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Interaction of human adenoviruses and coliphages with kaolinite and bentonite

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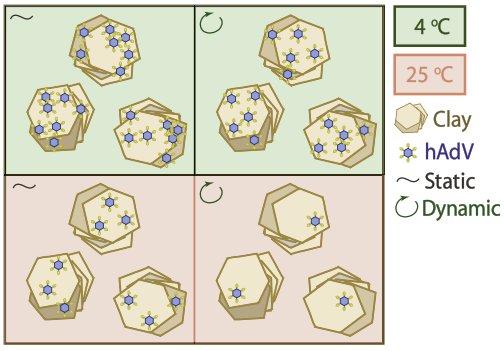
HIGHLIGHTS

- Temperature plays a significant role on virus adsorption onto clays.
- Adenovirus adsorption is higher under static than dynamic conditions.
- For most cases considered, hAdV adsorption is higher at the highest IS.
- The adsorption of both MS2 and ΦX174 is not similar to that of hAdV.

GRAPHICAL ABSTRACT



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ABSTRACT

Human adenoviruses (hAdVs) are pathogenic viruses responsible for public health problems worldwide. They have also been used as viral indicators in environmental systems. Coliphages (e.g., MS2, ΦX174) have also been studied as indicators of viral pollution in fecally contaminated water. Our objective was to evaluate the distribution of three viral fecal indicators (hAdVs, MS2, and ΦX174), between two different phyllosilicate clays (kaolinite and bentonite) and the aqueous phase. A series of static and dynamic experiments were conducted under two different temperatures (4, 25 °C) for a time period of seven days. HAdV adsorption was examined in DNase I reaction buffer (pH = 7.6, and ionic strength (IS) = 1.4 mM), whereas coliphage adsorption in phosphate buffered saline solution (pH = 7, IS = 2 mM). Moreover, the effect of IS on hAdV adsorption under static conditions was evaluated. The adsorption of hAdV was assessed by real-time PCR and its infectivity was tested by cultivation methods. The coliphages MS2 and ΦX174 were assayed by the double-layer overlay method. The experimental results have shown that coliphage adsorption onto both kaolinite and bentonite was higher for the dynamic than the static experiments; whereas hAdV adsorption was lower under dynamic conditions. The adsorption of hAdV increased with decreasing temperature, contrary to the results obtained for the coliphages. This study examines the combined effect of temperature, agitation, clay type, and IS on hAdV adsorption onto clays. The results provide useful new information on the effective removal of viral fecal indicators (MS2, ΦX174 and hAdV) from dilute aqueous solutions by adsorption onto kaolinite and bentonite. Factors enabling

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enteric viruses to penetrate soils, groundwater and travel long distances within aquifers are important public health issues. Because the observed adsorption behavior of surrogate coliphages MS2 and ΦX174 is substantially different to that of hAdV, neither MS2 nor ΦX174 is recommended as a suitable model for adenovirus.

1. Introduction

Pathogenic enteric viruses are frequently found in natural and wastewaters (Hundesa et al., 2006; Muscillo et al., 2008; Ogorzaly et al., 2009). The presence of pathogenic enteric viruses in the environmental waters poses a significant risk to human health. Their extremely small size (e.g., 23–25 nm for MS2, 28–30 nm for hepatitis A virus, 27–30 for poliovirus, 35–39 nm for norovirus, 65–85 nm for adenovirus) enables enteric viruses to penetrate soils and contaminate groundwater through wastewater discharges, sanitary landfills, septic tanks, and agricultural practices (Chrysikopoulos et al., 2010; Masciopinto et al., 2008; Sim and Chrysikopoulos, 2000; Syngouna and Chrysikopoulos, 2011). Frequently encountered pathogens in environmental systems include adenoviruses, enteroviruses, hepatitis A virus, noroviruses, and rotavirus (Fong et al., 2010; Pang et al., 2014). Among the waterborne viruses, human adenoviruses (hAdVs) are described as emerging pathogens (Jiang, 2006), and they are considered to be highly resistant in water (Ogorzaly et al., 2010). Nevertheless, there is still significant lack of data on the occurrence, persistence and stability of these viruses in groundwater (Mena and Gerba, 2009). To predict the presence of pathogenic viruses in natural waters and wastewater, microorganisms known as indicator organisms (e.g., hAdVs and coliphages), which are commonly associated with fecal contamination, are monitored. HAdVs have been suggested as preferred indicators of viral contamination (Mellou et al., 2014; Poma et al., 2012). Coliphages MS2 and ΦX174 have also been studied as indicators of viral pollution in fecally contaminated water (Grabow, 2001; Havelaar et al., 1986; Chrysikopoulos and Aravantinou, 2012). Compared to other pathogenic viruses, coliphages behave more conservatively (lower sorption); furthermore, they are capable to survive under significant periods of time in groundwater. For this reason, the structural resemblance to many human enteric viruses, coliphages are considered as good model viruses and have been extensively used in numerous studies focused on virus fate and transport in the subsurface (Anders and Chrysikopoulos, 2006; Chrysikopoulos and Aravantinou, 2014; Syngouna and Chrysikopoulos, 2011).

Viruses in natural waters and wastewaters are frequently found attached onto sand, clays, suspended colloids, etc. (Meschke and Sobsey, 1998). Naturally abundant clay minerals are a class of layered alumosilicates, which comprise of various layers of silica and alumina sheets (McBride, 1974), with good biocompatibility, strong adsorption, ion exchange ability and expansibility (Zhang et al., 2010; Zhou and Keeling, 2013; Zhou et al., 2012; Zhou, 2011; Vasiliadou et al., 2011; Vasiliadou and Chrysikopoulos, 2011). Kaolinite, montmorillonite and illite represent some of the main groups of clay minerals found within soils (Brennan et al., 2014). Montmorillonite is 2:1 (3 layer) clays, while kaolinite is 1:1 clay (2 layer) with relatively smaller expansion and adsorption capacity. Montmorillonite, commercially known as bentonite, is most commonly used as an additive to existing natural clay liner materials to decrease permeability (Kau et al., 1998). Bentonite typically displays very low permeability and high cation exchange capacity, which are known to aid in contaminant retardation (Hussin et al., 2011; Ralla et al., 2010).

