Coagulant residues' influence on virus enumeration as shown in a study on virus removal using aluminium, zirconium and chitosan

Ekaterina Christensen and Mette Myrmel

ABSTRACT

Research on microorganism reduction by physicochemical water treatment is often carried out under the assumption that the microbiological enumeration techniques are not affected by the presence of coagulants. Data presented here indicate that bacteriophage enumeration by plaque assay and RT-qPCR (reverse transcription quantitative polymerase chain reaction) can be affected by these water treatment chemicals. Treatment of water samples with an alkaline protein-rich solution prior to plaque assay and optimization of RNA extraction for RT-qPCR were implemented to minimize the interference. The improved procedures were used in order to investigate reduction of three viral pathogens and the MS2 model virus in the presence of three coagulants. A conventional aluminium coagulant was compared to alternative agents (zirconium and chitosan) in a coagulation-filtration system. The highest virus reduction, i.e., 99.9–99.99%, was provided by chitosan, while aluminium and zirconium reduced virus by 99.9% in colour-rich water and by 90% in water with less colour, implying an effect of coagulant type and raw water quality on virus reduction. Although charge characteristics of viruses were associated with virus reduction, the results reveal that the MS2 phage is a suitable model for aggregation and retention of the selected pathogens.

Key words | chitosan, coagulation, drinking water treatment, pathogen removal, virus quantification, zirconium

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INTRODUCTION

Treatment of sewage and drinking water often involves destabilization of the particulate and dissolved matter by a coagulation step, which usually precedes separation processes, e.g., sedimentation and filtration. Drinking water plants around the world use salts of aluminium (Al) or ferric (Fe) for coagulation; however, there are some concerns regarding their use. Aluminium is a known neurotoxin, and residual Al in treated water has been linked to neurological disease at concentrations $\geq$0.1 mg/L (Rondeau *et al.* 2009). Ferric salts tend to affect pH and alkalinity of the treated water which requires increased use of chemicals for stabilization and corrosion control (Matilainen *et al.* 2010). Both Al and Fe have also been associated with production of high amounts of sludge (Ødegaard *et al.* 2010). Several reports have acknowledged that compounds, such as zirconium (Zr) and chitosan, possess properties that may enhance organic matter removal and reduce sludge production compared to Al and Fe (Eikebrokk & Saltnes 2002; Jarvis *et al.* 2012; Christensen *et al.* 2016). However, their efficacy in reduction of waterborne pathogens is scarcely documented.

The main purposes of drinking water treatment are reduction and inactivation of pathogens. Traditionally, the effect of treatment is assessed in terms of turbidity (particle content), whereas the microbial reduction is intermittently evaluated using heterotrophic plate count (HPC). However,

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turbidity and HPC cannot predict the effect of treatment on other groups of microorganisms, like viruses (Hijnen & Medema 2010).

Most of the registered waterborne outbreaks in the Nordic countries, between 1998 and 2012, were caused by viruses, mainly norovirus (NV) (Guzman-Herrador *et al.* 2015). Enteric viruses are excreted in high numbers by infected individuals (10^7–10^9 per gram) and enter the environment via waste water (Rusinol & Girones 2017). Although 35% to 90% of viruses are removed by wastewater treatment, high levels can still enter the recipient, e.g., a source of drinking water (Myrmel *et al.* 2015). As ingestion of a few virus particles, like NV (Teunis *et al.* 2010), can cause infection, understanding the conditions for efficient virus reduction during drinking water treatment is an important task in preventing waterborne disease.

Optimal reduction of water pollutants during physico-chemical water treatment relies, among other things, on coagulation efficacy and factors like coagulant dose, pH and presence of other colloids (Ødegaard *et al.* 2010). The influence of these factors on viruses can be hard to confirm experimentally (Hendricks *et al.* 2005), making predictions on virus removal uncertain. Virus retention is often studied using model viruses, like bacteriophages, and not viral pathogens (Xagoraraki *et al.* 2004). The MS2 bacteriophage is extensively used as a model virus, due to similarities with enteric viral pathogens in size and structure (Dawson *et al.* 2005). Quantification of infective MS2 by plaque assay is simple, cheap and rapid; however, the enumeration can be affected by virus aggregation (Langlet *et al.* 2007). Virus clustering in water can be influenced by electrolytes (Floyd & Sharp 1978), including coagulants (Shirasaki *et al.* 2009), whereas it is unclear whether aggregation impacts enumeration of infectious viruses in treated water, which may contain coagulant residue. This knowledge is essential in quantitative microbial risk assessment (QMRA) for water safety management.

Another commonly used technique for virus quantification is RT-qPCR (reverse transcription quantitative polymerase chain reaction), which detects the total of infective and non-infective target virus. This methodology is sensitive to a variety of inhibitors, including metals (Schrader *et al.* 2012); however, previous studies do not give a clear answer as to whether coagulants can influence virus enumeration by RT-qPCR.

The aims of the present study were to investigate the influence of coagulants on quantitation of MS2 model virus, and to minimize any effect of these water treatment chemicals by method optimization (part I). For quantification of infective MS2 by plaque assay, beef extract (BE) solution was tested. The choice of this basic, proteinaceous solution was explained by its ability to reduce virus adsorption to solid surfaces and other virus particles (Moore *et al.* 1982). Two commonly utilized RNA extraction protocols were tested for quantification of total MS2 by RT-qPCR. The two extraction methods employ different silica matrices for RNA binding (Petrich *et al.* 2006).

