Key processes in the attenuation, inactivation and elimination of pathogenic viruses during passage through aquatic sediments

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Affidavit

I hereby declare that I am the sole author of this work. No assistance other than that which is permitted has been used. Ideas and quotes taken directly or indirectly from other sources are identified as such. This written work has not yet been submitted in any part.

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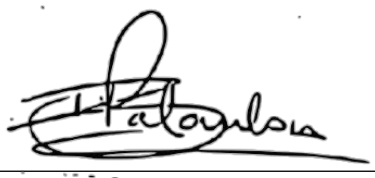

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Abstract in English

Pathogenic viruses entering river water can cause contamination of raw water when using riverbank filtration as a method for drinking water production. The efficiency of natural attenuation for pathogenic viruses during passage through aquatic sediments is controlled by a number of abiotic and biotic variables. Typically, transport and survival mechanisms of viruses during sediment passage are limited to interpretations based on concentration data of suspended and active viruses. What really happens with regards to inactivation of viral particles and viruses that become reversibly or irreversibly adsorbed to the sediment matrix is largely unknown. Objectives of this study were to explore virus attenuation mechanisms using uniquely combined detection methods and novel sampling techniques to differentiate between discrete fractions of free active (infectious) viruses, attached active viruses, and inactive (non-infectious) viral particles. A series of transport experiments were performed in small sediment columns to explore the effects of common subsurface gradients, including variations of flow velocity (1m d^{-1}, 0.5m d^{-1}), viral load (10^7, 10^5, 10^4 pfu mL^{-1}), dissolved oxygen conditions (oxic, hypoxic, anoxic), and sediment microbial activity (sterile, low, stimulated). The bacteriophage MS2 was used as a surrogate for pathogenic viruses, supplied to water saturated and sediment filled glass columns, accompanied by a conservative fluorescent tracer. The application of molecular analyses and culture-based assays were chosen to track the total number of viral particles as genome copies and the number of infectious viruses as plaque forming units (pfu). Replicate columns sacrificed sequentially over the duration of each experiment provided novel information on viruses adsorbed to the sediment matrix, indicating a temporal dynamic of the virus and sediment surface interaction.

Under standard experimental conditions, the majority of active viruses infiltrated into the columns were recovered when the free and attached fractions of particles were combined. When reducing the flow velocity, less attached infections viruses were recovered. Under the standard flow velocity and a lower viral load, the recovery of active viruses quantitatively shifted to the adsorbed fraction on the sediment matrix where they experienced lower rates of inactivation. In experiments performed under anoxic and hypoxic conditions, the main mechanism of attenuation was by elimination. This is in contrast to published findings which observed a high virus particles recovery in the absence of oxygen. Considerable recovery of inactive viral particles under hypoxic condition also points to additional mechanisms of inactivation specific to that environment. In the presence of stimulated bioactivity, a low recovery of active viruses but a high concentration of inactive viral particles in solution was obtained. Although, recovery of attached viruses also indicated that adsorption led to some preservation on bioactive sediment surfaces. In contrast, the absence of bioactivity led to high recovery of active viruses in solution and extremely limited removal by adsorption, supporting conclusions that the presence of a high microbial activity contributed to virus inactivation. These experiments demonstrated that monitoring concentrations of alternative viral fractions and matrices is necessary for an accurate characterisation of the transport, adsorption, and survival behaviour of viruses in saturated porous media.

Keywords: *Bacteriophage, MS2, viruses, groundwater, riverbank filtration, sediment column, transport, adsorption, elimination, inactivation, breakthrough curve, qPCR, recovery, flow velocity, viral load, dissolved oxygen, bioactivity.*

Abstract in Deutsch

Bei der Uferfiltration als Methode zur Trinkwassergewinnung können pathogene Viren, die in Flusswasser gelangen, zu einer Kontamination des Rohwassers führen. Die Effizienz des natürlichen Rückhaltes pathogener Viren während der Passage durch aquatische Sedimente wird durch eine Reihe abiotischer und biotischer Variablen gesteuert. Typischerweise sind Transport- und Überlebensmechanismen von Viren während der Sedimentpassage auf Erkenntnisse über im Wasser transportierte und auf aktive Viren beschränkt. Ob Viruspartikel während der Passage durch das Sediment inaktiviert werden und was mit Viren passiert, die reversibel oder irreversibel an der Sedimentmatrix adsorbiert werden, ist weitgehend unbekannt. Ziele dieser Studie war es neue Nachweismethoden heranzuziehen, um das Transportverhalten von freien aktiven (infektiöser) Viren, sorbierter aktiver Viren und inaktiver (nicht infektiöser) viraler Partikel unterscheiden und untersuchen zu können. Eine Reihe von Transportexperimenten wurde in kleinen Sedimentsäulen durchgeführt, um die Auswirkungen üblicher unterirdischer Variablen zu untersuchen, einschließlich Variationen der Fließgeschwindigkeit (1m d^{-1}, 0,5m d^{-1}), Viruslast (10^7, 10^5, 10^4 pfu mL^{-1}), Bedingungen für gelösten Sauerstoff (oxisch, hypoxisch, anoxisch) und mikrobielle Sedimentaktivität (steril, niedrig, stimuliert). Der Bakteriophage MS2 wurde als Surrogat für pathogene Viren verwendet, der in wassergesättigte und sedimentgefüllte Glassäulen zugeführt wurde zusammen mit einem konservativen fluoreszierenden Tracer. Die Anwendung molekularer Analysen und kulturbasierter Assays wurde gewählt, um die Gesamtzahl der Viruspartikel als Genomkopien und die Zahl der infektiösen Viren als Plaque-bildende Einheiten (pfu) zu verfolgen. Über die Dauer jedes Experiments wurden Replikatsäulen geschlachtet, um neue Informationen über an der Sedimentmatrix adsorbierte Viren zu erhalten, um die zeitliche Dynamik der Interaktion zwischen Virus und Sedimentoberfläche zu untersuchen.

