIM monolithic methacrylate columns. We found no difference in RoV or NoV levels before and after the pre-filter step with the 0.8 μm filter on seawater and brackish river water samples (data not shown). Additionally, for on-site applications, it is possible to include the 0.8 μm filter inline via a specially designed stainless steel housing (Gutiérrez-Aguirre et al., 2011), to ensure that the pre-filtration step does not increase the processing time for concentration of seawater samples. Previously, CIM QA columns have been operated at flow rate of 100 ml/min with environmental water samples with no loss in viral concentration capacity (Gutiérrez-Aguirre et al., 2009). Thus, higher flow rates to 100–120 ml/min could be employed to reduce the processing time by up to 50% for future viral monitoring studies. Empirical determination of the flow rate for samples based on water turbidity will allow for an optimized concentration performance with CIM C4 columns. As far as handling high viral loads is concerned, a combination of CIM OH and CIM QA columns was used in tandem to as a tool for waterborne virus removal with a capacity of 10^{11} RoV particles/ml in a complex wastewater effluent matrix (Rački et al., 2015). Thus, it would be feasible to handle complex and highly turbid sample using a combination of CIM column chemistries for efficient viral concentration and detection.

Downstream of RoV and NoV concentration by the CIM C4 hydrophobic methacrylate monolithic columns, virus detection and quantitation was carried out by RT-qPCR. The limit of detection of the RT-qPCR assay for both NoV and RoV was between Cq values of 35–37, which is consistent to earlier studies using these primer pairs and corresponding to less than 10 copies per reaction (Gutiérrez-Aguirre et al., 2008; Kageyama et al., 2003; Steyer et al., 2011). When testing with saline water samples with low viral loads where both RoV and NoV were beyond the detection limits for RT-qPCR, concentration with CIM C4 columns enabled the detection of both RoV and NoV in the concentrate eluate (Table 5). The CIM C4 protocol clearly extends the sensitivity of RoV and NoV detection by RT-qPCR and should allow for detection of as low as 10 RoV and 10–100 NoV particles/ml, considering a concentration factor of 300-fold for RoV and 40-fold for NoV using the CIM C4 8 ml column (Table 6). Since the dose of enteric virus exposure that manifests in gastrointestinal disease is around 10 particles (Haas et al., 1993), the CIM C4 protocol should be capable of detecting RoV and NoV threats around these levels. We use the Cq results of the RoV and/or NoV RT-qPCR assay and applying quantitation from Log_{10} dilution series standard curve to arrive at the concentration factor as a

metric of metric of the improvement of the qPCR-based detection of RoV/NoV. When the technique will be employed for enteric virus surveillance at environmental sites, usage of end-point metrics will be essential for comparison with results from studies using other concentration methods. This could be achieved by using an absolute standard such as armored RNA (Pasloske et al., 1998) or plasmid cDNA to represent RoV and NoV as genome copies. Recently, the RoV RT-qPCR assay was successfully adapted to a one-step digital PCR (RT-ddPCR) which results in an end-point and absolute metric that enables the determination of target copy number for RoV without the need for a standard (Rački et al., 2013). The RT-ddPCR assay matches the performance of RT-qPCR in terms of sensitivity and also has the advantage of better tolerance to matrix inhibition making it a good choice as a molecular detection tool for waterborne enteric virus quantitation in environmental water samples following concentration by methacrylate monolithic columns.

5. Conclusions

In this study, we evaluated the applicability of CIM C4 methacrylate monolithic column chromatography to concentrate rotavirus and norovirus particles from saline water matrices.

- CIM C4 hydrophobic interaction monolithic supports efficiently bind both rotavirus and norovirus particles simultaneously from saline water samples and both viruses can be eluted in a single step
- CIM C4 column performance in concentrating rotavirus and norovirus particles was congruent in saline seawater as well as brackish river water samples
- The use of an 8 ml CIM C4 column allows seamless scaling up of the method to process larger volumes of environmental saline water samples in a reasonably short time
- CIM C4 monolithic columns were successfully deployed in a portable setup for an on-site concentration of rotavirus particles from seawater samples
- The combination of concentration using CIM C4 columns paired with detection using RT-qPCR allows for an efficient estimation of rotavirus and norovirus contamination in saline water matrices

Overall, the results from successful deployment of CIM C4 hydrophobic interaction columns for concentration of rotavirus and norovirus in saline water samples reiterate the utility of CIM methacrylate monolithic supports as efficient, scalable and modular preparative tools for processing environmental water samples to enhance viral detection using molecular methods.

Author contributions

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2016.10.020.

References

Ahmed, W., Harwood, V.J., Gyawali, P., Sidhu, J.P.S., Toze, S., 2015. Comparison of concentration methods for quantitative detection of sewage-associated viral markers in environmental waters. Appl. Environ. Microbiol. 81, 2042–2049. http://dx.doi.org/10.1128/AEM.03851-14.