Second, optimized procedures were applied in a reduction study on viruses in water using Zr, chitosan and the extensively used PACl (polyaluminium chloride) coagulant (part II). Virus reduction was assessed in terms of raw and finished water turbidity and colour, type of coagulant and virus morphology. MS2 was introduced in order to assess its suitability as a model for enteric virus reduction by coagulation-filtration. The human, enteric pathogens hepatitis A virus (HAV), bovine NV (BNV) and bovine coronavirus (BCoV) were included as they represent various virus sizes, isoelectric points (pI) and structural properties.

MATERIAL AND METHODS

Raw water samples

Water was collected from Nedre Romerike Water Treatment Plant (WTP) in Strømmen (Glomma River, Norway) and Årnes WTP (Lake Dragsjøen, Norway). The samples were stored in a dark room at 4 °C. The water was analysed for turbidity, true colour and pH (Table 1) after spiking with the virus suspension.

Table 1 | Characteristics of Glomma and Dragsjøen raw water after spiking with the virus suspension

	Raw water parameters		
Water source	**Turbidity, NTU**	**Colour, mg Pt/L**	**pH**
Dragsjøen	1.1	93	6.6
Dragsjøen diluted	0.5	25	6.6
Glomma	0.8	26	7.3

Dragsjøen diluted, Dragsjøen water diluted 1:4 by distilled water.

Water analyses

Turbidity was measured using a 2100AN turbidimeter (Hach Company, USA). True colour was selected as a surrogate for natural organic matter (NOM), due to its extensive use in surveillance of water treatment in Norway (Norwegian Food Safety Authority 2016). The colour parameter was measured by a Shimadzu UV Visible Spectrophotometer UVmini-1240 (Shimadzu Corporation, Japan), following Standard APHA Method 2120C ($\lambda = 455$ nm) procedure. Prior to colour measurements, the samples were filtered through a 0.45 μm syringe polypropylene membrane (514-0065, VWR, USA), in order to avoid the influence of turbidity.

Coagulants

Polyaluminium chloride (PACl)

A ready-to-use PACl product, PAX-18, was obtained from Kemira Chemicals (Norway). The product has an Al content of 9% (17% as Al_2O_3), basicity of 42% and specific gravity of 1.37 g/mL.

Zirconium oxychloride

Zirconium (IV) oxychloride octahydrate powder was obtained from Teta Vannrensing Ltd (Norway). A working solution of 37% (w/w) was prepared in distilled water and gave a Zr concentration close to the Al content of 9%.

Chitosan

KitoFlokk™ (low molecular weight (MW, 100 kDa) and deacetylation degree (DD) close to 0.8) was obtained from Teta Vannrensing Ltd, Norway. The concentration of the working solution was 2% (w/v) in 0.1 M HCl. A higher chitosan concentration was avoided to prevent undissolved debris in the working solution. The chitosan solution was stored for no longer than 2 weeks. All working solutions were stored at room temperature as no change in coagulation properties had been observed under the selected storage condition.

Viruses

The size and pI characteristics of viruses included in the present study are given in Table 2. Propagation of MS2 bacteriophage was performed according to ISO 10705 (1995), using *Salmonella* Typhimurium WG49 (NCTC 12484) as host.

The apathogenic HAV strain pHM175 43c (kindly provided by Prof. Albert Bosch, University of Barcelona) was propagated in foetal rhesus monkey kidney cells (FRhK-4/R, ATCC® CRL-1688), as previously described (Flehmig 1980).

The BNV (genotype III2) originated from a transmission study (Jor *et al.* 2010). Faecal samples were diluted 1:10 in PBS, rocked for 15 min, and centrifuged at 1,000 g for 15 min at 4 °C. The supernatant was aliquoted, stored at −80 °C, and centrifuged at 12,000 g for 5 min at room temperature before use.

The BCoV stock was prepared in human rectal tumour cells (HRT-18G, ATCC® CRL-11663), according to the procedure described previously (Oma *et al.* 2016).

A spike suspension was prepared by mixing the virus stocks. The volume of individual viruses was defined by initial tests to ensure that processed samples would be virus positive. The culture medium gave a 5–10% increase in water turbidity and colour. The spike suspension was aliquoted and stored at −80 °C.

Table 2 | Virus characteristics; size and isoelectric point (pI)

Virus	Size, nm	pI	Reference
Bacteriophage MS2	30	3.9	Langlet *et al.* (2007)
Hepatitis A virus	28	2.8	Flehmig (1980), Michen & Graule (2010)
Bovine norovirus	30–35	6.0–6.3	Otto *et al.* (2011); theoretical pI, calculated using the ExPASy ProtParam tool, based on the capsid protein sequence
Bovine coronavirus	80–120	4.5–4.6	Kapil *et al.* (1999), King & Brian (1982)

MS2 plaque assay

Infectious MS2 was enumerated by plaque assay as previously described by Debartolomeist & Cabelli (1991). In order to study any impact of coagulants on infectious MS2 enumeration (part I), water samples were processed with (+BE) and without (−BE) beef extract (Shirasaki *et al.* 2009; Christensen *et al.* 2017).

For +BE, 1 mL of sample was mixed in 9 mL BE (13%) and stirred at 1,500 rpm for 5 h at +4 °C. The BE (211520, Becton-Dickinson and Company, USA) was prepared in sterile water, adjusted to pH 9.5–10.0 with 5 M NaOH, stored at +4 °C and used within 3 days. The quantity of MS2 is given as plaque forming units (PFU) per mL.

Extraction of RNA and RT-qPCR

In order to study any impact of coagulants on extraction of RNA and quantification of MS2 genomes by RT-qPCR (part I), two commercial RNA extraction protocols, used for processing of water samples, were compared: QIAamp® Viral RNA (Qiagen, Germany) and NucliSENS miniMAG® (Biomerieux, France), hereafter referred to as method Q, and N, respectively.

For method Q, 140 µL sample and 3.1 µg carrier RNA were added to lysis buffer (560 µL) and processed according to the manufacturer prior to RNA elution in 60 µL buffer and storage at −80 °C.

