Role of biofilm on virus inactivation in limestone aquifers: implications for managed aquifer recharge

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Abstract

Background Virus, as nano-sized microorganisms are prevalent in aquifers, which threaten groundwater quality and human health wellbeing. Virus inactivation by attachment onto the limestone surfaces is a determining factor in the transport and retention behavior of virus in carbonaceous aquifers.

Methods In the present study, the inactivation of MS2 -as a model virus- by attachment onto the surfaces of limestone grains was investigated in a series of batch experiments under different conditions such as limestone particle size distribution (0.25–0.50, 0.5–1 and 1–2 mm), treated wastewater and RO water, temperature (4 and 22 °C), initial MS2 concentrations (10^3 – 10^7 PFU/mL) and static and dynamic conditions. The experimental data of MS2 inactivation was also fitted to a non-linear kinetic model with shoulder and tailing. The characteristics of biofilm on the surfaces of limestone aquifer materials were assessed using scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM).

Results The inactivation rate of virus decreased with increasing the adsorbent diameter. Furthermore, virus inactivation was greater at room temperature (22 °C) than 4 °C, in both static and dynamic conditions. The inactivation of virus via attachment onto the limestone aquifer materials in dynamic conditions was higher than under static conditions. In addition, fitting the experimental data with a kinetic model showed that virus inactivation was high at higher temperature, smaller limestone grains and dynamic conditions. Moreover, the experiments with treated wastewater showed that in authentic aqueous media, the virus inactivation was considerably higher than in RO water, due to the presence of either monovalent or divalent cations and surface roughness created by biofilms.

Conclusion Finally, in terms of managed aquifer recharge systems, the presence of biofilm increases bacteria and virus retention onto the aquifer surfaces.

Keywords Virus inactivation · Biofilm · Batch experiment · MS2 · Limestone · Temperature

Introduction

Recently, the human demand for clean and safe drinking water resources is strongly increasing [1, 2]. Although groundwater resources, compared to the surface waters, are more tolerant of pollution, the discharge of treated wastewater and stormwater

containing microbial pollutants into the aquifers has been recently considered a great threat for subsurface water resources [3]. Human pathogenic viruses (and various surrogates, MS2, PRD1, ϕ X174) are quite ubiquitous in the subsurface environment, which may be derived from either intentional or accidental discharges [4]. Virus can enter groundwater resources through discharge of municipal wastewater, wide application of faecally contaminated water and sewage sludge for agricultural activities and leakage of septic tanks [5, 6]. In addition, the practice of artificial groundwater recharge has grown in order to compensate for the fast depletion of aquifers, which has led to the discharging of microbial-polluted water into the subsurface environment [7]. Indeed, the release of viruses into the environment is a potential danger for public health [8]. Furthermore, their presence in high concentrations

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in groundwater resources can be ascribed to a lack of efficient conventional water treatment techniques [9, 10]. Therefore, in order to prevent viral diseases, it is critical to identify the various environmental factors influencing virus transport and retention in aquifers.

In general, a large number of factors such as inactivation by attachment and detachment to/from solid collectors and environmental factors potentially influences on virus interactions with solid surfaces [11]. Additionally, the intensity of interaction between viruses and collector surfaces can be determined by different environmental factors such as pH of solution, temperature, ionic strength and composition (e.g., presence of mono or divalent cations), the amount of organic matter and metal oxides [12–15].

Furthermore, virus inactivation may demonstrate different behaviors depending upon the physical, chemical, mineralogical and surface characteristics of the porous media. Chrysikopoulos and Aravantinou (2014) [15] reported that quartz sand grains had an interaction with MS2 and ϕ X174 viruses under different experimental conditions such as temperature, adsorbent size distributions and agitation status. Clay particles are ubiquitous in the subsurface environment, which can adsorb viruses, due to their high specific surface areas, expandability and ion exchange capacity [16]. Bellou et al., (2015) and Zhou, (2011) [17, 18] showed, at different temperatures and ionic strengths, the significant effect of kaolinite and montmorillonite on the attachment of human adenoviruses and coliphages.

Carbonaceous (limestone and dolomite) aquifers are globally important resources for potable water [19]. In these aquifers, the groundwater pH is generally buffered at 7. However, the surface charges of aquifer minerals at either neutral or high pH values are negative which is quite unfavorable for virus removal. Therefore, these aquifers pose a risk for drinking water resources from the point of view of pathogenic pollution. In order to manage the risk of groundwater resources pollution in carbonaceous aquifers, the interaction between limestone material and viruses, under different experimental conditions, needs to be understood. In majority of studies on virus interaction with substrate, the surfaces of substrate have been considered physically clean and simple. However, natural porous media contains different microbes that can produce biofilm on the surfaces of aquifer grains. Biofilm consists of several layers of bacteria which can be accumulated on a surface and surrounded by a matrix of extracellular polymeric substances (EPS) [20]. Surprisingly, little attention has been paid by hydrogeologists to the interaction between microorganisms and biofilm in the subsurface environment. An exception was a laboratory study of the transport of colloidal particles through biofilm-conditioned porous media, where the biofilm was produced using a culture of *Pseudomonas putida* [21]. However, in real MAR sites, the natural biofilm derives from a plethora microorganisms with different

features. Therefore, in this study, we used treated wastewater obtained from a wastewater treatment plant in Mount Barker to produce natural biofilm on the limestone aquifer grains.