Barut, M., Podgornik, A., Brne, P., Štrancar, A., 2005. Convective Interaction Media short monolithic columns: enabling chromatographic supports for the separation and purification of large biomolecules. J. Sep. Sci. 28, 1876–1892. http://dx.doi.org/10.1002/jssc.200500246.

Bishop, R., 2009. Discovery of rotavirus: implications for child health. J. Gastroenterol. Hepatol. 24, S81–S85. http://dx.doi.org/10.1111/j.1440-1746.2009.06076.x.

Brown, S., Nicholls, R.J., Woodroffe, C.D., Hanson, S., Hinkel, J., Kebede, A.S., Neumann, B., Vafeidis, A.T., 2013. Sea-level rise impacts and responses: a global perspective. In: Finkl, C.W. (Ed.), Coastal Hazards. Coastal Research Library. Springer, Netherlands, pp. 117–149. http://dx.doi.org/10.1007/978-94-007-5234-4_5.

Calgua, B., Mengewein, A., Grunert, A., Bofill-Mas, S., Clemente-Casares, P., Hundesa, A., Wyn-Jones, A.P., López-Pila, J.M., Girones, R., 2008. Development and application of a one-step low cost procedure to concentrate viruses from seawater samples. J. Virol. Methods 153, 79–83. http://dx.doi.org/10.1016/j.jviromet.2008.08.003.

Cashdollar, J.L., Brinkman, N.E., Griffin, S.M., McMinn, B.R., Rhodes, E.R., Varughese, E.A., Grimm, A.C., Parshionikar, S.U., Wymer, L., Fout, G.S., 2013. Development and Evaluation of EPA Method 1615 for detection of enterovirus and norovirus in water. Appl. Environ. Microbiol. 79, 215–223. http://dx.doi.org/10.1128/AEM.02270-12.

Cashdollar, J.L., Wymer, L., 2013. Methods for primary concentration of viruses from water samples: a review and meta-analysis of recent studies. J. Appl. Microbiol. 115, 1–11. http://dx.doi.org/10.1111/jam.12143.

da Silva, A.K., Kavanagh, O.V., Estes, M.K., Elimelech, M., 2011. Adsorption and aggregation properties of norovirus GI and GII virus-like particles demonstrate differing responses to solution chemistry. Environ. Sci. Technol. 45, 520–526. http://dx.doi.org/10.1021/es102368d.

De Keuckelaere, A., Baert, L., Duarte, A., Stals, A., Uyttendaele, M., 2013. Evaluation of viral concentration methods from irrigation and processing water. J. Virol. Methods 187, 294–303. http://dx.doi.org/10.1016/j.jviromet.2012.11.028.

Fong, T.-T., Lipp, E.K., 2005. Enteric viruses of humans and animals in aquatic environments: health risks, detection, and potential water quality assessment tools. Microbiol. Mol. Biol. Rev. 69, 357–371. http://dx.doi.org/10.1128/MMBR.69.2.357-371.2005.

Gentry-Shields, J., Wang, A., Cory, R.M., Stewart, J.R., 2013. Determination of specific types and relative levels of QPCR inhibitors in environmental water samples using excitation–emission matrix spectroscopy and PARAFAC. Water Res. 47, 3467–3476. http://dx.doi.org/10.1016/j.watres.2013.03.049.

Gibbons, C.d, Rodríguez, R.a., Tallon, L., Sobsey, M.d., 2010. Evaluation of positively charged alumina nanofibre cartridge filters for the primary concentration of noroviruses, adenoviruses and male-specific coliphages from seawater. J. Appl. Microbiol. 109, 635–641. http://dx.doi.org/10.1111/j.1365-2672.2010.04691.x.

Girones, R., Ferrús, M.A., Alonso, J.L., Rodriguez-Manzano, J., Calgua, B., de Abreu Corrêa, A., Hundesa, A., Carratala, A., Bofill-Mas, S., 2010. Molecular detection of pathogens in water – the pros and cons of molecular techniques. Water Res. 44, 4325–4339. http://dx.doi.org/10.1016/j.watres.2010.06.030.