For N, an identical volume of 140 µL was treated with 2 mL lysis buffer and 50 µL magnetic beads. After several washing steps, RNA was eluted from the beads in 60 µL buffer and stored at −80 °C.

RT-qPCR was performed in a Stratagene AriaMx Real-Time PCR System (Agilent Technologies, Inc., USA), using the RNA UltraSense™ One-Step Quantitative RT-PCR System kit (Invitrogen, USA). Three µL RNA was used in a total volume of 20 µL, with primers, probe and RT-qPCR conditions as listed in Table 3. ROX was used as passive reference, and positive and negative controls were included in each run. Each sample was run in technical duplicates and the results analysed with Agilent AriaMx 1.1 Software.

Relative quantification of viral RNA was performed using standard curves prepared from ten-fold serial dilutions of homologous RNA in RT-qPCR triplicates. The amount of viral RNA was expressed as RT-PCR units (RT-PCRU) per mL: one RT-PCRU was defined as the amount of target RNA in the highest dilution of the standard, which gave a positive result. Aliquoted, homologous RNA was included

Table 3 | Primers/probe and RT-qPCR conditions used for detection of viruses

Virus	Primers and probes		RT-qPCR conditions	Reference or sequence
All			RT-step: 30 min at 55 °C, 2 min at 95 °C, then 40 cycles of:	
MS2	MS2-TM2-F (200 nM)	Forward	15 s at 95 °C, 30 s at 58 °C	Dreier *et al.* (2005)[a]
	MS2-TM2-R (200 nM)	Reverse		
	MS2-TM2 (100 nM)	Probe		
HAV	HAV 68 (500 nM)	Forward	15 s at 95 °C, 1 min at 60 °C, 8 s at 64 °C, 8 s at 68 °C	Costafreda *et al.* (2006)[a]
	HAV 240 (900 nM)	Reverse		
	HAV 150 (250 nM)	Probe		
BNV	BNVF1 (500 nM)	Forward	15 s at 95 °C, 1 min at 58 °C, 8 s at 62 °C, 8 s at 66 °C	5′-GATCTTTGTGCCATCACACC
	BNVR1 (500 nM)	Reverse		5′-CGACTACCTTCCCACAGTGA
	BNVP1 (500 nM)	Probe		5′- AACCTCATCCAAGCAAACATGGAGC-BHQ
BCoV	BCoV 1F20 (400 nM)	Forward	15 s at 95 °C, 30 s at 55 °C, 15 s at 60 °C	5′-TGGTGTCTATATTCATTTCTGCTG
	BCoV 1R89 (400 nM)	Reverse		5′- GGCCACTGCCTAGGATACA
	BCoV 1P48 (800 nM)	Probe		5′-ACACGTCCCTGGCTGAAAGCTG-BHQ

MS2, bacteriophage MS2; HAV, hepatitis A virus; BNV, bovine norovirus; BCoV, bovine coronavirus.
[a]Some modifications in cycling conditions and primers/probe concentrations.

in all plates and used as an inter-plate calibrator (IPC). The threshold in each run was adjusted manually in order to obtain identical Ct values for the IPC (Christensen *et al.* 2017).

Standard bench scale procedure

The outline of the protocol is given in Figure 1. The procedure was initially used to define suitable coagulation conditions, and later during part I and II setups. Further details are given under the relevant sections below. A water sample (400 mL) was spiked with the virus mix to achieve a final concentration of 3–6 $\log_{10}$ PFU/mL for MS2 and 4 $\log_{10}$ PCRU/mL for the other viruses. The sample was swirled prior to collection of a control (C), divided between three bottles (100 mL in each), and pH adjusted with 1 M NaOH or 0.3 M HCl. After adding coagulants, the bottles were immediately vortexed for 30 s ($G = 262\ s^{-1}$), left on a rocking table for 10 min at 50 rpm ($G = 5\ s^{-1}$), centrifuged at 112 g for 3 min, and the supernatant filtered through a Whatman membrane (glass fibre filter with 1.2 μm pore size) (Whatman GF/C, GE Healthcare, USA). Samples collected during processing were defined as under mixing (UM), supernatant (S) and filtrate (F).

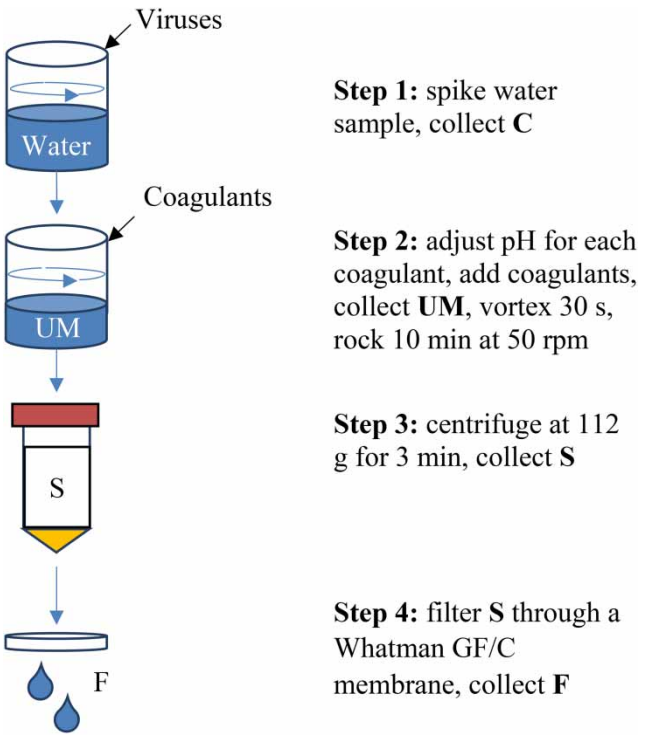

Figure 1 | Bench scale procedure used for initial optimization of coagulation conditions and during part I and II setups. C, control; UM, under mixing; S, supernatant; F, filtrate.