Unter Standard-Versuchsbedingungen (1m d^{-1}, oxisch) war die Mehrheit der Viren (frei und sorbiert) aktiv. Bei reduzierter Fließgeschwindigkeit wurde eine erhöhte Inaktivierung der sorbierten Viren gefunden. Wurden weniger Viren im Versuch benutzt, waren mehr Viren der adsorbierten Fraktion noch aktiv. Unter anoxischen und hypoxischen Bedingungen wurden die meisten Viren eliminiert. Die Rückgewinnung von inaktiven Viruspartikeln unter hypoxischen Bedingungen wies jedoch auf zusätzliche Mechanismen der Inaktivierung hin. In Gegenwart von erhöhter mikrobieller Biomasse wurde eine geringe Wiederfindung aktiver Viren, aber eine hohe Konzentration inaktiver viraler Partikel in Lösung gefunden, und sorbierte Viren waren weiterhin viral. Versuche mit geringerer Biomasse zeigten eine hohe Wiederfindung aktiver Viren in Lösung und eine begrenzte Entfernung durch Adsorption. Die Experimente zeigten, dass es wichtig ist zu unterscheiden, ob Viren während der Sedimentpassage ihre Aktivität weiterhin behalten oder verlieren, um somit auch Aussagen über das Transportverhalten von Viren bei der Uferfiltration machen zu können.

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1 Introduction

Access to safe and sustainable drinking water is a growing public health concern. Abstraction from groundwater reservoirs offers large volumes of clean fresh drinking water. However, aquifers are finite in capacity, therefore abstraction is often paired with groundwater recharge methods such as riverbank filtration (RBF) in order to replenish shrinking reservoir quantities (Jakeman, Barreteau and Rinaudo, 2016). Riverbank filtration is seen as a sustainable way for drinking water producers to harness natural processes and avoid the financial and logistical burdens involved with traditional water treatment methods. As such, RBF has been widely adopted as an affordable and environmentally sustainable water treatment alternative throughout the world (Umar *et al.*, 2017). However, rivers often serve as a passage for wastewater, whether by release of municipal and industrial effluents or as a catchment for agricultural runoff (Bosch *et al.*, 2008). Natural attenuation significantly lowers the concentration of many contaminants by percolating through distinct biogeochemical sedimentary zones (Bosch, Pintó and Abad, 2006). The purification of water by physical, chemical and biological processes has been characterised as a vital ecosystem service (Krauss and Griebler, 2011). Particular concerns are focused on the efficiency of control measures for biological contaminants, specifically because water has been well established as a main vehicle of transmission for pathogens (Bosch *et al.*, 2008).

Riverbank filtration technology is reliant on a functional assurance that subsurface removal processes can guarantee public health risks are adequately mitigated (Anders and Chrysikopoulos, 2005). Evaluating virus specific risks relies on quantifying pathogen travel distance and survival time relative to the impact of contaminant sources such as wastewater or risk scenarios such as storms, floods and droughts (Azadpour-Keeley, Faulkner and Chen, 2003). Various studies have previously reported the abundance of viral pathogens in surface water and groundwater (Bosch *et al.*, 2008; McMinn, Ashbolt and Korajkic, 2017; Pan *et al.*, 2017). Particular viral characteristics have been shown to make their removal by RBF especially difficult (Jin and Flury, 2002; Pinon and Vialette, 2018). For example, virus particles are highly resistant and extremely small allowing easy passage through sediment filters and affording them the capacity for long range transport (Ogorzaly *et al.*, 2010). Since viral particles can remain persistently infective and have the ability to cause infection at low infective doses, it is not surprising that they are also able to reach and infect susceptible hosts (Schijven, Pang and Ying, 2017). In fact, viruses have been linked to many waterborne outbreaks and suspected as the cause for many others (Krauss and Griebler, 2011; Schijven, Pang and Ying, 2017).

Water treatment provides a critical barrier to interrupting the faecal-oral route of pathogen transmission. Removal of pathogens by RBF occurs as a result of natural purification processes which, under favourable conditions, alters the mobility, concentration and infectivity of pathogenic viruses (Yates, Gerba and Kelley, 1985). As such, better systems of viral risk assessment are being developed including the viral indicator concept (Leclerc *et al.*, 2000; McMinn, Ashbolt and Korajkic, 2017; Schijven, Pang and Ying, 2017), viral transport modelling (Schijven and Hassanizadeh, 2000; Tufenkji, 2007), and delineation of reservoir protection zones qualified to site-specific environmental conditions (Schijven *et al.*, 2006; Frohnert *et al.*, 2014). Aquifer vulnerability is tied to the potential for entry of viral contaminants into the drinking water reservoir (Harvey and Ryan, 2004; Krauss and

Griebler, 2011). By default, RBF selects for the most persistent and least adsorbing viruses, as these are most likely to pass filtration processes (Schijven, Pang and Ying, 2017). Upon entry, steady conditions commonly seen within the aquifer itself, such as a water saturated environment, with low oxygen content, low temperatures, and low biological activity, are known to support survival of viral communities leading to fatal consequences for consumers should the aquifers become contaminated (Jin and Flury, 2002; John and Rose, 2005).