The MS2 virus, which is not a pathogenic agent and has also been studied in previous research [22–24], was used in this study as a surrogate for pathogenic viruses. It is an *F*-specific RNA virus with a size of 24–26 nm [25], a hydrophobic protein coat and an isoelectric point of 4.1. Its host is *Escherichia coli* (*E.coli*) [26]. Although the study of virus attachment onto the different solid materials was previously performed [15, 27, 28], to the best of our knowledge, no study focused on the role of limestone materials on virus inactivation at different experimental conditions. In addition, all previous studies used engineered, pure and synthetic materials to evaluate their role on virus removal. While, the novelty of present research is application of the authentic subsurface materials in batch tests of MS2 inactivation. Therefore, the principal aim of this research was to investigate the inactivation of MS2 virus onto the surfaces of limestone with different particle size distributions under different initial virus concentrations and temperatures under static and dynamic conditions. The obtained experimental data of virus inactivation were fitted with a log-linear with shoulder and tail kinetic model [29]. Additionally, to simulate the real environmental conditions, treated wastewater collected from a wastewater treatment plant in Mount Barker, South Australia was used as an aqueous medium to produce biofilm and to determine its influence on surface properties of limestone and consequently MS2 inactivation.

Materials and methods

Preparation of limestone

The aquifer material was obtained from Virginia, South Australia (−34° 42′ 44″, 138° 34′ 9″) at a depth of −102 to −108 m. The aquifer at this depth is part of the Tertiary-T2 Port Willunga Formation, which is extensively used for irrigation of horticulture. The obtained core material was rinsed with reverse osmosis (RO) water (Milli Q) and then dried in an oven at 40 °C. Afterwards, it was crushed by a hammer and then passed through stainless steel sieves to attain three different particle size distributions (e.g., small: 0.25–0.50, medium: 0.5–1.0 and coarse: 1–2 mm). The specific gravity of samples was obtained using pycnometer method [30]. Finally, limestone grains were stored in non-reactive containers in a cold room at 4 °C, prior to use in the experiments described below.

The chemical and mineralogical features of aquifer material were determined using an X-ray diffraction (XRD) instrument (Bruker Advance D8 Eco, Co-K α radiation, $\lambda=1.79$ Å) at 2θ range of 10–80°. In addition, the principal metal oxides in the structure of limestone aquifer material were measured

via X-ray fluorescence (XRF) technique (PANalytical Axios Advanced instrument). Moreover, the elemental analysis of limestone aquifer material was carried out using an energy dispersive X-ray spectroscope (EDS) installed on SEM. The presence of different functional groups on the surface of the limestone aquifer material was analyzed using Fourier transform infrared spectroscopy (FTIR, PerkinElmer/Frontier). Zeta potentials of MS2, pristine and conditioned limestone were measured in RO water and treated wastewater using a Malvern Zetasizer instrument (Malvern, Zetasizer Nano Series, Nano-ZS). The measurements were reported as the mean of triplicate determinations.

Preparation of virus suspension and enumeration

MS2 F-RNA bacteriophage (ATCC # 15597-B1) was used in all experiments to study its inactivation via attachment onto the substrate surfaces. MS2 host *Escherichia coli* (*E. coli*) was grown (37 °C, overnight) in a 10 mL suspension of tryptone soya broth (TSB) containing 0.15 g ampicillin sodium salt and streptomycin sulphate antibiotic stock, which was prepared in 100 mL RO water and filtered (0.22 µm). To prepare MS2 stock solution, 5 mL of *E. coli* suspension, grown in TSB and 5 mL of MS2 suspension was added to 10 mL double strength 1.5% tryptone soya agar (TSA) containing 1% of the ampicillin/streptomycin antibiotic stock solution. This suspension was added to a Petri dish containing a base layer of TSA, mixed, allowed to set and incubated overnight at 37 °C.

Following incubation, MS2 was harvested by flooding the plate with 5 mL 0.5% tryptone water (Oxoid), incubated for 45 min at 37 °C, over which time the plate was gently swirled 3–4 times. The resulted suspension was passed through a 0.22 µm syringe filter to remove *E. coli*. The obtained MS2 stock solution was kept in 100 mL solution containing 90 mL half strength solution and 10 mL glycerol at freezer at –20 °C.

The concentration of MS2 in the stock solution and in suspensions from experimental treatments was measured using the TSB double layer agar (DLA) method adapted from [31, 32]. Finally, the grown MS2 were reported as plaque forming units (PFU) per 1 mL.

Batch experiments

MS2 inactivation by attachment onto limestone grains was studied in a series of batch experiments at different particle size distributions with both treated wastewater and RO water under static and dynamic conditions at 4 and 22 °C temperatures. A working virus solution was prepared by addition of 1 mL of virus stock solution (above) to 1 L RO water and then shaken vigorously to create homogeneous virus suspension. Where a range of MS2 concentrations (10^3 – 10^7 PFU/mL) were used in the experiments they were prepared from the working solution diluted to the desired concentrations with

RO water. The pH of the suspension was adjusted to 7.5 using 0.1 M NaOH and 0.1 M HCl solutions. The concentration of virus into the solution was measured immediately after preparation of virus suspension.

To prevent or at least minimize virus inactivation by attachment onto the internal walls, acid washed (6 N HCl) sterile (121 °C for 16 min) Pyrex™ glass screw-cap tubes were used in all incubations. The sterilized glass tubes were filled with 2.5 g limestone and 25 mL virus suspension (1:10 w/v), to ensure absence of air bubbles to prevent the effect of air-water interface (AWI) on virus inactivation [33]. In addition, parallel control experiments, in the absence of limestone, were incubated under the same conditions using 25 mL virus suspension. All incubations were conducted in the dark [34]. The static experiments were conducted in quiescent conditions at both 4 and 22 °C. In contrast, in the dynamic experiments, the tubes containing virus suspension with limestone aquifer grains and the respective control incubations were gently shaken using a rotary shaker for 24 h to establish an equilibrium between the aquifer material and MS2. On completion of the incubations, the tubes were centrifuged (1000×g for 10 min; Phonix, Australia) to separate limestone from aqueous solution. Then, 1 mL virus suspension was pipetted from the tubes and the equilibrium MS2 concentration in the suspensions was determined using double layer agar method [31]. All experiments were carried out in triplicate and the mean values ± standard deviation were reported. The virus inactivation by attachment onto the limestone surfaces was reported as $\log_{10}$ fraction removal using Eq. 1 [35]:

$$\log_{10} \frac{N(t)}{N(0)} \quad (1)$$

where, $N(t)$ is the virus concentrations (PFU/mL) at time t in reactor and control tubes. While, $N(0)$ is the initial virus concentration (PFU/mL).