Optimization of coagulant doses

As optimal conditions for treatment of water from distinct sources could differ, individual optimization was performed. Criteria for filtrate turbidity (<0.2 NTU) and colour (<10 mg Pt/L) were set, as recommended by the Norwegian Institute of Public Health (Andersen 2016) for a coagulation-filtration step to be considered as a hygienic barrier. However, as filtration of small sample volumes with unsaturated Whatman filters was inefficient in meeting the turbidity criteria, coagulation conditions were selected in accordance with the lowest turbidity values obtained. For colour, the criteria were tightened (<5 mg Pt/L) for Al and Zr in order to avoid high metal residues, as colour and metals are often associated (Andersen 2016). Each coagulant was titrated at an optimal pH, as reported in the literature: 5.0–7.0 for PACl, 4.5–6.3 for Zr and 4.0–7.0 for chitosan (Ødegaard *et al.* 2010; Christensen *et al.* 2016). The coagulation conditions used for part I and II and treatment results are presented in Table 4.

Table 4 | Selected conditions used for water treatment and the quality of filtrates after treatment with aluminium (Al), zirconium (Zr) and chitosan coagulants

	Selected conditions		Water parameters, filtrate		
Water source	Coagulant	Dose, mg/L	pH	Turbidity, NTU	Colour, mg Pt/L
Dragsjøen	Al	8	5.9	0.8	5
	Zr	16	5.0	0.5	5
	Chit	15	5.0	0.5	13
Dragsjøen diluted	Al	3	5.6	0.6	5
	Zr	4	4.9	1.1	5
	Chit	4	4.9	0.3	6
Glomma	Al	3	6.6	ND	2
	Zr	9	6.1	ND	2
	Chit	2	5.1	ND	9

Dragsjøen diluted, Dragsjøen water diluted 1:4 by distilled water; ND, not done.
Coagulant concentrations were optimized relative to colour reduction, at a defined pH.

Part I: optimization of virus quantification by plaque assay and RT-qPCR

Initial experiments included Glomma and diluted Dragsjøen water (Dragsjøen D) with similar concentrations of particulate and organic matter (Table 1), in order to determine whether water constituents from different water sources could influence virus quantification.

After spiking with MS2, the water samples were processed using the standard bench-scale protocol, followed by collection of C, UM, S and F (Figure 1). MS2 was quantified with plaque assay (−/+ BE) to explore any influence of coagulants on enumeration of viable virus. For quantification of viral genome copies, RT-qPCR was used after RNA extraction with methods Q and N.

The applicability of the most sensitive methods was finally tested using undiluted Dragsjøen water, which was included in part II.

Water samples were processed in three biological replicates with virus enumeration in duplicates. An overview of the setup is given in Figure 2.

Part II: reduction of viruses in water; efficiency of Al, Zr and chitosan in different water types

Diluted and undiluted Dragsjøen water was spiked with MS2, HAV, BNV and BCoV, followed by the standard bench-scale procedure. Samples C, S and F were collected and processed immediately. Based on the results from part I, virus was quantified using the +BE plaque assay (MS2), and RT-qPCR using RNA extracted with method Q (Figure 2). Three biological replicates, with virus enumeration in duplicates, were conducted on each water type.

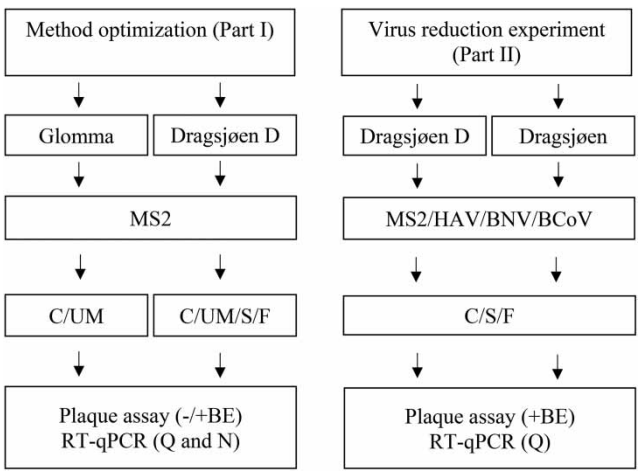

Figure 2 | Outline of part I and II of the study. Dragsjøen D, diluted Dragsjøen water; C, control; UM, under mixing; S, supernatant; F, filtrate; BE, beef extract; Q, Qiagen RNA extraction; N, Nuclisens RNA extraction; MS2, bacteriophage MS2; HAV, hepatitis A virus; BNV, bovine norovirus; BCoV, bovine coronavirus.

Data analysis

Data analysis was performed in STATA 8.0 (Stata Corporation, USA). Differences in effluent turbidity and colour between coagulants were calculated by using one-way analysis of variance (ANOVA). A linear regression model was applied to assess the association between virus reduction ($\Delta\log_{10}$) and the explanatory variables raw water quality, type of coagulant and virus morphology.

The required coagulant dosages were expected to be proportional to the level of NOM in Dragsjøen D and Dragsjøen samples (Ødegaard *et al.* 2010). Consequently, the independent variables raw water type and coagulant dosages were used interchangeably in the present work.