The aquatic sediment layers of a riverbank provide protection to adjacent groundwater reservoirs from the flow of exposed surface waters. Fluctuations in the hydrogeological interface between surface water and groundwater catchments can either promote or reduce the effectiveness of viral attenuation. This interface has been recognised as a distinct biogeochemical environment where properties such as temperature, flow velocity, dissolved oxygen, and bioactivity, occur along a gradient from the point of infiltration where hydrodynamic and mechanical processes dominate through dynamic biological and geochemical transitions with increased distance (Tufenkji, Ryan, and Elimelech, 2002). Traditionally, the length of protection zones around groundwater abstractions wells were defined by hydraulic transit times (Taylor *et al.*, 2004; McGuire and McDonnell, 2006). Although for viral hazards to be adequately delineated, reliable assessments must be based off more than just precautionary calculations. Reliance on groundwater reservoirs as a drinking water resource and the use of bank filtration technologies are considered some of the safest approaches to water consumption on earth (Krauss and Griebler, 2011). However, the outlook on water demands, such as increased water abstraction, more frequent and more diverse release of contaminants, and extreme hydrological events linked to climate change, emphasize the importance of elucidating mechanisms that govern viral attenuation; to ensure continued protection and production of safe drinking water into the future.

The goal of this study was to investigate the impact of various environmental conditions on virus transport and survival through aquatic sediments. Porous medium was aquifer sand sampled from Flehe waterworks operating with RBF in Düsseldorf, Germany and experimental conditions were chosen to simulate common subsurface gradients of this location. This study forms part of a larger project consisting of work already done on a meso-scale column experiment run in a pilot plant in Holthausen (Düsseldorf) and macro-scale field work at the waterworks in Flehe. Results are expected to be incorporated into an existing predictive model in relation to the larger project. Further developments in predictive capability will aid in optimizing water treatment methods, refining transport modelling scenarios, and informing viral risk-based analysis during the evaluation of protection measures.

2 Literature Review

2.1 Theoretical framework

2.1.1 Bacteriophage as viral indicators

Viruses are infectious agents with a core of genetic material surrounded by a protein capsid. A wide range of viruses represent significant risk to humans via waterborne contamination. Obvious examples include enteric viruses frequently detected in water sources (McMinn, Ashbolt and Korajkic, 2017), however additional threats are being identified such as those linked to respiratory disease (Cronin and Pedley, 2002), emerging zoonoses (Cotruvo, 2013), or even biowarfare agents (Wade *et al.*, 2010). It is common to employ model organisms, such as non-infectious bacteriophages, as surrogates to study the behaviour of human pathogenic viruses because they have similar properties but do not require additional safety regulations (Schijven, Pang and Ying, 2017). Bacteriophages are viruses that uniquely infect particular bacterial hosts. The bacteriophage MS2 was selected as a surrogate based on its charecterisation as a potential worst-case indicator (Schijven and Hassanizadeh, 2000). MS2 is an obligate lytic, non-enveloped, icosahedral, male-specific, RNA coliphage with a reported diameter of 20-25 nm (Xagoraraki, Yin and Svambayev, 2014). The relatively small size of MS2 and its low isoelectric point of 3.9 (defined as pH where the charge of the virus is zero), (Zerda *et al.*, 1985) allow MS2 to move through aquatic sediments with low adsorption affinity.

Coliform bacteria are well-known indicators of faecal contamination. As such coliphages, a type of bacteriophage that only infects coliform bacteria, are increasingly studied as surrogates for enteric virus transport and survival. Problems with using coliphage as an indicator of faecal contamination are linked to their presence as indicators of coliform bacteria, which are indicators in themselves making detection unreliable. Contradictions include coliphage that can replicate in other forms of *Escherichia coli* not found in faeces and findings showing coliphage presence in waters free of faecal pollution (Leclerc *et al.*, 2000). Although, coliphages are thought to share many morphological and survival characteristics to other pathogenic enteric viruses (Verbyla and Mihelcic, 2015). For example, in comparing MS2 to other enteric viruses, Schijven *et al.* (2003) showed that Coxsackievirus B4 had similar removal rates in laboratory columns of dune sand, although the same pattern was not found with Poliovirus 1. However, the inactivation of MS2 has rates comparable to most human pathogenic viruses in solutions of pH 6-8 and temperatures less than 10°C (Schijven and Hassanizadeh, 2000). Except MS2 is not as resistant as other bacteriophage such as PRD1, which shows longer survival times in oxic field conditions (Schijven *et al.*, 1999) and ΦX174 surviving longer than MS2 under anoxic field conditions (Van Der Wielen, Senden and Medema, 2008). Nevertheless, MS2 is easy to prepare and enumerate, which enhances the accuracy of quantitative measurements, and has garnered widespread use particularly in column and field transport experiments (Schijven and Hassanizadeh, 2000). These factors, combined with the relative stability and weak absorption of MS2, allow conclusions to be made with accuracy, comparability, and functionality towards a worst-case scenario.

2.1.2 **Adsorption and inactivation**

Replication of viruses is restricted by the presence of susceptible host cells, thus in an experimental setting where no hosts are present the concentration of an infectious virus may only decrease with time relative to the conditions present (Pinon and Vialette, 2018). Transport, attenuation or removal mechanisms are often described as advection-dispersion, dilution, straining, reversible and irreversible adsorption, inactivation, and decay. Active viral colloids generally enter aquatic environments either as free viruses suspend in the water column or attach viruses adsorbed to the surface of particles. Viruses are commonly believed to survive better in an adsorbed state than in suspension (Harvey and Ryan, 2004). Adsorption is also known to be reversible, where infectivity may be maintained during attachment and can present additional risks as viruses detach back into solution (Loveland *et al.*, 1996). The extent of retention and survival on sediment is often considered the governing factor in the transport of pathogens in subsurface environments (Bition and Harvey, 1992). Virus mobility was conventionally explored by means of hydrodynamic parameters, including advective-dispersive transport and dilution. With advances in detection and modelling techniques, the complex behaviour of inactivation and attachment-detachment kinetics were added to interpretations of survival and mobility. While hydrodynamic parameters become more significant in large column and field scale studies, it is commonly recognised that inactivation and adsorption are the two most significant processes controlling virus recovery in laboratory experiments (Schijven *et al.*, 2006).