Additional batch incubations were performed with limestone ‘conditioned’ by incubation with wastewater obtained from Mount Barker, South Australia, to encourage biofilm development. The wastewater was stored in non-reactive plastic containers in a cold room at 4 °C prior to use. 100 g of limestone grains were added to 1 L (10% w/v) conical flasks containing treated wastewater. Then, the flasks were sealed with aluminum foil and shaken (22 °C, 100 rpm, Innova 4) for two months to enable the formation of biofilm on the surfaces of limestone particles. In addition, in order to keep 10% w/v solid/solution ratio, the specific amounts of fresh treated wastewater were added into the bottles to compensate for evaporative loss. Following the two month incubation the wastewater was decanted and used to prepare MS2 suspensions (25 mL) for use in batch incubations with the conditioned limestone (2.5 g). The methods

where then as described above for the RO water incubations.

Chemical and biological parameters of treated wastewater were measured initially and after the 2 month incubation with limestone aquifer material. The pH of the wastewater was determined using a pH-meter (Jenway, United Kingdom) instrument. Nitrate (NO_3^- -N), nitrite (NO_2^- -N), ammonium (NH_4^+ -N) and phosphorous (PO_4 -P) were determined using a Foss nutrient analyzer (FIAstar 5000 Analyzer, Sweden), while total organic carbon (TOC) and total nitrogen were measured using a TOC analyzer (TOC-L, Shimadzu, Japan). Calcium carbonate was also measured using titration [36]. Ca^{2+} and Mg^{2+} concentrations were determined using an ion chromatograph (Metrohm, Switzerland). Finally, a TOC analyzer (SSM-5000A, Shimadzu, Japan) was applied for measurement of the total carbon of pristine and conditioned limestone grains.

Pristine and conditioned limestone grains were fixed using 2.5% (v/v) glutaraldehyde, dehydrated using a series of ethanol, (30–100%) which was continued by coating with platinum and examined using scanning electron microscopy (SEM, Inspect D50). In addition, to further demonstrate the production of biofilm on the limestone surfaces, confocal laser scanning microscopy (CLSM, Leica TCS SP5, Flinders Microscopy and Microanalysis) was applied. In this regards, the pristine and conditioned limestone grains were washed with phosphate buffered saline (PBS) solution, followed by staining with carbohydrate-recognizing lectin concanavalin A conjugated to Alexa Fluor 488 (ConA-Alexa488; Molecular Probes, Inc., Eugene, OR) for 30 min and washing with RO water to remove excess ConA. The samples were imaged with a 20x HC PL Apo 20 $\times$ 0.70 NA objective lens, using the 488 nm laser line of a Leica TCS SP5 confocal microscope with the PMT detector set to capture fluorescence from 492 to 561 nm. The processing and preparation of 3-dimensional (3-D) images of pristine and conditioned materials was carried out using Imaris software (version 8.3, Bitplane).

Kinetic studies

In order to study the kinetics of MS2 inactivation under various experimental conditions, the Geeraerd and Van Impe [29] inactivation model-fitting tool was applied (GinaFit, Version 1.5; KU Leuven, Belgium). Following inspection, a non-linear model with shoulder and tail was used as shown in Eq. 2 [29]:

$$N_t = (N_0 - N_{res})e^{-k_{max}t} \left(\frac{e^{k_{max}Sl}}{1 + (e^{k_{max}Sl} - 1)e^{-k_{max}t}} \right) + N_{res} \quad (2)$$

where, k_{max} is the virus inactivation rate (h^{-1}), N_0 and N_{res} are the initial and residual virus concentrations in the solution (PFU/mL), Sl is the shoulder length (h) which is the required

time for beginning the inactivation of virus and t is the reaction time (min).

Statistical analysis

Analysis of variance (ANOVA) was applied for evaluation of the role of agitation status, temperature, initial virus concentrations, adsorbent particle size distributions and biofilm on MS2 inactivation rate. Mean differences were compared through least significant difference (LSD) test ($p < 0.05$). The statistical analysis was performed by SPSS software (version 16) for Windows and the Figures were plotted using Microsoft Excel.

Results and discussion

Characterization of limestone aquifer material

The calculated specific gravity of limestone aquifer materials was 2.55 g/cm^3 . Results of XRD analysis showed that the aquifer materials consisted of a significant amount of calcite with negligible quantities of dolomite and quartz (Fig. 1a). In addition, based on XRF analysis, the aquifer materials contained 46.41% CaO, 9.59% SiO_2 , 2.84% MgO, 1.63% Fe_2O_3 , 0.41% Al_2O_3 , 0.21% K_2O , 0.20% SO_3 , 0.07% Na_2O , 0.03% MnO, 0.02% P_2O_5 and 38.56% loss on ignition. Furthermore, results of EDS analysis revealed the presence of O, Ca, C, Mg and Si in the body of aquifer grains (Fig. 1b). Finally, according to the FTIR spectrum of aquifer materials (Fig. 1c), three sharp peaks were observed in the region of absorption at 712.66 , 872.20 and 1404.32 cm^{-1} attributed to calcite as the highest proportion of aquifer material. A slight peak was also observed between 1000 and 1200 cm^{-1} , indicating the presence of quartz in the limestone aquifer material. Furthermore, the FTIR spectrum of dolomite can be characterized at 727 cm^{-1} . But, in this study no obvious peak was observed in this domain which may be attributed to the low amount of dolomite in the structure of limestone aquifer materials that was confirmed by the results of EDS and XRF techniques.