RESULTS

Optimization of coagulant doses

In general, turbidity was reduced less efficiently than colour. In water with low amounts of NOM, chitosan provided lower effluent turbidity than Al or Zr, whereas no significant difference in turbidity reduction was found for the three coagulants in high NOM water. Chitosan was less efficient in colour reduction than Al or Zr. The criteria for colour <5 mg Pt/L (and <10 mg Pt/L for chitosan) were fulfilled in all samples with the selected coagulant doses (Table 4), except for Dragsjøen water treated with chitosan.

Part I: optimization of virus quantification by plaque assay and RT-qPCR

In Glomma water, the number of MS2 plaques increased after treatment with BE, independently of coagulant type (Figure 3(a)). The efficiency of BE was specifically demonstrated for chitosan, for which a 5 $\log_{10}$ reduction in PFU

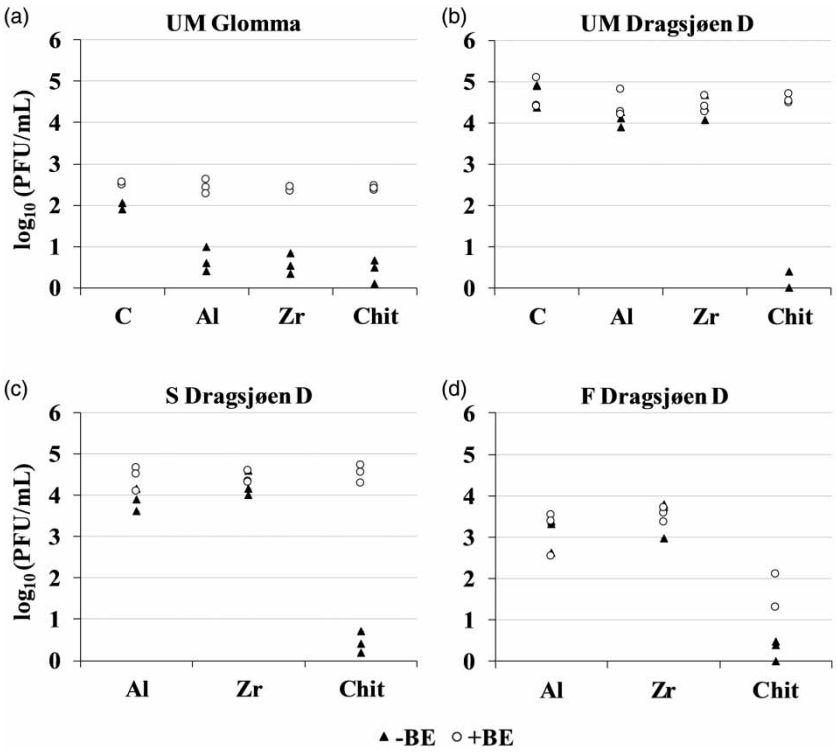

Figure 3 | Influence of coagulants on MS2 enumeration by plaque assay. Viable MS2 was quantified in collected UM, S and F after treatment of Glomma (a) and Dragsjøen (b, c and d) water with Al, Zr or chitosan. Collected samples were treated with (+BE) or without (−BE) beef extract. C, control; UM, under mixing; S, supernatant; F, filtrate; Dragsjøen D, Dragsjøen diluted.

was reversed with BE in UM and S samples from Dragsjøen (Figure 3(b) and 3(c)). An impact of chitosan was also observed in the filtered Dragsjøen sample, as the titre increased by 1.0–1.5 $\log_{10}$ after BE treatment (Figure 3(d)). For Al and Zr, the positive effect of BE was most significant in Glomma water.

Two RNA extraction methods (Q and N) were compared for RT-qPCR quantification of MS2 genomes in samples without (control C in Figure 4(a) and 4(b)) and with coagulants (Figure 4). In water samples without coagulants, the Q kit gave more than 1 $\log_{10}$ higher viral RNA titre, compared to N (Figure 4(a) and 4(b)). RNA dilution revealed the presence of RT-qPCR inhibitors from undiluted Dragsjøen in the RNA, extracted with method N. For water samples with coagulants, virus quantification was not affected when using method Q (Figure 4(a) and 4(b)). For N method, the greatest interference was registered for Glomma water with a 1.0–1.5 $\log_{10}$, reduction, depending on the coagulant. Overall, a high variability was found for virus titres in samples processed with method N, whereas a high reproducibility was seen for the level of viral RNA extracted with method Q.

Virus titres were reduced by no more than 0.4–0.5 $\log_{10}$, when the applicability of the +BE plaque assay and the Q extraction method was tested on undiluted Dragsjøen UM water (worst-case scenario). The two processing methods were, therefore, used in part II of the study.

Part II: virus reduction by coagulants

Reduction of MS2 and enteric viruses was assessed for coagulation-filtration treatment using three coagulants (Figure 5).

In the supernatants (S), 10–70% (0.1–0.5 $\log_{10}$) less virus was measured, compared with the control (C). Filtration was generally more efficient, but the results differed between the coagulants. For the metal coagulants, log-reductions were often similar and close to 90% (1 $\log_{10}$) and 99.9% (3 $\log_{10}$) for low and high NOM waters, respectively. Both metals showed a smaller reduction of BNV, compared to