Virus transport in porous media has found to be influenced by clay colloid presence (Katzourakis and Chrysikopoulos, 2014; Syngouna and Chrysikopoulos, 2013). Moreover, clays have been reported to affect the growth and metabolic activity of viruses. Numerous studies have investigated the interaction between viruses and clays and the effect of clay minerals on virus survival and infectivity (Jin and Flury, 2002; Kimura et al., 2008). Most literature has focused on the effect of clay type (Christian et al., 2006; Lipson and Stotzky, 1985; Templeton et al., 2008), pH (Zhuang and Jin, 2008; Walshe et al., 2010), ionic strength (IS) (Tong et al., 2012), buffer composition (Gutierrez et al., 2010; Zhuang and Jin, 2008), cation exchange capacity (Lipson and Stotzky, 1983; Vettori et al., 1999), virus surface morphology (Block et al., 2014), temperature, and agitation (Syngouna and Chrysikopoulos, 2010). Although Syngouna and Chrysikopoulos (2010) conducted batch experiments to investigate bacteriophage (MS2 and ΦX174) sorption onto various clay minerals at two different temperatures under static and dynamic conditions, to our knowledge, there is no published study that investigated temperature, agitation, IS, and clay type synergistic effects on the interaction of hAdV with clay minerals (kaolinite, and bentonite). Thus, the main objective of this study was to evaluate and compare the adsorption of three viral fecal indicators (hAdVs, MS2, and ΦX174), onto two different phyllosilicate clays (kaolinite and bentonite) under different experimental conditions.

2. Materials and methods

2.1. Cell lines and hAdV stock preparation

The hAdV serotype 35 strain was cultivated in human lung carcinoma cell line A549 growing in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, U.S.) containing 4.5 g/L D-glucose, L-glutamine and pyruvate with 10% heat inactivated fetal bovine serum (FBS; Gibco). A549 cells support the replication of most human adenovirus serotypes, except of the fastidious serotypes 40 and 41. For the preparation of hAdV stocks, A549 cells were cultured confluently (80–90%) in 175-cm^2 flasks, in a CO_2 incubator (5% CO_2) at 37 °C, and infected with hAdV serotype 35 (kindly donated by Dr Annika Allard, University of Umea, Sweden). HAdVs were released from cells by freezing and thawing the culturing flasks for 3 times. A centrifugation step at 3000 ×*g* for 20 min was applied to eliminate cell debris. The supernatant was ultracentrifuged for 1 h at 34,500 ×*g*, re-suspended in PBS, quantified and stored in 10 mL aliquots at −80 °C until use. The initial concentration of each hAdV stock was quantified by real-time PCR (qPCR), and recorded at 10^6 genome copies/mL.

2.2. HAdV nucleic acid extraction

The analytical approach was designed to contain a step of an enzymatic treatment by DNase I, aiming at reducing the detection of false positives by qPCR. DNase I should degrade any viral DNA that is no longer protected by the viral capsid. A volume of 2.5 μL of DNase I (RNase — free, 2000 units/mL, New England BioLabs, Inc.) was added to 137.5 μL of each sample and then all aliquots were incubated at 37 °C for 2 h. Following this incubation period, all samples were immediately placed in a freezer at −80 °C for storage prior to nucleic acid extraction and molecular assay. The samples were thawed immediately prior to the nucleic acid extraction step in which 140 μL of each sample was added (separately) to 560 μL of the Buffer AVL and 5.6 μL of the carrier RNA of a QIAamp viral RNA mini kit (Qiagen). The samples are first lysed under the highly denaturing conditions provided by Buffer AVL to inactivate RNases and to ensure isolation of intact viral RNA. Carrier RNA, added to Buffer AVL, improves the binding of viral RNA to the QIAamp membrane especially in the case of low-titer samples, and limits possible degradation of the viral RNA due to any residual RNase activity. The DNase I enzyme presumably denatures in this buffer. The viral DNA

was extracted from each sample using this kit according to the manufacturer's protocol; the final volume was 100 μL. The extracts were immediately stored at −80 °C.

2.3. HAdV real-time PCR (qPCR) assay

For the molecular detection of hAdVs, the conserved region of the hexon gene was used as the target area. HAdVs were quantified by qPCR (TaqMan Universal PCR Master Mix, Applied Biosystems) and a carry-over contamination prevention system (uracil N-glycosylase). A neat and a 10-fold dilution of the virus nucleic acid extract were tested. All samples were tested in duplicates (two neat and two diluted). The primers and probes for quantification of hAdVs were adopted from Hernroth et al. (2002). In each assay, 10 μL sample of nucleic acid extract was added to a final reaction volume of 25 μL. For each plate, the genome copies (GC/mL) were measured. Ultra-pure water was used as the non-template control for each assay. QPCR was carried out for 2 min at 50 °C, with preheating for 10 min at 95 °C, followed by 45 cycles of PCR amplification (denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 1 min) (Hernroth et al., 2002). The assay was designed to quantify all common human adenoviruses, and was proven to be highly efficient (Bofill-Mas et al., 2013), with lower detection limit of 10 GC/mL (Bofill-Mas et al., 2006; Carducci and Verani, 2013). The calibration curve equation employed in this study was: FAM, $Y = -3.376 * \log(X) + 38.39$, eff = 97.8%.

2.4. Coliphage plaque forming unit (pfu) assay

Bacteriophages are viruses that infect bacteria, whereas coliphages are bacteriophages that specifically infect coliform bacteria (e.g., *Escherichia coli*). The coliphage MS2 is a F-specific, single-stranded RNA phage with 31% nucleic acid content, whose host bacterium is *E. coli* (ATTC 15597-B1); whereas, the coliphage ΦX174 is an icosahedral, single-stranded DNA phage with 26% nucleic acid content, whose host bacterium is *E. coli* (ATTC 13706-B1). Both coliphages (MS2, ΦX174) were assayed by the double-layer overlay method (Adams, 1959), where 0.1 mL of the appropriate host bacterium and 0.1 mL of diluted virus sample solution were mixed in a centrifuge tube. The mixture was combined with molten soft-agar medium (4.5 mL), maintained at 45 °C in a tube, and poured onto a Petri dish containing solid agar medium. The plates were solidified for 10 min and incubated overnight at 37 °C. Viable coliphage concentrations were determined by counting the number of plaques in each host lawn, and reported as plaque-forming units per milliliter (pfu/mL). Only dilutions that resulted in the range of 20–300 plaques per plate were accepted for quantification. All virus concentrations reported are the average of two replicate plates (Syngouna and Chrysikopoulos, 2010).

2.5. Clay minerals

Two different types of clay minerals were used in this study: kaolinite (03584 Kaolinite, Sigma), and bentonite (18609 Bentonite, Riedel de Haen) with chemical compositions described by Syngouna and Chrysikopoulos (2010). The main structural elements of the clay minerals employed are two-dimensional arrays of silicon–oxygen tetrahedra (tetrahedral silica sheet), and that of aluminum– or magnesium–oxygen–hydroxyl octahedra (octahedral, alumina, or magnesia sheet). The sharing of oxygen atoms between silica and alumina sheets results in two-layer minerals (TO) or three-layer minerals (TOT) (Van Olphen, 1963). Kaolinite is a two-layer or TO clay, whereas montmorillonite, the predominant clay mineral in bentonite, is a three-layer or TOT clay.