Effect of agitation and temperature

The agitation status and temperature are two most critical factors affecting virus inactivation in the presence of solid surfaces [15]. Figure 2 shows the $\log_{10}$ removal of MS2 over the reaction time, due to the inactivation by attachment onto the surfaces of small particle size (0.25 – 0.50 mm) aquifer material under static and dynamic (mixed) incubation conditions. MS2 inactivation rate in the presence of limestone was significantly higher than those of the controls in the absence of limestone. For example, in 4°C static treatment, MS2 inactivation rate in

Fig. 1 XRD diagram (a), EDS (b) and FTIR spectra (c) of limestone aquifer materials (C: calcite, Q: quartz and D: dolomite)

Fig. 2 Log_{10} removal of MS2 due to attachment on small particle size (0.25–0.50 mm) limestone aquifer materials incubated under static and dynamic conditions and at 4 and 22 °C (initial MS2 concentration: 10^3 PFU/mL), in the presence (○) and absence (●) of limestone substrate. The error bars smaller than dots are not shown

the control incubation (-0.03) was significantly lower than incubations in the presence of limestone (-0.07 ; $p < 0.05$). Indeed, in the control experiments, the most likely causes of virus inactivation are dark die off inactivation and attachment to the internal surfaces of reactors. However, in reactive experiments with the presence of solid surfaces, the removal of virus is the sum of the above-mentioned processes together with inactivation by attachment onto the surfaces of substrate. The maximum $\log_{10} (N_t/N_0)$ removal of MS2 at 22 °C in the presence of limestone was -0.38 in the dynamic incubation, compared with -0.15 when incubated statically. Agitation in the dynamic incubation increases contact between virus particles and the limestone surfaces thereby enhancing the chance of collision between these particles and reducing the resistance to mass transfer [11]. Furthermore, this observation can be ascribed to the presence of air-liquid and air-solid interfaces in dynamic conditions, which were not effective in virus inactivation in static conditions. Similar findings have been reported in other studies [5, 33]. In addition, Fig. 2 shows that virus inactivation in both static and dynamic experiments was higher at the higher temperature. Accordingly, the maximum virus inactivation was observed with $\log_{10}$ removal of -0.38 at 22 °C under dynamic incubation. While, at 4 °C under dynamic incubation, the maximum virus $\log_{10}$ removal was -0.12 . This may be damage due to high temperature on some viral components that are necessary for infection [37].

Bellou et al., (2015) [18] similarly reported a considerable increase in MS2 inactivation when increasing the incubation temperature from 4 to 22 °C.

Results of kinetic studies of MS2 inactivation on aquifer material in both control and reactive experiments under static and dynamic incubation conditions, at both 4 and 22 °C are reported in Table 1. As can be seen from Table 1, the significantly high correlation coefficients (R^2) and low root mean square error ($RMSE$) and sum of squared error (SSE) demonstrate the accuracy of the model applied to fit the experimental data of MS2 inactivation. Furthermore, k_{max} , which is a reliable virus inactivation index, of the control experiments, in the absence of limestone, is lower than reactive experiments, where limestone is present. In addition, although no trend was observed for SI (h), by increasing the temperature and agitation, in both control and reactive experiments, the k_{max} (h^{-1}) was higher at room temperature than at 4 °C and in dynamic, compared with static incubations.

Effect of particle size distributions and initial virus concentrations

In a typical aquifer, the medium consists of materials of different particle sizes, which may influence on the inactivation of micro-organisms. This study determined the inactivation of MS2 at five different initial MS2 concentrations (10^3 – 10^7 PFU/mL) by

Table 1 Kinetic studies of MS2 inactivation in the presence (reactive) and absence (control) of limestone aquifer material at 4 and 22 °C under static and dynamic conditions in RO water (particle size; small (0.25–0.50 mm); medium (0.5–1.0 mm) and coarse (1–2 mm))