Inactivation is a one-way process by which viruses lose their ability to infect hosts and replicate (Azadpour-Keeley, Faulkner and Chen, 2003). Environmental factors can inactivate viruses by disrupting or degrading part of the viral structure, like the capsid or genome (Pinon and Vialette, 2018). To complicate interpretation, viruses can be inactivated at one rate in bulk solution and at another rate attached to the sediment matrix (Harvey and Ryan, 2004). Since the rate of inactivation for free and attached viruses can be so different, Azadpour-Keeley, Faulkner and Chen (2003) suggest that researchers should clearly distinguish between inactivation rates on sediment surfaces and in solution. However, sampling of attached viruses in transport experiments has typically been limited to spatial variations (Bradford and Bettahar, 2006), giving no insight into the changes of attached virus concentrations with time. In a seminal modelling approach, a sensitivity analysis established that the height of the desorption tail in a breakthrough curve of free active viruses is mainly determined by the desorption rate while the tail slope is substantially affected by altering the inactivation rate of attached viruses (Schijven *et al.*, 1999). However, the inactivation rate of attached viruses is yet to be measured directly. It has been suggested that inactivation rates may even differ with close proximity to a mineral surface during attachment-detachment, when compared to inactivation rates of suspended or attached viruses (Harvey and Ryan, 2004). In fact, the effect of virus adsorption on the rate of inactivation is also not definitive, emphasized by inactivation which can accelerate when attached to some mineral surfaces and be inhibited thus prolonging survival (i.e., preservation) on other surfaces (Pinon and Vialette, 2018). Mechanisms of inactivation specific to the processes occurring at the sediment surface (i.e., surface inactivation) are thought to require strong electrostatic attraction. It is theorized that strongly (i.e., irreversibly) adsorbed viruses are

consequently inactivated as the force is likely sufficient enough to disrupt the viral structure (Harvey and Ryan, 2004). However irreversible adsorption, while conventionally considered as viruses removed from the aquatic system, may be dependent on mechanisms of attachment that are relative to environmental conditions leading to detachment should those conditions change while the virus is still active (Schijven *et al.*, 2006).

In adsorption, transport and attachment are the two sequential steps required to deposit viruses onto grain surfaces. On the small scale and in a non-flowing system, transport is typically described by gravitational sedimentation and Brownian motion (i.e., diffusion), both of which are highly dependent on virus size (Bition and Harvey, 1992). The kinetics of viral attachment are controlled by colloid interaction forces operating at short separation distances between the grain and virus surfaces (Pinon and Vialette, 2018). These include hydrodynamic interactions, hydration (i.e., structural) forces, hydrophobic interactions, electrical double layer and van der Waals interactions, and macromolecular adsorbed layer (i.e. steric) interactions (Jin and Flury, 2002). The sum of the colloidal interactions yields the total interaction energy. The nature of these interactions are driven by three main factors; (1) the surface charge of the virus which is characterised by the isoelectric point derived from ionization of functional groups on the surface of the protein capsid (Verbyla and Mihelcic, 2015); (2) the surface charge density of the aquifer mineral also defined by the isoelectric point derived from isomorphic substitution, amphoteric (i.e., pH and ionic strength dependent) functional groups, and ionized functional groups on adsorbed organic matter surfaces (Harvey and Ryan, 2004); and (3) the chemistry of the solution which depends on pH, ionic strength, ionic composition and dissolved organic matter content (Azadpour-Keeley, Faulkner and Chen, 2003).

2.2 Conceptual framework

2.2.1 Composition of the porous medium

Physicochemical and hydrologic filtration properties are determined by the composition and distribution of sediment grains in the porous medium. The sedimentary profile of aquifer material often includes a major component of primary minerals, such as quartz and feldspar, and minor components of secondary minerals such as clays, metal oxides, and attached amorphous substances like organic matter. The attachment of organic matter onto sediment particles has been shown to change their structural and surface properties (Han, 2008). The presence of organic matter inhibits virus attachment by competing for sorption sites and/or producing hydrophobic virus-particle interactions, thus promoting viral transport (Armanious, 2014). Hydrophobic interactions are recognised as the dominant determinant of attachment during flow through porous media (Xagoraraki, Yin and Svambayev, 2014). The importance of electrostatic interactions, such as hydrophobicity, are particular to the structural organisation of the protein capsid and thus dependent on the virus type (Harvey and Ryan, 2004). Penrod, Olson and Grant (1996) concluded that loops protruding from the MS2 surface played a significant role in steric repulsion inhibiting attachment to quartz sand. In contrast, other bacteriophages like PRD1 have hydrophilic amino acids that dominate the composition of their protein exterior (Harvey and Ryan, 2004).