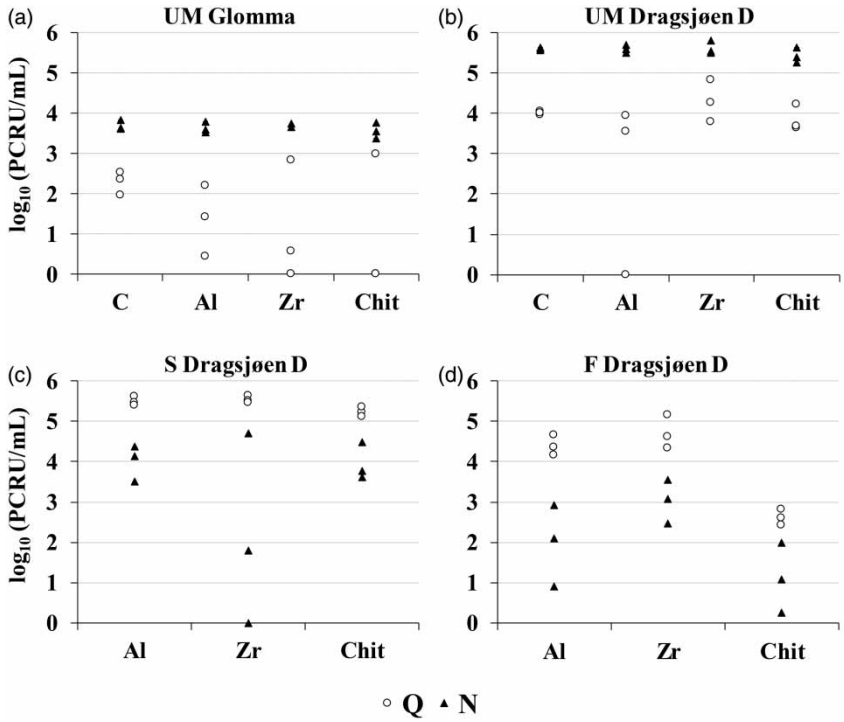

Figure 4 | Influence of RNA extraction method on virus enumeration by RT-qPCR. Total MS2 was quantified in UM, S and F samples after treatment of Glomma (a) and Dragsjøen (b, c and d) water with Al, Zr or chitosan. RNA was extracted with method Q (Qiagen) and N (Nuclisense). C, control; UM, under mixing; S, supernatant; F, filtrate; Dragsjøen D, Dragsjøen diluted.

chitosan. Chitosan treatment provided at least 99.9–99.99% (3–4 $\log_{10}$) reduction of all viruses for low and high NOM water samples.

Regression analysis (Table 5) revealed an association between virus reduction and the independent variables, such as raw water origin, type of coagulant and virus. NOM content and, subsequently, coagulant dose, was positively associated with virus reduction after the centrifugation and filtration steps. The supernatants revealed no difference in virus reduction between the coagulants, whereas virus numbers in filtrates indicated an increased efficacy of chitosan. The reduction pattern for BNV was distinct from the other viruses. The highest virus reductions were observed for Dragsjøen water processed by Zr-centrifugation or chitosan-centrifugation-filtration. The model fit has a coefficient of determination (R^2) of 44% for supernatants and 70% for filtrates, implying that 70% of the variance in virus reduction by coagulation-filtration could be explained by the type of water, coagulant and virus.

DISCUSSION

Effect of coagulants on enumeration of viable MS2 by plaque assay (aggregation effect)

The present study demonstrated that coagulants in water samples interfered reversibly with the plaque assay. The number of PFUs decreased not only in samples recently mixed with the coagulants (UM), as previously reported (Shirasaki *et al.* 2009), but also in supernatant and filtrates, especially when chitosan was the coagulant present.

An apparent fall in plaque number, due to coagulant presence, could be explained by several mechanisms. Apart from virus aggregation (Floyd & Sharp 1978), coagulants could prevent virus–host interaction electrostatically (Puck *et al.* 1951), or physically (Tanneru *et al.* 2013). Phage entrapment on the flocs might also result in temporary conformational changes in the virus capsid (Taylor *et al.* 1980).

Treatment of the samples with BE reversed reduction in infective MS2 plaque counts almost entirely, despite high