Experimental conditions	^a C ₀ (PFU/mL)	Control experiments					Particle size	Reactive experiments				
		^b k _{max} (h ⁻¹)	^c SI (h)	^d R ²	^e RMSE	^f SSE		k _{max} (h ⁻¹)	SI (h)	R ²	RMSE	SSE
4 °C static	2.48 × 10 ³	0.74 ± 0.13	0.43 ± 0.34	0.982	0.0024	0.0000	Small	0.64 ± 0.23	0.48 ± 0.28	0.975	0.005	0.0000
4 °C dynamic	2.80 × 10 ³	0.78 ± 0.07	0.68 ± 0.07	0.955	0.0038	0.0000	Small	0.69 ± 0.03	0.88 ± 0.75	0.956	0.0130	0.0002
22 °C static	3.71 × 10 ³	0.86 ± 0.37	1.05 ± 0.67	0.975	0.0044	0.0000	Small	1.22 ± 0.39	0.65 ± 0.26	0.990	0.0069	0.0001
22 °C dynamic	3.28 × 10 ³	0.95 ± 0.65	0.87 ± 0.44	0.983	0.0054	0.0000	Small	1.38 ± 0.72	1.34 ± 0.28	0.982	0.0276	0.0008
22 °C dynamic	3.31 × 10 ⁴	0.73 ± 0.32	0.57 ± 0.68	0.974	0.0061	0.0000	Small	1.35 ± 0.49	2.06 ± 0.25	0.969	0.0246	0.0006
22 °C dynamic	3.53 × 10 ⁵	0.68 ± 0.15	0.71 ± 0.44	0.951	0.0083	0.0001	Small	1.25 ± 0.52	2.08 ± 0.30	0.972	0.0221	0.0004
22 °C dynamic	2.73 × 10 ⁶	0.79 ± 0.14	0.62 ± 0.10	0.935	0.0087	0.0001	Small	1.23 ± 0.25	2.15 ± 0.08	0.960	0.0230	0.0006
22 °C dynamic	3.24 × 10 ⁷	0.66 ± 0.08	0.72 ± 0.48	0.948	0.0069	0.0001	Small	1.13 ± 0.23	2.24 ± 0.12	0.970	0.0186	0.0003
22 °C dynamic	3.19 × 10 ³	1.05 ± 0.31	1.38 ± 0.08	0.980	0.0065	0.0000	Medium	1.31 ± 0.08	1.45 ± 0.19	0.988	0.0136	0.0001
22 °C dynamic	4.21 × 10 ⁴	1.03 ± 0.36	1.14 ± 0.39	0.979	0.0055	0.0000	Medium	1.27 ± 0.24	1.55 ± 0.04	0.979	0.0168	0.0003
22 °C dynamic	3.48 × 10 ⁵	0.94 ± 0.03	1.43 ± 0.09	0.974	0.0060	0.0000	Medium	1.18 ± 0.26	1.58 ± 0.14	0.974	0.0164	0.0003
22 °C dynamic	4.29 × 10 ⁶	0.90 ± 0.08	1.35 ± 0.05	0.964	0.0064	0.0000	Medium	1.13 ± 0.11	1.70 ± 0.13	0.980	0.0122	0.0001
22 °C dynamic	3.37 × 10 ⁷	0.89 ± 0.17	1.50 ± 0.75	0.945	0.3236	0.0001	Medium	1.03 ± 0.22	2.02 ± 0.23	0.985	0.0110	0.0001
22 °C dynamic	3.50 × 10 ³	0.97 ± 0.15	0.91 ± 0.17	0.975	0.0064	0.0001	Coarse	1.20 ± 0.15	1.72 ± 0.09	0.965	0.0192	0.0004
22 °C dynamic	3.93 × 10 ⁴	0.95 ± 0.31	0.91 ± 0.35	0.983	0.0048	0.0000	Coarse	1.15 ± 0.12	1.04 ± 0.12	0.984	0.0110	0.0001
22 °C dynamic	2.80 × 10 ⁵	0.91 ± 0.31	1.20 ± 0.31	0.962	0.0065	0.0001	Coarse	1.14 ± 0.15	1.83 ± 0.28	0.985	0.0101	0.0001
22 °C dynamic	3.18 × 10 ⁶	0.86 ± 0.09	1.46 ± 0.45	0.972	0.0045	0.0000	Coarse	1.13 ± 0.37	1.81 ± 0.30	0.969	0.0137	0.0002
22 °C dynamic	4.40 × 10 ⁷	0.78 ± 0.06	1.68 ± 0.22	0.940	0.0069	0.0001	Coarse	1.04 ± 0.12	1.73 ± 0.37	0.968	0.0121	0.0001

^a Initial MS2 concentration. ^b Virus inactivation rate. ^c Shoulder length. ^d Coefficient of determination. ^e Root mean square error. ^f Sum of squared error

attachment onto the surfaces of aquifer materials of different particle size distributions (i.e., small, medium and coarse). The results confirmed that the MS2 inactivation in the presence of limestone particles was significantly higher than in those incubations where limestone was absent (Fig. 3, control), irrespective of the initial concentration of MS2. In addition, an indirect relationship was observed between the particle size and MS2 inactivation rate. At initial virus concentration of 10^3 PFU/mL in three different particle size distributions 0.25–0.50, 0.5–1 and 1–2 mm, the MS2 $\log_{10}$ fraction removal were obtained -0.38 , -0.24 and -0.19 , respectively. Increasing particle size likely reduces, the available reactive sites decreasing virus inactivation via attachment onto the adsorbent surfaces. Although the grain shape of the aquifer materials with different size distribution was similar since they were obtained from the same source, the number of attachment sites on smaller particles is higher than larger ones. Chrysikopoulos and Aravantinou (2014) [15] in a research on interaction of two bacteriophages MS2 and Φ X174 with quartz sand grains with different diameters also reported higher virus inactivation rates in smaller particle size distributions. Results of kinetic studies of MS2 inactivation using limestone with different particle size distribution are illustrated in Table 1. Higher values of k_{max} (h^{-1}) were observed for reactive treatments than the controls, indicating more MS2 inactivation in reactive treatments, compared to the control ones at all initial MS2 concentrations and limestone size distributions. Furthermore, by decreasing the particle size of aquifer materials, an increase in the value of k_{max} (h^{-1}) was observed.

In addition, MS2 inactivation rate was high at lower initial virus concentrations in both control and reactive experiments. Indeed, when incubated with the small particle size material, the mean MS2 inactivation rate showed a statistically

significant decrease from -0.38 to -0.21 , ($p = 0.000361$) when the initial MS2 concentration was increased from 10^3 to 10^7 PFU/mL. This observation can be ascribed to increasing virus aggregation in high virus concentrations [38]. Increasing the initial virus concentrations in the solution, increases the likelihood of creating a virus sub-population, which leads to the presence of virus with high resistance to inactivation [38]. The findings of kinetic studies also showed that k_{max} (h^{-1}) at all particle size distributions, showed a decreasing trend with increasing initial MS2 concentration. However, an indirect relationship was observed between k_{max} (h^{-1}) and SI (h). Where, a sharp increase was observed by increasing initial MS2 concentrations. Similar results were reported by Ng et al., (2016) [39] who investigated the effect of bromide on the photocatalytic inactivation of bacteria in aqueous media.