Electrostatic interaction between particles and viruses are influenced by the variable surface charges that occur between the sediment and the virus capsid as a function of solution pH (Templeton, Andrews and Hofmann, 2008). Changes in the net charge ratio between the virus isoelectric point and solution pH have a controlling factor in adsorption (Banzhaf and Hebig, 2016). The isoelectric point for aquifer minerals ranges between 2-3 for quartz, 5-6 for feldspar (Schulze-Makuch, Guan and Pillai, 2003), and can be as high as 8-9 for ferric (Fe^{3+}) and aluminium (Al^{3+}) oxides (Harvey and Ryan, 2004). While even the most common clay minerals encountered as suspended matter in surface waters, montmorillonite, illite, and chlorite, have isoelectric point's that are difficult to define due to their amphoteric nature (i.e., change relative to solution pH and ionic strength) (Templeton, Andrews and Hofmann, 2008). Despite this, the highly adsorptive potential of clay minerals is well established owing to their extremely high surface area (Gerba *et al.*, 1981). MS2 at pH values of 7-8 is expected to have a net negative surface charge and thus should be repelled from negatively charged matrix surfaces such as pure sand (Zhuang and Jin, 2003). Although, natural sand grains typically have impurities such as metal oxides and clays known to attract negatively charged viruses like MS2. Many studies have attributed virus removal to the existence of metal oxides on the surface of sand grains (e.g., Jin *et al.*, 1997; Zhuang and Jin, 2003; Cao, Tsai and Rusch, 2010), and other researchers have used clay coated grains to preferentially remove viruses from solution (e.g., Tong *et al.*, 2012). Although, the contribution of clays is variable, for example research has shown that the presence of clay particles enhanced transport distance of MS2 and ΦX174 by their cotransport on clay colloids (Syngouna and Chrysikopoulos, 2013) and Babich and Stotzky (1980) showed that the rate of inactivation was greatly reduced for Φ11M15 in river water in the presence of clays.

2.2.2 **Fluid mechanisms and velocity**

Grain and pore size distribution of aquatic sediments are the structural components controlling permeability and filtration efficiency (Tufenkji, Ryan and Elimelech, 2002). Changes in pore water velocity and thus retention time are generated by changes in hydraulic conductivity of the aquatic sediment and the hydraulic gradient between the river and the aquifer (Jaramillo, 2012). Distribution of viruses in a saturated environment are therefore driven primarily by hydrodynamic mechanisms such as advection-dispersion and diffusion (Krauss and Griebler, 2011). It is generally accepted that virus removal rates decrease with increasing hydraulic conductivity, hydraulic loading, and infiltration rates (Sharma *et al.*, 2012). However, due to heterogeneity of the subsurface sediments, hydraulic properties are temporally and spatially variable leading to macro and micro-scale differences in flow velocity (Han, 2008).

The size of a pore space can exhibit different effects depending on the size of the virus and the flow velocity. For example, colloid exclusion effects can be characterised as an inability to access pore spaces by larger colloids relative to the pore throat. Straining effects have been observed on various size dimensions of bacteriophage PRD1 (63 nm) showing increased retardation over smaller viruses like MS2 (Dowd *et al.*, 1998). A separate mechanism based on the same colloid exclusion principle is described by the existence of differential advective velocities in pore spaces used to explain shorter residence times for virus particles when compared to some conservative tracers. It is thought viral colloids are excluded by size from slower streamlines that occur in small pores or at the pore wall,

which impart fluid drag on tracer ions that do disperse into these flow paths (Keller, Sirivithayapakorn and Chrysikopoulos, 2004). Viral particles are thus assisted by the availability of preferential flow paths in the central pore channel resulting in straighter flow or larger pores with higher advective velocity (Keller, Sirivithayapakorn and Chrysikopoulos, 2004). For example, Schulze-Makuch, Guan and Pillai (2003) observed enhanced MS2 transport in relation to bromide, while Frohnert *et al.* (2014) observed earlier arrivals of MS2 peaks compared to sodium and chloride ions. The relative size of tracer ions also makes them more likely to disperse into smaller dead-end pore spaces, dependent on the effective porosity and fine-grained particle content. Thus, higher velocity generally increases the speed of preferential flow paths that can assist colloid transport or depending on the colloid to ion size and effective porosity, may enhance straining effects.

In modelling done by Han (2008), a decrease in fluid flow lead to a significant increase in colloid deposition on grain surfaces. This was explained by mechanisms of Brownian motion and reduced fluid drag, where lowering fluid velocities resulted in longer residence times which subsequently increased contact efficiency with grains and thus removal by adsorption (Han, 2008). Another mechanism most likely to occur at low groundwater velocities is settling or sedimentation in pores, determined by Stoke's settling velocity, which depends on the mass density difference between the particle, the fluid, gravity force and the inverse of the fluid viscosity (Jaramillo, 2012). Hydraulic conductivity decreases as tortuosity increases, in such cases survival is often assumed to simply decrease as a function of time (Azadpour-Keeley, Faulkner and Chen, 2003). Longer residence times under slower flow velocities also allow for longer exposure to developing biogeochemical conditions which influence survivability (Verbyla and Mihelcic, 2015). Hydraulic conductivity is a function of sediment permeability and properties like fluid viscosity, which have a relationship to climatic effects such as temperature and rainfall (Jaramillo, 2012). Temperature was observed to have a relative importance on the enhanced retention of latex nanoparticles as a function of pore water velocity, where Sasidharan *et al.*, (2017) showed that as temperature increased the percentage of the sand surface area that contributed to attachment also increased, but attachment was greater when the pore water velocity was lower. However, the conclusions from both Han (2008) and Sasidharan *et al.*, (2017) are based on modelling from recovery of solution concentrations. The effect of decreasing flow velocity on attached active viruses remains to be tested on sediment sampled directly.