2.6. Zeta potentials

The zeta potentials of hAdV and clay particles (kaolinite, bentonite) used in this study were measured at pH 7.6 in the DNase I reaction buffer solution of IS = 1.4, 14, and 140 mM by a zetasizer (Nano ZS90, Malvern Instruments, Southborough, MA) at 25 °C. Moreover, the zeta potentials of MS2, ΦX174, kaolinite, and at pH 7 in the PBS reaction solution of IS = 2 mM were obtained from previous studies (Syngouna and Chrysikopoulos, 2010, 2011). All zeta potentials were obtained in triplicates and are listed in Table 1.

2.7. Adenovirus and coliphage adsorption onto clays

The adsorption of hAdV, MS2 and ΦX174 onto kaolinite and bentonite was examined at two different temperatures (4 and 25 °C), under static and dynamic batch conditions following the procedure described by Syngouna and Chrysikopoulos (2010). A schematic illustration of the experimental procedures is presented in Fig. 1. The adenovirus stock solution was added into a 2 mL centrifuge tube containing 0.02 g of the clay. A separate reactor tube was used for each type of clay, at a concentration of 10 mg clay per mL of DNase I reaction buffer solution (solids to solution ratio: 1 to 100). Control tubes (without clays) were prepared by adding hAdV stock solution into a 2 mL centrifuge tube containing DNase I reaction buffer solution in order to monitor the hAdV time-depended inactivation and inactivation due to sorption onto tube walls. The DNase I reaction buffer solution was prepared with 100 mM Tris–HCl, 25 mM $MgCl_2$ and 5 mM $CaCl_2$ at pH 7.6. The final hAdV suspension had an IS of 140 mM. The adsorption of hAdV, under conditions, was also determined at IS = 14, and 1.4 mM. Four different hAdV concentrations (10^6, 10^7, 10^8, 10^9 copies/mL) in DNase I reaction buffer were prepared. The initial and final hAdV concentrations were quantified by qPCR, as described above. Additional quality control analyses were performed by a cultivation method (Carratalà et al., 2013). The infectivity of hAdVs adsorbed onto clays was investigated by molecular methods. Moreover, cytotoxicity effects were determined by visual inspection under the optical microscope. All selected hAdV samples are presented in Table 2.

For the coliphages, the batch equilibration method used involved the addition of coliphage stock solution into a 50 mL glass reactor tube containing 0.5 g of the clay (separate reactor tubes were used for each type of clay), at a concentration of 10 mg clay per mL of PBS solution (solids to solution ratio: 1 to 100). The PBS solution was prepared with 1.2 mM NaCl, 0.027 mM KCl, and 0.010 mM phosphate buffer salts in UV-disinfected distilled water with a specific conductance of 17.8 μS/cm. The specific conductance of the final coliphage suspension was 212 μS/cm, which corresponds to IS ≈ 2 mM. Different coliphage stock concentrations ranging from 10^3 to 10^9 pfu/mL were used. Control tubes contained coliphage in PBS solution without the presence of clay. The initial and final coliphage concentrations were quantified by the double agar layer method, as described above. All selected coliphage samples are presented in Table 2.

Glass tubes, instead of polypropylene tubes, were used in this study, so that MS2 phage sorption and inactivation would not occur onto the tube surface. Thompson and Yates (1999) reported that the inactivation of MS2 and R17 increased with increasing IS during mixing in polypropylene tubes, while ΦX174 remained unaffected. Also, it was reported

Table 1
Zeta potential measurements.

Zeta potential (mV) ± SD				
Particle	Ionic strength (mM)			
	1.4 mM[a]	2 mM[b]	14 mM[a]	140 mM[a]
hAdV	−21.78 ± 1.39	–	−9.18 ± 1.35	−7.19 ± 0.37
MS2	–	−33.5 ± 1.80	–	–
ΦX174	–	−31.15 ± 0.25	–	–
Kaolinite	−19.95 ± 0.50	−45 ± 1.49	−16.73 ± 0.48	−14.68 ± 0.25
Bentonite	−29.76 ± 0.78	−31.00 ± 0.5	−14.80 ± 1.34	−12.36 ± 0.88

[a] pH = 7.6 in the DNase I reaction buffer.
[b] pH = 7 in the PBS reaction solution.

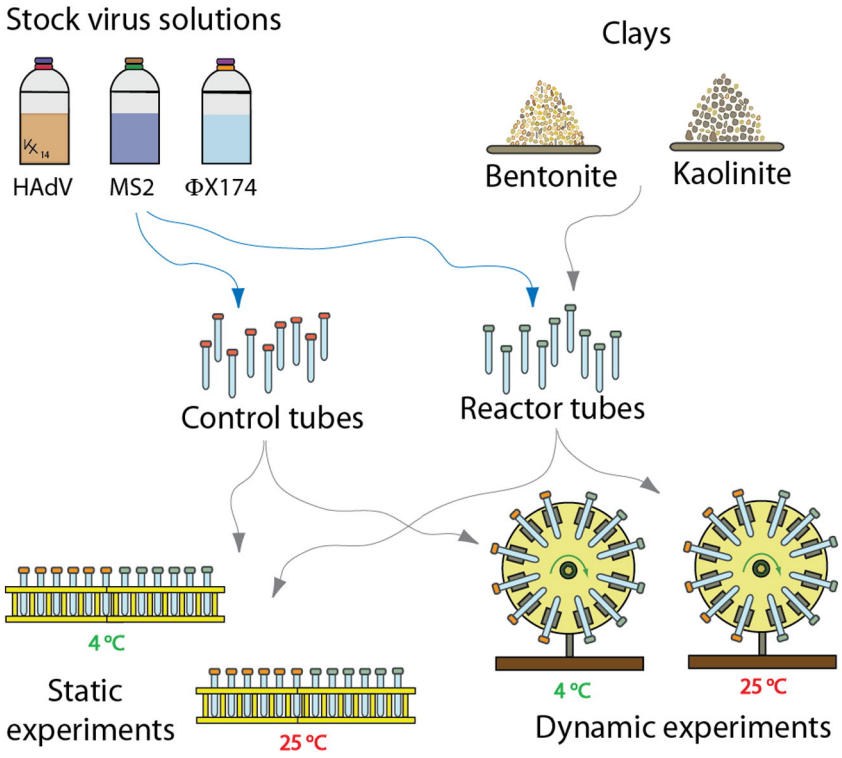

Fig. 1. Schematic illustration of the experimental procedure.

in the literature that inactivation is eliminated in the absence of headspace in the liquid phase for both MS2 in glass tubes (Thompson et al., 1998), and for hAdV in polypropylene vials (Wong et al., 2013). Consequently, in this study, both glass (for coliphages), and polypropylene (for hAdV) tubes were completely filled with solution.