Furthermore, contact time is a very crucial factor for the removal of adsorbate molecules onto the solid surfaces [40–43]. Depending on the physical and chemical features of adsorbents and intrinsic nature of viruses, the equilibrium contact time of virus inactivation is quite different. In this study, the inactivation of MS2 by attachment on the limestone surfaces attained equilibrium at 4 h after which the changes in MS2 inactivation were negligible. However, results of Bellou et al., (2015) [18] showed that hAdV, MS2 and Φ X174 inactivation using the kaolinite and bentonite was a slower process and equilibrated after 7 days.

Effect of biofilm

Limestone aquifer substrate (0.25–0.50 mm) was pre-incubated for 2 months with secondary treated wastewater

Fig. 3 MS2 inactivation determined simultaneously in the presence of limestone aquifer materials of different particle size distributions (reactive), small particles (0.25–0.50 mm; ●), medium (0.5–1.0 mm; ◆) and coarse (1–2 mm; ■) and in the respective controls in the

absence of limestone material. The inactivation rates were determined at five initial MS2 concentrations at 22 °C under dynamic conditions. The error bars smaller than dots are not shown

Table 2 Chemical and biological parameters of studied treated wastewater, before and after conditioning limestone aquifer material

Parameter	Before conditioning <i>Treated wastewater</i>	After conditioning
pH	7.21 ± 0.04	8.43 ± 0.02
Total nitrogen (mg/L)	74.33 ± 0.36	23.87 ± 0.21
NH ₄ -N (mg/L)	64.40 ± 0.1	Below detection limit
NO ₂ -N (mg/L)	0.028 ± 0	0.005 ± 0
NO ₃ -N (mg/L)	0.243 ± 0.001	16.85 ± 0.08
PO ₄ -P (mg/L)	4.31 ± 0.08	0.54 ± 0.001
TOC (mg/L)	24.13 ± 0.9	4.70 ± 0.02
Calcium carbonate (mg/L)	124 ± 7.0	151 ± 10.0
MS2 (PFU/ml)	Below detection limit	Below detection limit
Ca ²⁺ (mg/L)	9.55 ± 0.17	42.55 ± 0.27
Mg ²⁺ (mg/L)	10.63 ± 0.24	26.13 ± 0.18
	<i>Limestone aquifer material</i>	
Total carbon (mg C/g)	5.22 ± 0.78	5.48 ± 0.07

obtained from Mount Barker Wastewater Treatment Plant, South Australia to condition the surface with biofilm. The chemical and biological properties of wastewater, before and after incubation with the limestone, are presented in Table 2. Batch incubation with MS2 were performed to determine the effect of conditioning the limestone on MS2 inactivation under static and dynamic conditions at 4 and 22 °C. Figure 4 shows that, irrespective of other incubation conditions, incubation at 22 °C increased the MS2 inactivation rate, which was further increased by agitation. -0.46 and -1.88 MS2 log₁₀ removal were observed in the presence of the conditioned limestone substrate at 4 °C static and 22 °C dynamic treatments, respectively. Furthermore, results of kinetic studies of MS2 inactivation in conditioned limestone show that the lowest k_{max} 1.53 (h⁻¹) was obtained at 4 °C in the static

incubation (Table 3). While, dynamic treatment at 22 °C showed the highest k_{max} 1.94 (h⁻¹). In addition, the inactivation rate of virus in limestone conditioned with treated wastewater was higher than those determined with pristine limestone in RO water. Accordingly, in the dynamic treatment at 22 °C, the difference in the mean MS2 inactivation rate in pristine limestone with RO water (-0.38) and conditioned limestone with treated wastewater (-1.88) was statistically significant ($p = 0.000002$). In the same experimental conditions, the minimum and maximum values of SI (h) were observed at 22 °C dynamic and 4 °C static treatments, respectively. A most likely explanation of this phenomenon is the presence of irregularities and roughness onto the surfaces of conditioned limestone which were created by biofilm. Previous research have reported the role of biofilm in

Fig. 4 MS2 inactivation in the biofilm-conditioned, small particle size (0.25–0.50 mm) limestone aquifer materials incubated in treated wastewater for 2 months under static and dynamic conditions at 4 and 22 °C (initial MS2 concentration 10³ PFU/mL), in the presence (○) and absence (●) of limestone substrate. The error bars smaller than dots are not shown

Table 3 Kinetic studies of MS2 inactivation by attachment onto the surfaces of conditioned limestone aquifer material at different temperatures (4 and 22 °C) and agitation (static and dynamic) conditions in RO water and treated wastewater aqueous solution (particle size; small (0.25–0.50 mm))