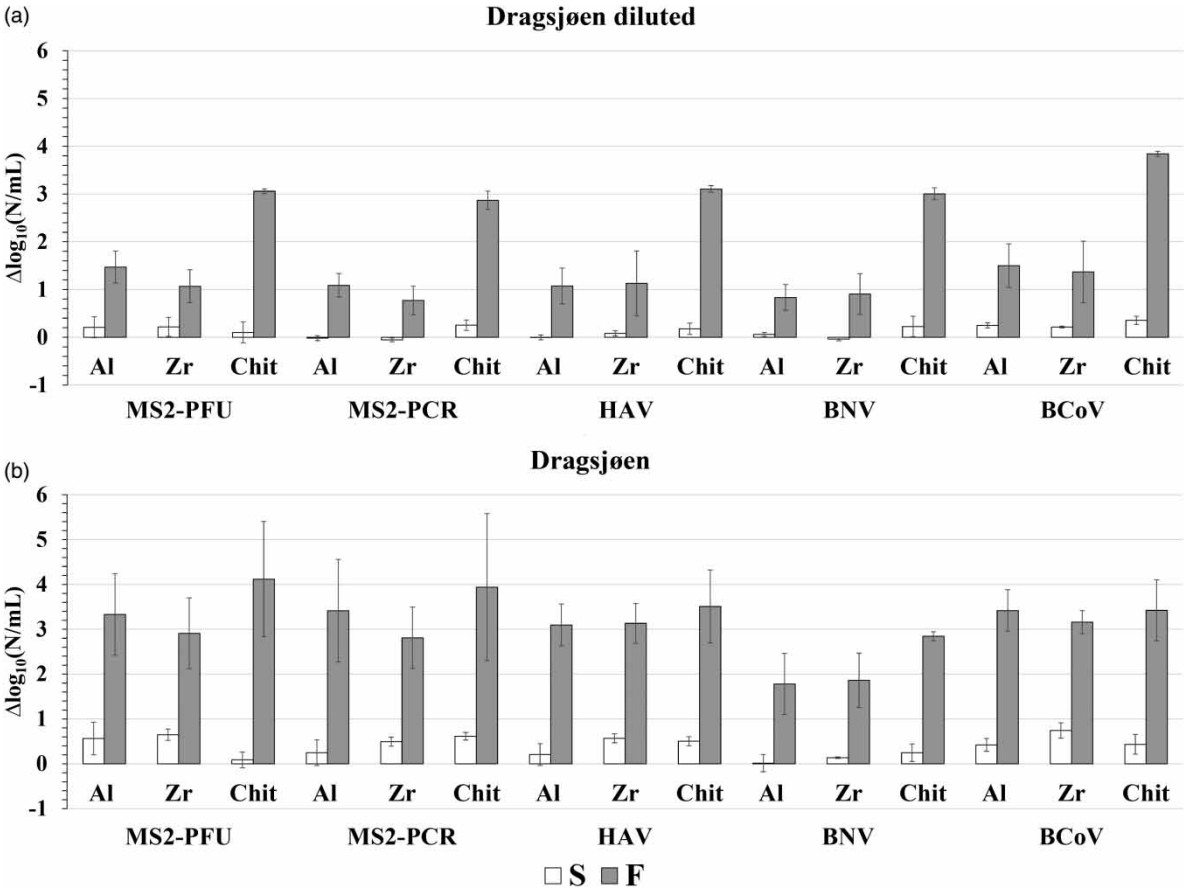

Figure 5 | Log-reductions of MS2, hepatitis A virus (HAV), bovine norovirus (BNV) and bovine coronavirus (BCoV) in diluted (a) and undiluted (b) Dragsjøen water during treatment with Al, Zr and chitosan coagulants. Viable MS2 was enumerated by plaque assay including beef extract. Viral RNA was extracted from supernatant (S) and filtrate (F) with the Qiagen method and quantified by RT-qPCR. The results are expressed as mean and SD ($n = 3$).

coagulant doses. The effect of BE could be caused by a synergetic effect of high pH and excess of proteins. While a solution with alkaline pH dissolves the coagulant flocs and enhances repulsion between virus–virus and virus–coagulant complexes, excess of proteins displaces viruses from the active attachment sites on undissolved flocs and naturally present suspended solids.

The impact of Al and Zr on viable MS2 quantification increased in Glomma water, compared to Dragsjøen D. For chitosan, the opposite was observed. The applied coagulation conditions varied for the two water samples. A higher amount of Zr and a lower amount of chitosan were used for Glomma compared to Dragsjøen D water. However, similar PACl doses were applied for the two waters, whereas the pH conditions varied for Al and Zr only. Therefore, the aggregation was probably caused by a combined effect of water components, coagulant dose and pH.

The results in the present study indicate that enumeration of viable MS2 in water with coagulants and without BE treatment can result in artificially high estimates on virus reduction. This finding may provide useful insights for future studies with model viruses. For enteric virus, the BE treatment might be employed to study reversible aggregation of viruses with solids in raw water (Hejkal *et al.* 1981) or to improve the sensitivity of the single-hit dose–response models, which are essential in QMRA (Nilsen & Wyller 2016).

Effect of coagulants on virus enumeration by RT-qPCR

The present study demonstrates a difference between the two extraction methods regarding purification and/or

Table 5 | Regression coefficients for the estimated virus reduction ($\Delta\log_{10}$)

	Coefficients (Std. error)	
	Supernatant	**Filtrate**
Water type		
Dragsjøen diluted	0.00	0.00
Dragsjøen	0.21* (0.08)	1.78** (0.24)
Coagulant type		
Al	0.00	0.00
Zr	−0.01 (0.08)	−0.15 (0.18)
Chitosan	0.12 (0.08)	2.04** (0.14)
Virus type		
MS2-PFU	0.00	0.00
MS2-PCR	−0.07 (0.07)	−0.27 (0.28)
HAV	−0.07 (0.07)	−0.16 (0.22)
BNV	−0.21** (0.07)	−0.73** (0.23)
BCoV	0.09 (0.07)	−0.08 (0.22)
Coagulant/water type		
Al-Dragsjøen	0.00	0.00
Zr-Dragsjøen	0.24* (0.11)	−0.08 (0.33)
Chitosan-Dragsjøen	−0.06 (0.12)	−1.49** (0.38)
Intercept	0.14 (0.07)	1.44 (0.19)

Standard error is in parentheses.
Dragsjøen diluted, Dragsjøen water diluted 1:4 by distilled water.
* and ** indicate significance at the 95% and 99% level, respectively.

recovery of RNA from water samples. The efficacy of methods Q and N was influenced differently by water quality and coagulants. While Q provided stable levels of pure viral RNA, regardless of water quality and presence of coagulants, the efficacy of N was significantly reduced by both.

The difference between the two methods might be explained by inclusion of silica in a gel membrane for method Q, while N uses silica-covered magnetic beads. Furthermore, method Q uses carrier RNA to increase RNA binding to the silica (Boom *et al.* 1990). On the other hand, other studies have demonstrated the adverse effect of high metal concentrations on the sensitivity of method Q (Chen & Chang 2012). Therefore, it might be necessary to assess the sensitivity of a selected extraction kit prior to an experiment.

Virus removal by coagulant and filtration

In the present study, the extent of virus–floc association, and subsequent virus removal, were assessed with respect to raw water quality, coagulant type and dosage, and virus characteristics. According to Edwards & Amirtharajah (1985), flocculation performance increases with increasing concentrations of particles and humic molecules in raw water and/or coagulant dosing. Moreover, selected coagulants may exhibit different destabilization properties and affect floc characteristics, which play a fundamental role in operation of physical separation processes (Hussain *et al.* 2014). Finally, efficient removal involves transport and attachment behaviour of colloids, determined by their size and charge characteristics (Yao *et al.* 1971).