2.2.3 **Viral load and occurrence**

Water temperature has been correlated to the seasonal distribution of pathogens detected in aquatic systems, and also linked to the seasonal occurrence of waterborne outbreaks (Bosch, Pintó and Abad, 2006). The load of enteric viruses released into the environment is often contingent on the prevalence of infected patients and their shedding rate (Krauss and Griebler, 2011). The disturbance of riverbeds by recreational water activities such boating or by the movement of wildlife can also lead to an increase in viral loads by resuspending sediments and physically desorbing attached viruses potentially moving them back into the water column. Variations in pathogen concentrations can also be linked to spills, leaks, and runoffs, all of which are exacerbated by climatic events such as irregular storms and flooding. Studies commonly report faecal pollution in urban stormwater and watersheds, with high storm-loading rates of faecal indicator bacteria (Surbeck *et al.*, 2006). Although, flooding

and rainfall can influence virus concentrations both as a transport mechanism and by dilution. It has been suggested that fluctuations in the viral load combined with the hydrogeological setting through which they filter will control the potential for viral transport to a much greater extent than the survival of the pathogen itself (Azadpour-Keeley, Faulkner and Chen, 2003). However, if the viral load is high enough, the probability of contaminant migration is increased regardless of the hydrogeological setting (Pang, 2009). This mechanism could be explained by repulsive colloid-colloid interactions, such as blocking by previously deposited particles (Bradford and Bettahar, 2006). Although, higher concentrations also lead to a higher collision frequency among virus particles, where virus aggregation is known to enhance survival (Galasso and Sharp, 1965). In fact, researchers often consider the majority of viruses suspended in water to exist in an aggregated state (Pinon and Vialette, 2018).

A review that compiled pathogenic viral occurrence in dewatered sludge and biosolids, showed that across many studies, Adenovirus, Enterovirus and Norovirus were frequently detected at ~10^4-10^5 copies g^{-1} (Xagoraraki, Yin and Svambayev, 2014). In the format of a solid matrix like biosolids these low amounts are measured as a concentrate of wastewater, suggesting that numbers could be much lower. Smaller viral loads are not likely to overwhelm adsorption spaces like higher concentrations would. However, in calculating sample recovery of low viral loads, while the total number of adsorbed viruses could be the same as would occur from higher viral loads, in relative terms smaller concentrations may lead to a higher overall percentage of adsorption. Concerns are raised with particle-associated viruses because, while conventionally seen as being removed from the aquatic system, they have been found to maintain activity longer during adsorption than in solution thus forming an infectious reservoir (Templeton, Andrews and Hofmann, 2008). With smaller viral loads, key drivers like the length of the flow path may be more important to consider. For example, virus removal can be non-linear with distance, in which concentrations decrease most strongly during the early distance of infiltration (Kvitsand, Ilyas and Østerhus, 2015; Betancourt *et al.*, 2019), while a small portion of the viral population have been shown to continue to transport without further decrease (Blanford *et al.*, 2005). For low viral loads it is also important to consider the structural features of adsorptive grains. For example, research has shown that some grain sizes and grain types can completely shield viruses from inactivating mechanisms such as UV disinfection but this effect is limited to loads that do not exceed the grain structure (Templeton, Andrews and Hofmann, 2008).

2.2.4 **Dissolved oxygen and redox reactions**

Molecular oxygen has a limited solubility in water as described by Henry's law, directly related to atmospheric pressure and inversely related to water temperature and salinity. The reactive molecules and free radicals derived from molecular oxygen in aerobic systems are collectively known as reactive oxygen species and have been well established as a mechanism of microbial inactivation. This is commonly cited as an explanation for increased virus removal and inactivation rates recorded under oxic conditions when compared to the absence of oxygen (Jin and Flury, 2002). The proposed mode of action is thought to be capsid oxidation (Xagoraraki, Yin and Svambayev, 2014), while other authors have noted that lipids from enveloped viruses like influenza can be disrupted by hydroxyl radicals (Pinon and Vialette, 2018). Dissolved oxygen (DO) is taken up by the exposure of surface

waters to the atmosphere and/or by phototrophic activity relative to exposure of sunlight. Groundwater aeration occurs by entrapment of oxygenated surface waters during bank filtration. Dissolved oxygen variations are correlated with hydrogeological and geochemical boundary conditions, where the oxic-anoxic interface moves according to seasonal variations, aquifer aging, fluctuations in the water table, and the hydraulic connection between riverbanks and groundwater (Tufenkji, Ryan and Elimelech, 2002).

Reduction/oxidation (i.e., redox) processes affect the chemical quality of groundwater by regulating the concentration and behaviour of metals and other organic and inorganic compounds in solution. Redox processes are also dependent on pH, temperature, and decomposition of organic compounds (Jaramillo, 2012). In anoxic sediment, metals are often solubilised but in oxic sediments, iron and other metals are present in their oxidised form as precipitates such as ferric oxyhydroxides. Increased virus removal and inactivation rates recorded under oxic conditions have been linked to the presence of ferric oxyhydroxides, thought to play a significant role in virus adsorption due to the negative surface charge and surface complexation kinetics (Zhang *et al.*, 2010a). Under strong changes in redox-potential and pH values, the accumulation of precipitates can also limit viral transport by clogging of aquifer pores (Jaramillo, 2012). In addition to the lower inactivation rates that occur in the absence of reactive oxygen species, solubilised metals leave fewer attachment sites, both contributing to a general trend that sees higher recovery of viruses under anoxic conditions (e.g., Schijven and Hassanizadeh, 2000; Van Der Wielen, Senden and Medema, 2008; Frohnert *et al.*, 2014). Consequently, researchers have suggested extending microbial protection zones around anoxic aquifers to account for longer virus transport ranges and survival rates in the field (Van Der Wielen, Senden and Medema, 2008).