The control tubes (without clays) were treated in the same fashion as the reactor tubes (with clays). For the dynamic batch experiments, the reactor and control tubes were placed on a small orbit tube rotator (J.P. SELECTA S.A.), operated at 12 rpm. For both static and dynamic batch experiments, sub-samples were withdrawn from each tube at regular intervals of 24 h for a time period of 7 days and centrifuged at 2000 ×*g* (6000 rpm) for 10 min in a Hermle Labortechnik Z160M. Centrifugation was performed in order to separate hAdVs and coliphages attached onto clays from those suspended in the liquid phase (Syngouna and Chrysikopoulos, 2013). It should be noted that the supernatant contained none of the clays. A 7-day experimental time-period was found to be sufficient for the virus–clay systems to reach equilibrium (Syngouna and Chrysikopoulos, 2010). All assays were performed in triplicate and negative and positive controls were included.

The adsorption of hAdV, MS2 and ΦX174 onto clays was determinated as:

$$\mathrm{Log}_{10}\frac{N_s(t)}{N_c(0)} = \mathrm{Log}_{10}\frac{N_c(t)-N_r(t)}{N_c(0)} \quad (1)$$

where $N_c(t)$ is the virus concentration at time t in the control tubes (copies/mL or pfu/mL), $N_c(0)$ is the initial virus concentration at $t = 0$ in the control tubes (copies/mL or pfu/mL) $N_r(t)$ is the virus concentration at time t in the reactor tubes (copies/mL or pfu/mL), and $N_s(t)$ is the concentration of viruses sorbed onto clays at time t (copies/mL or pfu/mL). Eq. (1) provides the combined concentration of viable and inactivated (non-functional) sorbed viruses. Moreover, the inactivation rates of suspended (i.e., those present in the bulk solution, non-sorbed onto surfaces) and adsorbed viruses are usually distinguished and may

Table 2
Experimental conditions of collected samples.

Batch conditions	Temperature (°C)	IS (mM)	Virus	Type of clay[a] (No. of samples)[b]	Total no. of samples
Static	4	1.4	hAdV	K (11) & B (11)	22
Static	25	1.4	hAdV	K (11) & B (11)	22
Static	25	14	hAdV	K (11) & B (11)	22
Static	25	140	hAdV	K (11) & B (11)	22
Static	25	2	MS2	K (11) & B (11)	22
Static	4	2	MS2	K (11) & B (11)	22
Static	4	2	ΦX174	K (11) & B (11)	22
Static	25	2	ΦX174	K (11) & B (11)	22
Dynamic	4	1.4	hAdV	K (11) & B (11)	22
Dynamic	25	1.4	hAdV	K (11) & B (11)	22
Dynamic	4	2	MS2	K (11) & B (11)	22
Dynamic	25	2	MS2	K (11) & B (11)	22
Dynamic	4	2	ΦX174	K (11) & B (11)	22
Dynamic	25	2	ΦX174	K (11) & B (11)	22

[a] K — kaolinite, B — bentonite.
[b] The first four samples were collected every 30 min and the remaining seven once per day.

be different (Gerba, 1984; Hurst et al., 1980; Sim and Chrysikopoulos, 1995, 1998; Yates and Yates, 1988). Virus survival characteristics under the various experimental conditions in the control and reactor tubes were also evaluated.

2.8. Statistical analysis

The effect of temperature, IS, experimental conditions (static or dynamic), and type of clay on virus adsorption were statistically analyzed by parametric statistical tests (paired t-test, ANOVA) after the test of normal distribution by Kolmogorov–Smirnov (K–S test) by the SPSS Software (ver. 18). It should be noted that $\alpha = 5\%$.

3. Results and discussion

3.1. Adenovirus and coliphage survival characteristics

Figs. 2 to 4 present the decay of virus concentration in the control and reactor tubes under the various experimental conditions of this study for hAdV, MS2 and ΦX174, respectively. The experimental results have shown that the decay of virus concentration in the control and reactor tubes is quite similar. However, as we expected, viral stability was significantly affected by temperature in both control and reactor tubes (Chrysikopoulos and Aravantinou, 2012). All viruses were systematically more persistent at 4 than 25 °C. Moreover, at 4 °C hAdV was more persistent than ΦX174, and ΦX174 more persistent than MS2, in both control and reactor tubes. However, at 25 °C under both static and dynamic conditions, virus persistence is following the trend ΦX174 > MS2 > hAdV (Figs. 2–4). Note that inactivation of suspended viruses depends on both time and the experimental physicochemical conditions. High temperatures can accelerate damage to specific viral components that are required for infection (Harvey and Ryan, 2004).

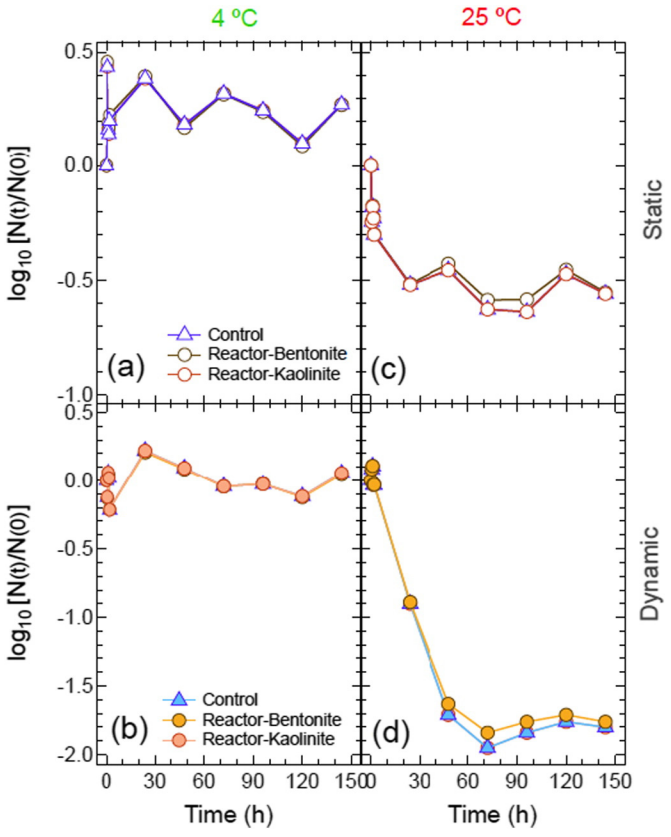

Fig. 2. Decay of hAdV concentration in control (triangles) and reactor (circles) tubes, expressed as $\log_{10}$ N(t)/N(0), under: (a) static conditions at 4 °C, (b) static conditions at 25 °C, (c) dynamic conditions at 4 °C, and (d) dynamic conditions at 25 °C.