Glass, R.I., Parashar, U., Patel, M., Gentsch, J., Jiang, B., 2014. Rotavirus vaccines:

successes and challenges. J. Infect. 68, S9–S18. http://dx.doi.org/10.1016/j.jinf.2013.09.010.
Gutiérrez-Aguirre, I., Banjac, M., Steyer, A., Poljšak-Prijatelj, M., Peterka, M., Štrancar, A., Ravnikar, M., 2009. Concentrating rotaviruses from water samples using monolithic chromatographic supports. J. Chromatogr. A 1216, 2700–2704. http://dx.doi.org/10.1016/j.chroma.2008.10.106, 3rd Summer School on Monolith Technology for Biochromatography, Bioconversion and Solid-Phase Synthesis 3rd Summer School on Monolith Technology for Biochromatography, Bioconversion and Solid-Phase Synthesis.
Gutiérrez-Aguirre, I., Steyer, A., Banjac, M., Kramberger, P., Poljšak-Prijatelj, M., Ravnikar, M., 2011. On-site reverse transcription-quantitative polymerase chain reaction detection of rotaviruses concentrated from environmental water samples using methacrylate monolithic supports. J. Chromatogr. A 1218, 2368–2373. http://dx.doi.org/10.1016/j.chroma.2010.10.048. Selected Papers of the 4th Summer School of Monolith Technology for Biochromatography, Bioconversion and Phase State Synthesis.
Gutiérrez-Aguirre, I., Steyer, A., Boben, J., Gruden, K., Poljšak-Prijatelj, M., Ravnikar, M., 2008. Sensitive detection of multiple rotavirus genotypes with a single reverse transcription-real-time quantitative PCR assay. J. Clin. Microbiol. 46, 2547–2554. http://dx.doi.org/10.1128/JCM.02428-07.
Haas, C.N., Rose, J.B., Gerba, C., Regli, S., 1993. Risk assessment of virus in drinking water. Risk Anal. 13, 545–552. http://dx.doi.org/10.1111/j.1539-6924.1993.tb00013.x.
Ikner, L.A., Gerba, C.P., Bright, K.R., 2012. Concentration and recovery of viruses from water: a comprehensive review. Food Environ. Virol. 4, 41–67. http://dx.doi.org/10.1007/s12560-012-9080-2.
Imai, T., Sano, D., Miura, T., Okabe, S., Wada, K., Masago, Y., Omura, T., 2011. Adsorption characteristics of an enteric virus-binding protein to norovirus, rotavirus and poliovirus. BMC Biotechnol. 11, 123. http://dx.doi.org/10.1186/1472-6750-11-123.
Kageyama, T., Kojima, S., Shinohara, M., Uchida, K., Fukushi, S., Hoshino, F.B., Takeda, N., Katayama, K., 2003. Broadly reactive and highly sensitive assay for norwalk-like viruses based on real-time quantitative reverse transcription-PCR. J. Clin. Microbiol. 41, 1548–1557. http://dx.doi.org/10.1128/JCM.41.4.1548-1557.2003.
Katayama, H., Shimasaki, A., Ohgaki, S., 2002. Development of a virus concentration method and its application to detection of enterovirus and norwalk virus from coastal seawater. Appl. Environ. Microbiol. 68, 1033–1039. http://dx.doi.org/10.1128/AEM.68.3.1033-1039.2002.
Kovač, K., Gutiérrez-Aguirre, I., Banjac, M., Peterka, M., Poljšak-Prijatelj, M., Ravnikar, M., Mijovski, J.Z., Schultz, A.C., Raspor, P., 2009. A novel method for concentrating hepatitis A virus and caliciviruses from bottled water. J. Virol. Methods 162, 272–275. http://dx.doi.org/10.1016/j.jviromet.2009.07.013.
Lees, D., 2000. Viruses and bivalve shellfish. Int. J. Food Microbiol. 59, 81–116. http://dx.doi.org/10.1016/S0168-1605(00)00248-8.
Lukasik, J., Scott, T.M., Andryshak, D., Farrah, S.R., 2000. Influence of salts on virus adsorption to microporous filters. Appl. Environ. Microbiol. 66, 2914–2920. http://dx.doi.org/10.1128/AEM.66.7.2914-2920.2000.
McCue, J.T., 2009. Chapter 25 Theory and use of hydrophobic interaction chromatography in protein purification applications. In: Deutscher, M.P., Burgess, R.R. (Eds.), Methods in Enzymology, Guide to Protein Purification, second ed. Academic Press, pp. 405–414. http://dx.doi.org/10.1016/S0076-6879(09)63025-1.
Mertens, B.S., Velev, O.D., 2015. Characterization and control of surfactant-mediated norovirus interactions. Soft Matter 11, 8621–8631. http://dx.doi.org/10.1039/C5SM01778E.
Michen, B., Graule, T., 2010. Isoelectric points of viruses. J. Appl. Microbiol. 109, 388–397. http://dx.doi.org/10.1111/j.1365-2672.2010.04663.x.
Monroe, S., 2011. Control and prevention of viral gastroenteritis. Emerg. Infect. Dis. http://dx.doi.org/10.3201/eid1708.110824.