The results of the present study revealed that the combination of flocculation and sedimentation induced 10% to 70% reduction in virus titre. At drinking water plants, sedimentation accounts for 27% to 74% decrease in virus amount (Gimbel & Clasen 1998), implying that the selected bench-scale procedure was capable of simulating a full-scale process. Consequently, chemical pre-treatment and sedimentation did not have a substantial virus reducing effect. Without attachment to flocs, virus would remain suspended in the solution. Apparently, impaired performance could result from small size and deficient settling characteristics of the formed aggregates.

The setup with a Whatman membrane aimed to reproduce a microfiltration process, for which straining and cake filtration are considered the predominant retention mechanisms (LeChevallier & Au 2004). The pore size of a Whatman filter (1.2 μm) exceeds the size range for monodispersed virus particles. The filter surface is also electronegative (Blass *et al.* 2013), and not likely to favour adsorption of MS2, HAV and BCoV at the pH conditions used. Consequently, viruses and colloids smaller than 1.2 μm, and presumably even 0.4 μm (Hickel 2013), were not expected to be efficiently retained on the membrane, unless their size and charge properties were modified by coagulation pre-treatment.

The filtration step showed an association between virus reduction and raw water quality. Addition of coagulants to water is followed by formation of hydrolysis species and increase of suspended solids. In low NOM water, a relatively low dosing of the metal coagulants could restrict formation of complexes between colloids, hydroxide flocs and pathogens (Chang *et al.* 1958). One can also assume that under the conditions produced with Al and Zr, the

size of the formed flocs was below the 1.2 μm pore size. This is consistent with the deficient turbidity reduction in Dragsjøen D samples. In undiluted Dragsjøen water, increased coagulant dosages were assumed to enhance collision rates between colloids, flocs and microbes, and induce formation of numerous and larger aggregates. This could favour retention of suspended solids and, associated with it, virus.

On the other side, chitosan was more efficient in reducing viruses during filtration, compared to Al and Zr (Table 5). Destabilization with chitosan could possibly result in formation of larger and denser flocs, but then lower virus titres should have been seen for this coagulant after the centrifugation step. Besides, chitosan does not form hydroxides and produces less suspended matter in the influent than the metal coagulants (Christensen *et al.* 2016). Consequently, a cake layer formed by chitosan on the membrane surface is expected to be thinner, giving a lower retention of particles and virus. Chitosan exhibits unique destabilization mechanisms, e.g., bridging or 'electrostatic patching' (Bolto & Gregory 2007). Therefore, it could be suggested that chitosan chains protrude from the aggregated suspended matter and bind strongly to the filter material.

In the present study, regression analysis showed a slightly deficient reduction of BNV, in comparison to viruses with more acidic pI. Under the selected coagulation pH, the positive charge of BNV could prevent it from approaching positively charged coagulants. Deficient BNV reduction, however, was not observed for chitosan, which could be caused by the distinct ability of chitosan to bind the filter membrane, as discussed earlier.

The influence of particle size in the 30–120 nm range was negligible for virus retention on the membrane and the results presented imply that aggregation and retention of MS2 and enteric viruses were generally similar.

Turbidity reduction often serves as an indicator of treatment performance for (oo)cysts, which have sizes in the same range as particulate matter (Nieminski & Ongerth 1995). The size of viruses is similar to that of humic compounds (Österberg *et al.* 1993), of which concentrations can be assessed with colour. Thereby, treatment conditions, which are beneficial for low effluent turbidity and colour, are believed to affect most microorganisms and maximize pathogen removal during coagulation and filtration treatment.

In the present work, treatment conditions were satisfying mostly with regard to colour criteria, whereas turbidity values were above 0.2 NTU. However, efficient virus removal could still be achieved, but depended on coagulant type and initial water quality.

In Dragsjøen D water, the performance of both Al and Zr was associated with deficient turbidity and virus reduction (Table 4 and Figure 5(a)). In Dragsjøen water, virus removal by the same coagulants was improved, despite high effluent turbidity (Table 4 and Figure 5(b)). Due to higher coagulant dosing, greater increase of solid matter was expected in Dragsjøen water, compared to Dragsjøen D water. As filtrate turbidity in both Dragsjøen and Dragsjøen D waters was rather similar, it was reasonable to assume that solid retention, as well as virus removal, was higher for Dragsjøen water. Consequently, retention of solids that can be assessed with turbidity parameters was assumed to be connected to removal of viruses.

In contrast, a straightforward association between reduction in colour and virus was not observed. A high NOM residue in the filtrate, accompanied by low virus titre, could be caused by adhesion of the chitosan chains to the membrane. Alternatively, the association was influenced by high turbidity, and presumably if low turbidity was achieved for Al and Zr, these two coagulants could demonstrate similar or greater virus reduction, compared to chitosan.

CONCLUSIONS

The present study shows that enumeration of the model virus MS2, by plaque assay and RT-qPCR (RNA extraction), was sensitive to coagulant contents and other constituents in water. Treatment of water samples with BE tended to reduce the interference on enumeration by plaque assay. The sensitivity of the RT-qPCR assay relied on the RNA extraction method. The results show that the suitability of virus quantification methods should be evaluated for water studies, especially when using coagulants.

Chitosan showed a high ability to retain suspended matter on the membrane in water with low NOM content.

Moreover, chitosan contributed to a higher hygienic performance in this type of water than Al or Zr coagulants. The efficacy of the metal coagulants to reduce both suspended matter and viruses was improved as a result of the enhanced contact opportunities between viruses and other particles in solution. Virus retention by the three coagulants could be somewhat predicted with turbidity reduction in the filtrates. In contrast, an association between effluent colour and virus reduction was not established.

In the present study, charge characteristics of viruses influenced virus reduction, however, the removal patterns for the model virus MS2 resembled the reduction of pathogenic viruses.

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