Conclusions on viral transport and survival are often based on the distinct occurrence of either oxic or anoxic conditions, however the impact of transitional hypoxic processes on virus attenuation is as yet undefined. In the absence of a balanced equilibrium, low DO concentrations will allow simultaneous reduction and oxidation processes resulting in mass transfer limitations for DO diffusion and utilization, the partial generation of precipitated intermediate products, and the accumulation and partial hydrolysis of soluble residual metals (Rose and Long, 1988). Such dynamic biogeochemical environments may promote unique removal processes not seen in either oxic or anoxic conditions. Redox processes in subsurface environments are largely catalysed by microbial metabolism which typically drive the rapid oxygen consumption. As oxidised recharge waters reach the groundwater table they become isolated from the atmosphere and DO tends to deplete along the flow path associated with the transition from aerobic to anerobic activity of the microbial community (Jaramillo, 2012). Viruses are neither aerobic nor anaerobic since they have a host-dependent metabolism. However, studies have shown infection rates of viruses tend to vary in response to hypoxia, for example via upregulation in the reproduction of Hepatitis C virus or downregulation of E1A Adenovirus type 5 (Morinet *et al.*, 2015). *E. coli*, the host of MS2, is classified as a facultative anaerobe however no specific study could be found that addressed the oxidative preferences of MS2.

2.2.5 **Microbial antagonism and subsurface ecology**

Biological processes are directly dependent on the type of microbes present, while the average metabolic activity of microbial communities largely determines the final quality of filtered water (Jaramillo, 2012). Subsurface communities vary widely in distribution, activity, and composition, reflective of gradients in temperature, pH, DO, nutrients and organic matter content (Tufenkji, Ryan and Elimelech, 2002). Since the interface between surface water and groundwater has the greatest regulation on nutrients, organisms, and energy, this zone is often characterised by intense bioactivity (Krauss and Griebler, 2011). At a certain distance from the river, microbial activity substantially diminishes as a response to depleted electron donors, also responsible for the sequence of redox reactions (Tufenkji, Ryan and Elimelech, 2002). The effects of autochthonous communities on viral contaminants can be explored by considering both direct and indirect influences, some of which can be interpreted as inhibitory to viral particles while others may be preservatory.

The most common evidence for the influence of indigenous microbes comes from the increased viral decay frequently observed under natural, unaltered waters when compared to high virus recovery from sterile water and sediments (Xagoraraki, Yin and Svambayev, 2014). Although some published results show inconsistencies, for example one study found no significant differences in bacteriophage survival in natural, autoclaved, or filtered lake waters (Babich and Stotzky, 1980), and another study reported slightly higher inactivation rates in sterile groundwater (Alvarez *et al.*, 2000). Reasons for these variations may be due to the co-regulatory effects between favourable conditions for indigenous microbes and their impact on virus removal. For example, researchers found that the effects of temperature, oxygen and nutrient levels were only significant for virus decay in the presence of native groundwater organisms, showing no significant influence when they were absent (Gordon and Toze, 2003). There also appears to be differences between the effects of physiological guilds, for example indigenous microbes in aerobic soil were shown to significantly reduce virus survival although indigenous anaerobes did not (Hurst, 1988).

Various antagonistic mechanisms are recognised between autochthonous microbes and introduced pathogens in aquatic environments that may play an important role in virus removal. Direct antagonisms include the grazing of viruses by protozoa similar to the predation seen by heterotrophic flagellates capable of consuming and digesting MS2 (Deng *et al.*, 2014). However, internalisation of viruses has also been shown to protect them from inactivation, with another study observing human adenoviruses taken up by ciliates in wastewater able to persist up to 35 days (Battistini *et al.*, 2013). Conversely, indirect antagonisms like the production of antiviral substances may also influence virus survival. The production of proteolytic enzymes capable of destroying the viral capsid has been suggested as a mechanism of inactivation, observed from some bacteria and protozoa (Azadpour-Keeley, Faulkner and Chen, 2003). Metabolic and enzymatic activity may also increase or decrease relative to temperature and DO, both of which are also factors directly influencing virus survival.

Indirect removal mechanisms could be related to viral adhesion, like adsorption but to organic particles such as suspended bacteria or biofilms (Pinon and Vialette, 2018). Enmeshment within suspended biogranules, such as amoeba cysts, fungal spores, bacterial biofilms or even seeds (Pinon and Vialette, 2018), could be followed by sedimentation which also removes viruses from solution

(Liu, Liu and Tay, 2004; Templeton, Andrews and Hofmann, 2008). While these mechanisms contribute to the immobilisation of viral particles, the potential for reversibility and thus resuspension back into the water column under changing conditions such as temperature or DO has also been observed (Liu, Liu and Tay, 2004; Verbyla and Mihelcic, 2015). Bio-clogging can also reduce transport by accumulation of excessive biomass in the form of plugs restricting the water flow (Jaramillo, 2012). Although viral transport may also be promoted by uniform biofilms covering adsorptive grain surfaces that would otherwise remove viruses from solution. The association with particles, whether adsorbed or inside bio-structures has also been shown to reduce vulnerability to sunlight inactivation and protect viruses from disinfection by chlorination (Templeton, Andrews and Hofmann, 2008; Verbyla and Mihelcic, 2015), and may even act as a shield from biological degradation by free nucleases and proteases (Bradford, Schijven and Harter, 2015).Regardless, conflicting results on the topic of viral attenuation relative to bioactivity may remain unresolved since microbial communities in laboratory and especially field experiments are entirely unstandardised, and tend not to be explicitly characterised in research studies.