Experimental conditions	^a C ₀ (PFU/mL)	Control experiments					Particle size	Reactive experiments				
		^b k _{max} (h ⁻¹)	^c SI (h)	^d R ²	^e RMSE	^f SSE		^b k _{max} (h ⁻¹)	^c SI (h)	^d R ²	^e RMSE	^f SSE
RO water												
4 °C static	2.77 × 10 ³	0.83 ± 0.05	1.76 ± 0.25	0.959	0.0042	0.0000	Small	1.34 ± 0.20	2.08 ± 0.21	0.952	0.0347	0.0012
4 °C dynamic	3.58 × 10 ³	0.88 ± 0.40	0.92 ± 0.60	0.945	0.0060	0.0000	Small	1.46 ± 0.27	1.42 ± 0.20	0.982	0.0423	0.0020
22 °C static	2.91 × 10 ³	0.91 ± 0.25	0.92 ± 0.45	0.947	0.0082	0.0001	Small	1.58 ± 0.34	1.38 ± 0.38	0.989	0.0513	0.0026
22 °C dynamic	3.30 × 10 ³	0.96 ± 0.18	0.57 ± 0.28	0.984	0.0056	0.0000	Small	1.63 ± 0.09	0.87 ± 0.15	0.989	0.0684	0.0052
Treated wastewater												
4 °C static	3.63 × 10 ³	1.25 ± 0.25	1.73 ± 0.28	0.991	0.0109	0.0001	Small	1.53 ± 0.38	1.95 ± 0.11	0.988	0.0277	0.0008
4 °C dynamic	2.72 × 10 ³	1.38 ± 0.62	1.60 ± 0.26	0.990	0.0136	0.0002	Small	1.57 ± 0.32	1.92 ± 0.22	0.968	0.0899	0.0092
22 °C static	4.15 × 10 ³	1.41 ± 0.17	1.64 ± 0.13	0.988	0.0162	0.0003	Small	1.63 ± 0.16	1.49 ± 0.18	0.989	0.0686	0.0049
22 °C dynamic	3.15 × 10 ³	1.46 ± 0.26	1.39 ± 0.37	0.980	0.0244	0.0006	Small	1.94 ± 0.25	1.34 ± 0.14	0.983	0.1281	0.0191

^a Initial MS2 concentration. ^b Virus inactivation rate. ^c Shoulder length. ^d Coefficient of determination. ^e Root mean square error. ^f Sum of squared error

changing the surface properties of granular substrates which provide low velocity regions with less hydrodynamic forces and torques to adsorb particles, even in unfavorable conditions [44]. SEM images of limestone aquifer materials, before and after conditioning with treated wastewater are shown in Plate 1. It is clear that conditioning of limestone in treated wastewater caused an enhancement in surface roughness of the limestone, which is attributed to the microbial growth onto the limestone surfaces by creation of a biofilm layer of relatively large thickness. CLSM images of pristine and biofilm-conditioned limestone grains are shown in Plate 2. A very thin layer of fluorescent can be seen around the pristine limestone, which is attributed to the negligible amounts of microbial mass. However, the CLSM image of biofilm-conditioned limestone grains shows a thick layer of fluorescent around the substrate, denoting biofilm production.

Dika et al., (2013) [45] similarly observed higher virus attachment on the surfaces of adsorbents by increasing the attachment sites originating from surface irregularities. Previous research has shown that the deposition of micro

and nano-sized colloidal particles was enhanced in the presence of surface roughness [46–48]. By conditioning limestone particles with treated wastewater, the amount of total carbon increased from 5.22 ± 0.78 to 5.48 ± 0.07 mg/g (Table 2), which was due to the production of biofilm on the surfaces of limestone grains, originating from bacterial growth. In some cases, an increase in organic matter can potentially cause a negative relationship with virus removal onto the substrates, due to blocking the attachment sites. For example, results of Mayotte et al., (2017) [49] showed that increasing organic carbon on sand particles decreased their adsorption capacity towards bacteriophage, because of the blockage of surface attachment sites of sand grains which prevented further MS2 attachment. In contrast, the results presented here showed increasing virus inactivation in conditioned limestone, which contained higher organic matter. It can be implied that the role of surface irregularities and roughness outweigh the blocking of surface attachment sites with organic matter on virus inactivation. To further elucidate the potential relationship between the presence of biofilm and enhanced virus inactivation,

Plate 1 SEM images of pristine (a) and biofilm-conditioned limestone particles (b)

Plate 2 CLSM images of (left) pristine and (right) biofilm-conditioned limestone with treated wastewater (a) and (b); confocal slice images through limestone grains, (c) and (d); transmitted light images of limestone shown in (a) and (b), respectively, (e) and (f): 3D view of confocal z-stack data set in Imaris software)

batch experiments were carried out incubating MS2 with conditioned limestone in RO water at 4 and 22 °C under static and dynamic agitation conditions. The lowest and highest MS2 inactivation rates were recorded in 4 °C static and 22 °C dynamic conditions, respectively (Fig. 5). Comparison of k_{max} (h^{-1}) and $\log_{10}$ removal of MS2 onto either pristine or conditioned limestone in RO or treated wastewater are shown in Fig. 6. The minimum and maximum for MS2 $\log_{10}$ removal were -0.32 and -1.31 at 4 °C static and 22 °C dynamic

conditions, respectively, which were apparently higher for the pristine limestone incubated in RO water in the same temperature and agitation status. Statically incubating MS2 at 4 °C with limestone conditioned with a biofilm led to a statistically significant increase in MS2 inactivation, when compared to similarly incubated pristine limestone. This result confirmed that the conditioning of limestone increased its potential to adsorb more MS2 from aqueous solution, because of enhanced surface irregularities by biofilm growth.

Fig. 5 MS2 inactivation in the biofilm-conditioned, small particle size (0.25–0.50 mm) limestone aquifer materials in RO water incubated under static and dynamic conditions and at 4 and 22 °C (initial MS2 concentration 10^3 PFU/mL); in the presence (○) and absence (●) of limestone substrate. The error bars smaller than dots are not shown

Fig. 6 Comparison between MS2 inactivation rate (K_{max}) and log removal under different treatments

Zeta potential is another essential parameter which governs the inactivation of MS2 on the substrate surfaces. The obtained zeta potentials of MS2 in RO water and treated wastewater were -20.3 ± 1.44 and -9.1 ± 0.6 mV, respectively, which shows the role of presence of different cations in treated wastewater on charge neutralization of MS2. The zeta potentials of pristine and conditioned limestone were -16.9 ± 1.3 and -13.3 ± 3.2 mV in RO water and -11.2 ± 2.7 and -5.1 ± 0.1 mV in treated wastewater. As can be seen, the zeta potentials of conditioned limestone in both solutions were slightly less negative than pristine limestone, indicating the role of biofilm on decreasing negative surface charges of limestone and consequently decreasing the electrostatic double layer. Tripathi et al., (2011) [46] observed that biofilm-coated sand particles had less negative values of zeta potential than clean sand grains, which enhanced nanoparticle retention in columns.