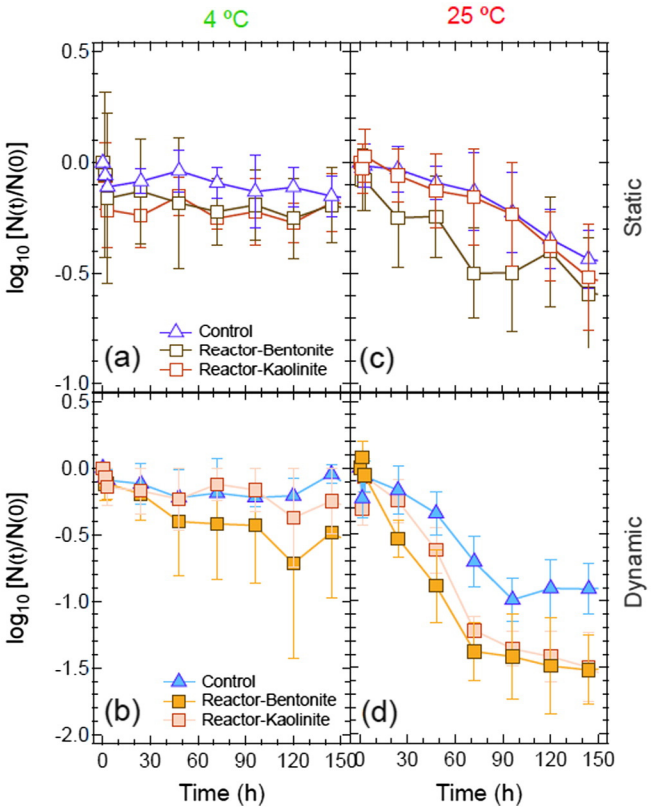

Fig. 3. Decay of MS2 concentration in control (triangles) and reactor (squares) tubes, expressed as $\log_{10}$ N(t)/N(0), under: (a) static conditions at 4 °C, (b) static conditions at 25 °C, (c) dynamic conditions at 4 °C, and (d) dynamic conditions at 25 °C.

Moreover, inactivation of clay-adsorbed viruses can either be enhanced (distortion and unfolding of protein structure due to strong electrostatic attraction) or reduced (virus protection) (Ryan et al., 2002; Schijven and Hassanizadeh, 2000). However, the exact role of virus attachment in virus inactivation is not clear.

3.2. Effect of temperature and batch conditions on hAdV adsorption

The data from hAdV adsorption experiments onto both kaolinite and bentonite at 4 and 25 °C, under static and dynamic conditions, are presented in Fig. 5, where the data were derived from qPCR analyses. As expected, a strong effect of temperature on hAdV adsorption was observed. The adsorption of hAdVs onto the selected clays was shown to be time depended (paired t-test, $P < 0.05$), because hAdVs were not uniformly equilibrated in each sample, causing fluctuations in the extractability of hAdV over time (Davidson et al., 2013). The experimental data for hAdV adsorption onto both kaolinite and bentonite, under static and dynamic conditions, suggested that hAdV adsorption is higher at low temperatures (Fig. 5a,b). However, hAdV adsorption was significantly different at the two different temperatures examined (there was a statistically significant decrease in adsorption between 4 and 25 °C, mean values: 1.32 ± 0.51 and 0.48 ± 0.41, with paired t-test, $P < 0.0005$). Although very similar trends are shown in the data for bentonite and kaolinite (see Fig. 5), statistical analysis revealed that hAdV adsorption was noticeably affected by the type of clay mineral (mean values kaolinite and bentonite: 1.03 ± 0.69 and 1.07 ± 0.73, respectively, with paired t-test, $P = 0.002$). Virus particles may adsorb to clays tightly during an initial pseudo-equilibrium period, but subsequently gradually allow them to detach from the clay particles (Davidson et al., 2013; Syngouna and Chrysikopoulos, 2010). Different trends were observed at 4 and 25 °C, suggesting that hAdV was more tightly adsorbed at 4 than 25 °C (compare Fig. 5a,b with Fig. 5c,d). The hAdV persistence

and abundance in clays could be significantly influenced by temperature (Davidson et al., 2013; Duboise et al., 1979; Wen et al., 2004). Moreover, the low temperature of 4 °C fostered the persistence of hAdV, while the higher temperature of 25 °C promoted thermal decay of hAdV particles (see Fig. 2). In the reactor tubes, it is unclear whether the hAdV is inactivated and adsorbed onto clays, or if the virus populations are not homogeneous, where some viruses were more resistant than others. Also, in the presence of the clays under dynamic conditions, hAdV adsorption was lower compared to that under static conditions (mean values for static and dynamic conditions were respectively: 1.05 ± 0.70 and 0.75 ± 0.48 with $P = 0.001 < 0.05$) (compare solid symbols and open symbols in Fig. 5). This finding contradicts the common belief that agitation enhances adsorption by maximizing the contact between viruses and clays. Although we cannot fully explain this observation, it is evident that the shaking caused by the dynamic conditions either prevents the adsorption of hAdV particles or promotes the desorption of previously adsorbed hAdV particles. The experimental data from the dynamic experiments at 25 °C with both kaolinite and bentonite suggest that the observed reduction of adsorbed hAdV particles was approximately 1.7-$\log_{10}$, for exposure time in the range between 0.50 and 144 h. In contrast, at 4 °C the adsorbed hAdV concentration was stable and the observed reduction of the adsorbed hAdV particles was about 0.2 $\log_{10}$. In the static experiments, the observed reduction of the adsorbed hAdV particles was 0.4-$\log_{10}$, and 0.6-$\log_{10}$ at 4 and 25 °C, respectively. For the dynamic conditions at 25 °C, hAdV adsorption markedly decreased over the time period between 0 and 60 h, and subsequently remained constant (see Fig. 5c,d); while for the static conditions at 25 °C, adsorption occurred almost instantaneously, and then remained constant. Although it is not unrealistic to hypothesize that under the dynamic conditions agitation promoted desorption of reversibly adsorbed hAdV particles over the time period between 0 and 60 h, additional research is required to fully describe the mechanisms responsible for hAdV desorption or detachment. Virus adsorption onto clay minerals and organic particulate occurs either by physical means, as a result of van der Waals forces and hydrogen bonding (Schaub and Sagik, 1975), or through the formation of a cation bridge between negatively surface-charged viruses and negatively charged platelet faces of clays. The adsorption of viruses onto clay minerals is enhanced in the presence of neutral electrolytes or buffers with the salts of polyvalent cations being more effective than those of monovalent cations (Moore et al., 1982; Schaub and Sagik, 1975). In addition, polyvalent cations may act as a bridge between the anionic groups on the virus and the negatively charged sites at the clay surface. Furthermore, contact time is an important parameter that controls adsorption (Akar and Uysal, 2010). The differences observed in the hAdV adsorption at different time intervals may be attributed to several factors, including virus type and structure, viral strain employed, virus aggregation tendency, and virus adsorption tendency onto particulate material or container walls (Ogorzaly et al., 2010). Moreover, hAdV has been reported in the literature to be more stable than F-specific RNA phage (MS2 and GA phage) (Ogorzaly et al., 2010). Also, Charles et al. (2009) reported a rapid decrease in the infectivity of hAdV at 12 °C, with a reduction of 4.2-$\log_{10}$ over 21 days; however, infectious hAdVs could still be detected over a 364-day period.