Nwachuku, N., Gerba, C.P., 2006. Health risks of enteric viral infections in children. Rev. Environ. Contam. Toxicol. 186, 1–56. http://dx.doi.org/10.1007/0-387-32883-1_1.
Paerl, H.W., Dyble, J., Moisander, P.H., Noble, R.T., Piehler, M.F., Pinckney, J.L., Steppe, T.F., Twomey, L., Valdes, L.M., 2003. Microbial indicators of aquatic ecosystem change: current applications to eutrophication studies. FEMS Microbiol. Ecol. 46, 233–246. http://dx.doi.org/10.1016/S0168-6496(03)00200-9.
Pasloske, B.L., Walkerpeach, C.R., Obermoeller, R.D., Winkler, M., DuBois, D.B., 1998. Armored RNA technology for production of ribonuclease-resistant viral RNA controls and standards. J. Clin. Microbiol. 36, 3590–3594.
Rački, N., Kramberger, P., Steyer, A., Gašperšič, J., Štrancar, A., Ravnikar, M., Gutierrez-Aguirre, I., 2015. Methacrylate monolith chromatography as a tool for waterborne virus removal. J. Chromatogr. A 1381, 118–124. http://dx.doi.org/10.1016/j.chroma.2015.01.003.
Rački, N., Morisset, D., Gutierrez-Aguirre, I., Ravnikar, M., 2013. One-step RT-droplet digital PCR: a breakthrough in the quantification of waterborne RNA viruses. Anal. Bioanal. Chem. 406, 661–667. http://dx.doi.org/10.1007/s00216-013-7476-y.
Riddle, M.S., Walker, R.I., 2016. Status of vaccine research and development for norovirus. Vaccine 34, 2895–2899. http://dx.doi.org/10.1016/j.vaccine.2016.03.077. WHO Product Development for Vaccines Advisory Committee (PDVAC) Pipeline Analyses for 25 Pathogens.
Robilotti, E., Deresinski, S., Pinsky, B.A., 2015. Norovirus. Clin. Microbiol. Rev. 28, 134–164. http://dx.doi.org/10.1128/CMR.00075-14.
Schoen, M.E., Soller, J.A., Ashbolt, N.J., 2011. Evaluating the importance of faecal sources in human-impacted waters. Water Res. 45, 2670–2680. http://dx.doi.org/10.1016/j.watres.2011.02.025.
Shipley, G.L., 2011. The MIQE guidelines uncloaked. In: Kennedy, S., Oswald, N. (Eds.), PCR Troubleshooting and Optimization: the Essential Guide. Caister Academic Press pp. viii–236.
Small, C., Nicholls, R.J., 2003. A global analysis of human settlement in coastal zones. J. Coast. Res. 19, 584–599.
Steyer, A., Gutiérrez-Aguirre, I., Rački, N., Glaser, S.B., Humar, B.B., Stražar, M., Škrjanc, I., Poljšak-Prijatelj, M., Ravnikar, M., Rupnik, M., 2015. The detection rate of enteric viruses and clostridium difficile in a waste water treatment plant effluent. Food Environ. Virol. 7, 164–172. http://dx.doi.org/10.1007/s12560-015-9183-7.
Steyer, A., Torkar, K.G., Gutiérrez-Aguirre, I., Poljšak-Prijatelj, M., 2011. High prevalence of enteric viruses in untreated individual drinking water sources and surface water in Slovenia. Int. J. Hyg. Environ. Health 214, 392–398. http://dx.doi.org/10.1016/j.ijheh.2011.05.006.
Thomas, A.S., Elcock, A.H., 2006. Direct observation of salt effects on molecular interactions through explicit-solvent molecular dynamics simulations: differential effects on electrostatic and hydrophobic interactions and comparisons to Poisson–Boltzmann theory. J. Am. Chem. Soc. 128, 7796–7806. http://dx.doi.org/10.1021/ja058637b.
Toplak, N., Okršlar, V., Stanič-Racman, D., Gruden, K., Žel, J., 2004. A high-throughput method for quantifying transgene expression in transformed plants with real-time PCR analysis. Plant Mol. Biol. Rep. 22, 237–250. http://dx.doi.org/10.1007/BF02773134.
Updyke, E.A., Wang, Z., Sun, S., Connell, C., Kirs, M., Wong, M., Lu, Y., 2015. Human enteric viruses—potential indicators for enhanced monitoring of recreational water quality. Virol. Sin. 30, 344–353. http://dx.doi.org/10.1007/s12250-015-3644-x.
Wyn-Jones, A.P., Carducci, A., Cook, N., D'Agostino, M., Divizia, M., Fleischer, J., Gantzer, C., Gawler, A., Girones, R., Höller, C., de Roda Husman, A.M., Kay, D., Kozyra, I., López-Pila, J., Muscillo, M., José Nascimento, M.S., Papageorgiou, G., Rutjes, S., Sellwood, J., Szewzyk, R., Wyer, M., 2011. Surveillance of adenoviruses and noroviruses in European recreational waters. Water Res. 45, 1025–1038. http://dx.doi.org/10.1016/j.watres.2010.10.015.