The observed increase in MS2 inactivation using the surfaces of the conditioned limestone in the presence of wastewater compared with the RO water may be associated with the higher ionic strength of the treated wastewater, due to the presence of various cations (e.g. Ca^{2+} and Mg^{2+}). This may contribute to decreasing the thickness of electrostatic double layer, cation bridging, neutralization of negative charges of

adsorbent and virus particles and binding between calcium and some carboxyl functional groups onto the virus surfaces [37, 50–52]. Sasidharan et al., (2016) [12] reported that the presence of Ca^{2+} in solution increased significantly virus inactivation in the surfaces of sand grains. In addition, Stevenson et al., (2015) [53] showed that the addition of Ca^{2+} led to higher virus inactivation in the surfaces of granular limestone aquifer materials. To test this hypothesis, the amounts of soluble calcium ions (Ca^{2+}) and calcium carbonate in the wastewater conditioning solution were measured. Table 2 shows that Ca^{2+} and calcium carbonate concentrations were increased in conditioned limestone incubation, confirming that the limestone dissolution into the aqueous solution was a dominating process. Indeed, the concentrations of Ca^{2+} and calcium carbonate in treated wastewater after incubation with limestone particles showed an increasing trend from 9.55 to 42.55 mg/L and 124 to 151 mg/L, respectively. The concentrations of Ca^{2+} and calcium carbonate in the incubations with treated wastewater were higher than for similar RO water incubations (data not shown). That is attributed to microbial activities increasing the dissolution of limestone. Rinck-Pfeiffer et al., (2000) [54] similarly attributed Ca^{2+} dissolution in limestone-packed columns to the microbial activity. The concentration of Mg^{2+} in treated wastewater

also increased from 10.63 to 26.13 mg/L, which may be due to the dissolution of dolomite. pH is influential on calcite dissolution. In this study, the initial pH of treated wastewater was 7.21 (Table 2), which is quite favorable for dissolution of limestone grains. After conditioning of the limestone particles with treated wastewater, the solution pH increased to 8.43, which can be ascribed to increasing cations in aqueous media. Another effectual process on limestone dissolution in treated wastewater is nitrification, due to this process releasing hydrogen ions (H^+) into the aqueous media. Nitrification was observed during limestone conditioning. NO_3^- -N concentration increased from 0.243 to 16.85 mg NO_3^- -N/L. While, a simultaneous reduction was observed in NO_2^- -N and NH_4^+ -N concentrations from 0.028 to 0.005 mg NO_2^- -N/L and 64.4 mg NH_4^+ -N/L to below detection limit, respectively. Total nitrogen, NH_4^+ , NO_3^- and NO_2^- concentrations were determined, before and after limestone conditioning in wastewater (Table 2). It is observed that the amount of total nitrogen declined from 74.33 to 23.87 mg N/L, which may be due either to volatilization of NH_4^+ -N or more likely to transformation to microbial biomass on the surfaces of limestone grains.

Environmental implications

The understanding of MS2 inactivation in the aquifer materials is required to use different water resources such as stormwater and treated wastewater in a managed aquifer recharge site. Much research focuses on physically simple and chemically clean substrates like sand grains and different clay minerals interactions with viruses [18, 33]. However, the interaction between authentic limestone aquifer materials, which are representative of an authentic limestone-based MAR site with MS2 has not previously been presented. In addition, the presence of biofilm in a MAR site is possible, due to the application of stormwater and treated wastewater containing high TOC and nutrient concentrations. Recent studies have shown the significant effect of biofilm on enhanced retention of nanoparticles and *Escherichia coli* (*E.coli*) [55, 56]. However, no study has paid attention to the virus inactivation behavior of biofilm grown onto the surfaces of authentic aquifer materials. The present study mainly focused on MS2 inactivation using pristine and biofilm-coated aquifer materials in RO water and treated wastewater, to mimic the inactivation of virus in a MAR site. Our results revealed the enhanced MS2 inactivation in biofilm-coated aquifer materials compared to pristine one, even when RO water was considered as aqueous media. Therefore, although application of different water streams in MAR may pose the groundwater resources to the risk of pathogenic pollution, the production of biofilm may enhance virus inactivation in groundwater and aquifers.

Conclusion

In this study, virus inactivation in the limestone grains was studied at different experimental conditions. Results revealed that virus inactivation was completely dependent on adsorbent particle size distribution. As aquifer limestone particle size increased, a sharp decrease occurred in virus inactivation. Furthermore, more virus in RO water or wastewater were inactivated in the presence of limestone grains incubated at 22 °C than 4 °C, indicating a firm relationship between virus inactivation and temperature. Additionally, incubation of limestone aquifer material with treated wastewater caused a significant increase in virus inactivation, due to the enhancement of adsorbent surface roughness and increasing solution ionic strength. Biofilm production onto the substrate surfaces increased the height of roughness that changes the mass transfer rate and lever arms associated with torque balance. These findings were confirmed by the SEM images of pristine and conditioned substrate. Results of the present research provide a valuable insight about the interaction between MS2 bacteriophage with biofilm in natural aquifers.

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Compliance with ethical standards

Conflict of interest The authors confirm that they have no real or perceived conflict of interest which might influence the results of the research. The final version of manuscript has been approved by all authors.

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