The experimental data presented in Fig. 5, suggesting that hAdV adsorption was affected by the type of clay mineral (kaolinite and bentonite), are in general agreement with the results from several previous studies. Schiffenbauer and Stotzky (1982) reported that the attachment of coliphages (T7, T1) was greater onto kaolinite than montmorillonite, while Lipson and Stotzky (1983) reported that more reovirus particles

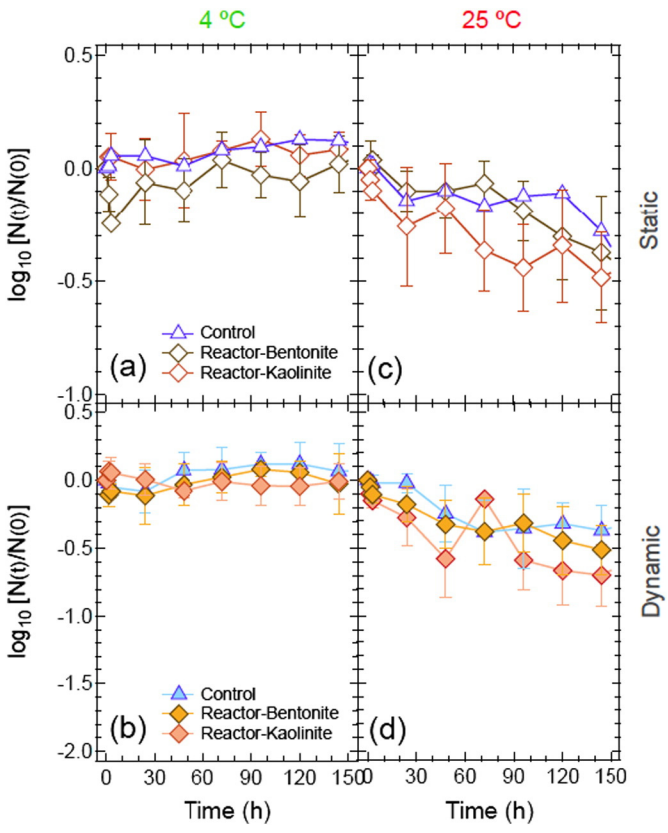

Fig. 4. Decay of ΦX174 concentration in control (triangles) and reactor (diamonds) tubes, expressed as $\log_{10} N(t)/N(0)$, under: (a) static conditions at 4 °C, (b) static conditions at 25 °C, (c) dynamic conditions at 4 °C, and (d) dynamic conditions at 25 °C.

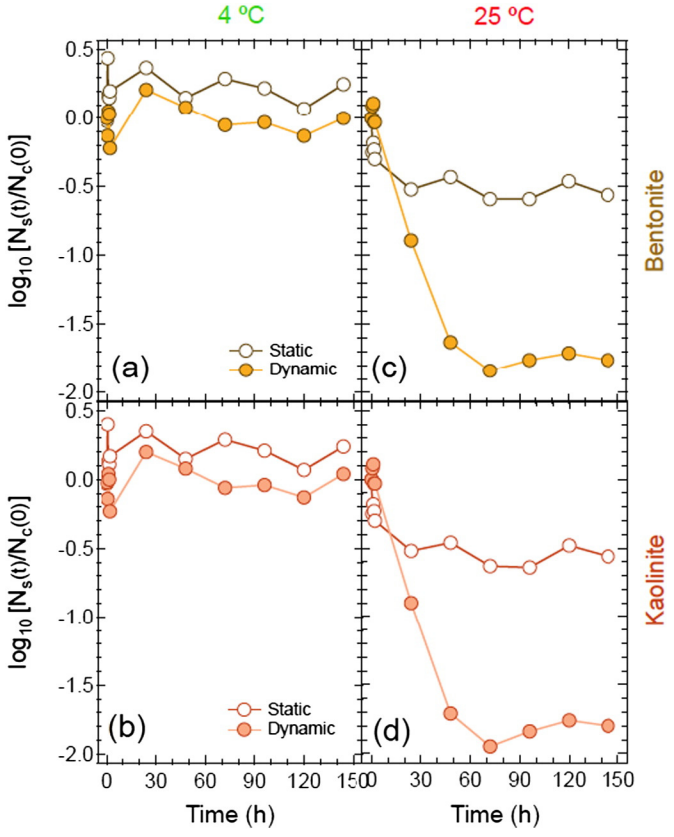

Fig. 5. Adsorption of hAdV, expressed as $\log_{10} N_s(t) / N_c(0)$, under static (open circles) and dynamic (solid circles) conditions onto: (a) bentonite at 4 °C, (b) kaolinite at 4 °C, (c) bentonite at 25 °C, and (d) kaolinite at 25 °C.

were attached onto montmorillonite than onto kaolinite, suggesting that the mechanisms of attachment differ for different viruses (Block et al., 2014; Chrysikopoulos and Syngouna, 2012). Lipson and Stotzky (1983, 1986) showed that viruses (e.g., poliovirus, coxsackie virus, reovirus) were adsorbed onto clays with enhanced survivability, and that the adsorption of reovirus onto montmorillonite or kaolinite was almost immediate and correlated with the cation exchange capacity of the clays. Lipson and Stotzky (1985) found that coliphage T1 and reovirus type 3 were adsorbed onto different sites on kaolinite and montmorillonite. Vilker et al. (1983a,b) investigated the interaction of poliovirus with montmorillonite, and determined that the negatively charged virions adhere to the positively charged montmorillonite edges.

3.3. Effect of temperature and batch conditions on coliphage adsorption

The data from MS2 and ΦX174 adsorption experiments onto both kaolinite and bentonite at 4 and 25 °C, under static and dynamic conditions, are presented in Figs. 6 and 7, respectively. The experimental data for coliphages were derived from cultivation analyses, according to an ATTC procedure. For most of the cases examined, the adsorption of both MS2 and ΦX174 was shown to increase with increasing temperature (compare Fig. 6c,d with Figs. 6a,b, and 7c,d with Fig. 7a,b for MS2 and ΦX174, respectively) except for the static experiments with ΦX174-kaolinite (compare open symbols Fig. 7d vs open symbols Fig. 7b) and MS2-kaolinite (open symbols in Fig. 6d with open symbols in Fig. 6b). Also, adsorption was higher under dynamic than static conditions (compare solid symbols with open symbols) except for ΦX174-kaolinite (see Fig. 7b,d). For the dynamic experiments at 25 °C, the observed reduction of the adsorbed MS2 was about 0.3-$\log_{10}$, for both kaolinite and bentonite. In contrast, at 4 °C the observed reduction of the adsorbed MS2 was 0.10-$\log_{10}$ and 0.45-$\log_{10}$, for bentonite and kaolinite, respectively. For the dynamic experiments at 25 °C, the observed reduction of the adsorbed ΦX174 was about 0.6-$\log_{10}$, for both clays. In contrast, at 4 °C the observed reductions of the adsorbed ΦX174-kaolinite and ΦX174-bentonite were 0.26-$\log_{10}$ and 0.56-$\log_{10}$, respectively. Due to the agitation, the number of accessible sites for attachment is much higher in dynamic than static experiments. Agitation improves the contact of particles with the liquid and decreases the resistance to mass transfer (Moore et al., 1981). Therefore, attachment rates were lower for static conditions. This finding has also been observed in previous studies (Anders and Chrysikopoulos, 2009; Syngouna and Chrysikopoulos, 2010). With the exception of kaolinite at 25 °C under static conditions, the adsorption onto both clays was greater for MS2 than ΦX174. This result is consistent with previous studies reported in the literature (Chrysikopoulos and Syngouna, 2012). Moreover, Chrysikopoulos and Syngouna (2012) showed that the affinity of both coliphages (ΦX174 and MS2) was greater for kaolinite than montmorillonite, and suggested that hydrophobic interactions played a significant role. Generally, hydrophobic interactions are more stable at higher temperatures. Furthermore, if sorption is controlled by partitioning of the hydrophobic part of a coliphage onto a clay particle, the sorption process is expected to increase with temperature (Bales et al., 1991). Chattopadhyay and Puls (1999) studied the different forces being applied between bacteriophages (T2, MS2 and ΦX174) and clay particles (hectorite, kaolinite and Norman clay), and suggested that van der Waals attraction was dominated over electrostatic repulsion. However, bacteriophage adsorption onto clays has been often attributed to the large surface area and high cation exchange capacity of

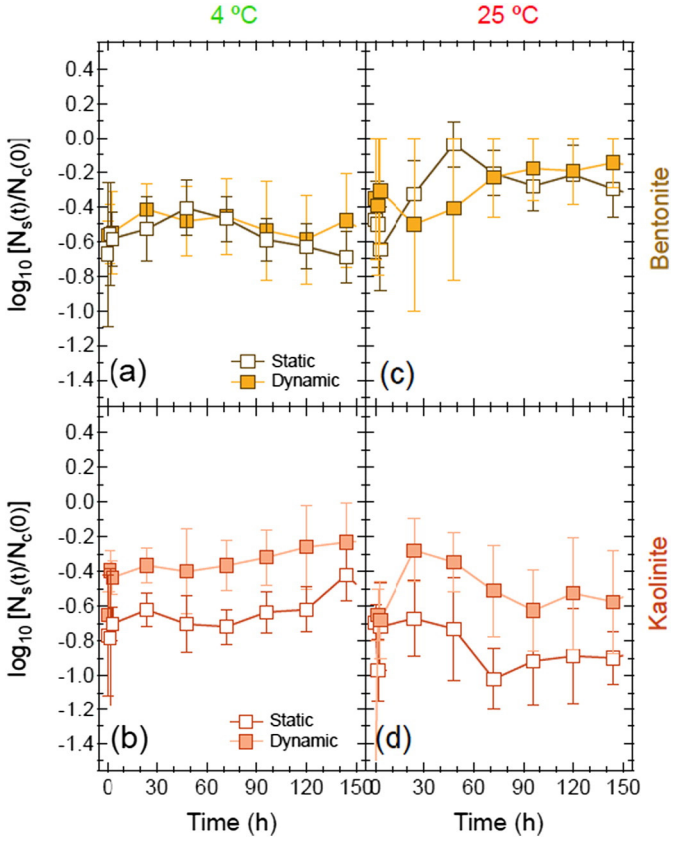

Fig. 6. Adsorption of MS2, expressed as $\log_{10} N_s(t) / N_c(0)$, under static (open squares) and dynamic (solid squares) conditions onto: (a) bentonite at 4 °C, (b) kaolinite at 4 °C, (c) bentonite at 25 °C, and (d) kaolinite at 25 °C.

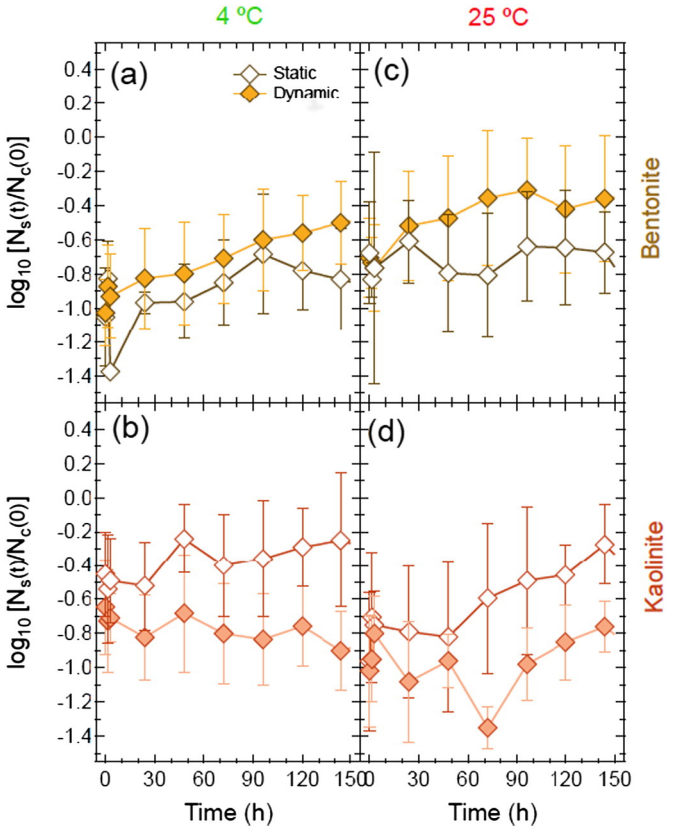

Fig. 7. Adsorption of ΦX174, expressed as $\log_{10} N_s(t) / N_c(0)$, under static (open diamonds) and dynamic (solid diamonds) conditions onto: (a) bentonite at 4 °C, (b) kaolinite at 4 °C, (c) bentonite at 25 °C, and (d) kaolinite at 25 °C.

clays (Chu et al., 2003). Furthermore, many researchers have related virus attachment onto cation-exchanged clays to positively charged sites on clay edges (Chattopadhyay and Puls, 1999; Schiffenbauer and Stotzky, 1982), while others to negatively charged sites (Lipson and Stotzky, 1983). However, the correlation of the observed virus adsorption with clay properties was beyond the scope of this paper.

3.4. Effect of IS on hAdV adsorption

Fig. 8 presents the data from experiments examining hAdV adsorption under various values of IS, with data derived from qPCR analyses. As expected, for most of the cases examined hAdV adsorption was found to be higher at the highest IS = 140 mM (paired t-test, $P < 0.05$) except for the case of kaolinite at 4 °C, where adsorption was higher at IS = 1.4. Under the experimental conditions of this study (DNase I reaction buffer: pH 7.6 and IS = 1.4, 14, 140 mM) the zeta potentials of hAdV, kaolinite, and bentonite were found to be negative and became less negative with increasing IS (see Table 1). Similar zeta potentials of hAdV under identical conditions were also reported in the literature (Wong et al., 2014). Moreover, Wong et al. (2012) documented that the isoelectric point (IEP) of hAdV was in the range of 3.5–4.0, and varied minimally with IS. The surface potentials of the variably-charged surfaces of viruses and clays depend on the solution IS, due to electric field compression and reduction of the electrostatic repulsion between identical charged particles caused by the shielding effect of counter ions in solution. Thus, viruses tend to strongly adsorb to various materials at high IS values (Grant et al., 1993; Lipson and Stotzky, 1983; Walshe et al., 2010) and, in general, virus attachment increases with increasing IS (Schijven and Hassanizadeh, 2000). However, quantitative relationships between attachment parameters and IS are not currently available. Shi et al. (2012) suggested that the observed strong attachment of hAdV was due to the high IEP of hAdV fibers. However, Wong et al. (2014) shown that at pH ~ 6, high deposition of hAdV onto sand surfaces was observed only at 10 and 100 mM NaCl, but not at 1 mM NaCl, supporting the hypothesis that fibers enhance hAdV deposition because hAdV can approach a sand surface close enough for fiber attachment at 10 and 100 mM, but not at 1 mM, where most hAdV and sand particles are likely separated beyond the fiber's length (Favier et al., 2002).

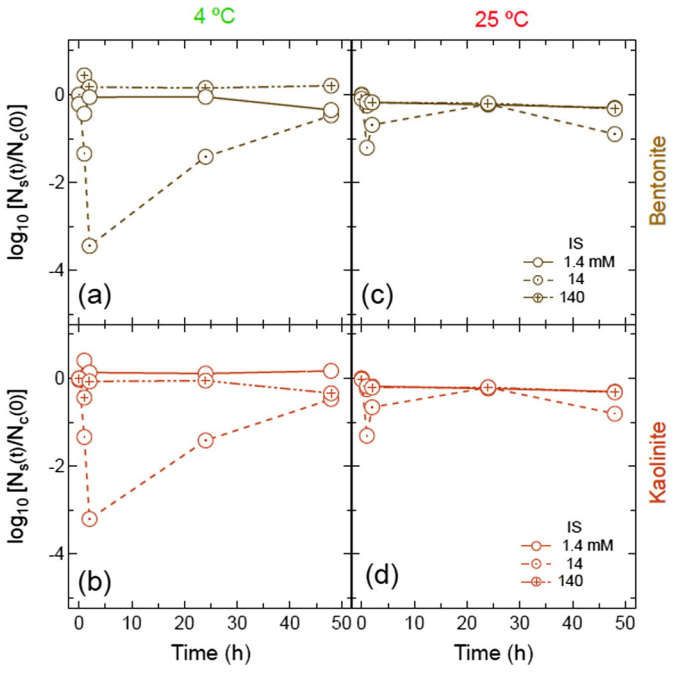

Fig. 8. Adsorption of hAdV, expressed as $\log_{10} N_s(t) / N_c(0)$, under static experimental conditions and different ionic strengths (IS = 1.4, 14, and 140 mM) onto: (a) bentonite at 4 °C, (b) kaolinite at 4 °C, (c) bentonite at 25 °C, and (d) kaolinite at 25 °C.

4. Conclusions

The experimental data presented in this study suggested that human adenoviruses and coliphages, and possibly other enteric viruses, adsorb to naturally occurring particles (e.g., clay minerals). The persistence of enteric viruses in aquatic and terrestrial environments has been related to their adsorption onto naturally occurring inorganic particulates, such as clay minerals. Temperature and the batch experimental conditions play a significant role on virus adsorption onto clay minerals. Additionally, the adsorption of hAdV was found to increase with decreasing temperature, under both static and dynamic batch conditions. For the most of the cases examined, the adsorption of coliphages (MS2 and ΦX174) was shown to increase with increasing temperature, and was higher under dynamic than static conditions. Generally, dynamic conditions improve the contact between viruses and clays, and possibly increase virus adsorption, because the number of accessible sites for attachment is higher. However, hAdV adsorption was lower under dynamic than static conditions, suggesting that agitation either prevents adsorption of hAdV particles or promotes desorption of previously adsorbed hAdV particles. Moreover, for most of the examined cases, hAdV adsorption was found to be higher at IS = 140 mM, except for the case of kaolinite at 4 °C, where adsorption was higher at IS = 1.4. HAdV adsorption at three different IS values had a statistically significant difference. The increased reduction of infectious viral numbers due to their contact with clays could play an important role in the prevention of infectious viral waterborne diseases. Finally, the adsorption behavior of surrogate coliphages MS2 and ΦX174 is not similar to that of hAdV. Consequently, neither MS2 nor ΦX174 is a suitable model for adenovirus.

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