3 Research Significance & Objectives

Despite increasing research on virus transport and survival during passage through aquatic sediments, studies routinely make conclusions based on detecting infectious, free-floating viral agents, recovered in the effluent alone. Very few studies have combined virus detection methods to explore the proportion of infectious and non-infectious viruses (e.g., Lodder *et al.*, 2013; Deng *et al.*, 2014; Kokkinos *et al.*, 2015). Mechanisms responsible for inactivation of free viruses are traditionally interpreted by performing batch experiments, often run in parallel with column transport experiments. It has been found that free viruses typically exhibit such low decreases of concentration in batch studies, that authors tend to assume this means little to no inactivation of free viruses also occurs in the porewater of sediment columns (John and Rose, 2005; Pinon and Vialette, 2018). Mechanisms involved in viral infectivity, persistence, and elimination (i.e., decay beyond detection levels) become more complex under porewater conditions due to the interaction between sediment interfaces and complicated flow paths. A true representation of how these mechanisms change with time inside sediment columns during transport is yet to be determined.

Though previous studies have provided extensive information on the factors affecting transport and survival of free viruses, many of the interpretations on how free viruses interact with sediment surfaces are based on detecting changes in the concentration of viruses in solution rather than sampling sediment directly. Often researchers assume that a decrease in free virus recovery in the effluent of transport experiments, which cannot be accounted for by corresponding inactivation of free viruses (i.e., batch experiments), must be attributed to adsorption. This inhibits the capacity to distinguish between reversible adsorption (i.e., desorption) and true elimination. Furthermore, studies make assumptions on the rate of desorption and inactivation for attached viruses based on the modelling of free virus recovery alone. This means mechanisms responsible for adsorption, desorption, and inactivation of attached viruses are described without confirming that viruses have indeed become particle associated. Researchers that do sample sediment often analyse relative to spatial variance in columns following termination of a transport experiment (e.g., Bradford and Bettahar, 2006) due to the difficulty of sampling during flow-through activity. To this authors knowledge, there are no reported studies showing the temporal variation of active viruses attached to sediment.

The movement of pathogenic viruses through subsurface environments and into groundwater catchments is dependent on fluctuating properties of the hydrological and biogeochemical interface, capable of either promoting or reducing the effectiveness of viral attenuation. Studies normally set flow velocity as a secondary feature of transport experiments, to between 1-1.5 m d^{-1} (e.g., 1 m d^{-1}: Frohnert *et al.*, 2014, 1.4 m d^{-1}: Keller, Sirivithayapakorn and Chrysikopoulos, 2004), rather than testing it as a primary variable. Researchers that specifically investigated flow velocity focused on the effects of rapid groundwater flow (Taylor *et al.*, 2004) and velocities from 1 m d^{-1} upwards (Han, 2008), with limited information available on the effects of slower flow conditions (e.g., 0.1m d^{-1} (Sasidharan *et al.*, 2017) ; 0.33-0.56 m d^{-1}: Van Der Wielen, Senden and Medema, 2008). Viral load in experimental settings is also a secondary feature of most transport experiments. Model viruses are often inoculated in high concentrations (~10^{7-9} particles mL^{-1}) to maintain detectability throughout

experiments, however pathogenic viruses tend to occur in the environment in smaller loads. It has been found that higher viral loads can overwhelm adsorption spaces (Pang, 2009), although processes involved in viral colloid interactions and adsorption using more natural concentrations rather than ideal experimental systems are yet to be determined.

Other key features of the biogeochemical interface are variations in dissolved oxygen (DO) and bioactivity, both of which are under-represented in the literature. When experimenting with DO, it is difficult to control concentrations that are below ambient levels, as such many studies have focused on experiments performed under oxic conditions. Although, John and Rose (2005) noted that laboratory experiments in ambient DO levels are unintentionally using concentrations that exceed natural aquifer conditions. Previous studies have been mainly focused on the behaviour of viruses in either oxic or anoxic conditions, while knowledge on the transport and survival of viruses through the transitional hypoxic zone is very limited. In fact, no specific study could be found that performed a transport experiment under hypoxic conditions. Large subsurface areas generally become naturally depleted in oxygen due to the action of highly bioactive zones. However, as mentioned earlier, the importance of autochthonous bioactivity can be incredibly varied, suggested as a feature capable of both enhancing and/or inhibiting virus transport and survival. Microbial activity commonly occurs near the sediment surface as substrates can support more extensive growth, although knowledge on adsorption and inactivation of attached viruses in the presence and absence of sediment bioactivity remains to be investigated directly.

Experiments in this study simulated various environmental conditions that commonly occur during passage through aquatic sediments, namely variations in; (i) flow velocity including standard flow and slow flow; (ii) viral load including low, medium, and high input concentrations; (iii) concentrations of DO including anoxic, hypoxic, and oxic conditions; and (iv) sediment microbial bioactivity including sterile conditions, nutrient stimulated bioactivity, and background sediment bioactivity typical for an oligotrophic aquifer. Objectives were to explore:

- → the mechanisms of infectivity, persistence, and elimination for free viruses in porewater during passage through aquatic sediments
- → the charecterisation of inactivation rates for both free and attached viruses
- → and the partitioning of viruses between the reversibly adsorbed (i.e., desorbed active viruses), irreversibly adsorbed (i.e., attached active viruses) and inactivated states.

These objectives were achieved by uniquely combining viral detection methods and novel sampling techniques. To detect free viruses in effluent, molecular analyses provided the enumeration of total viral genome copies and viral assays selecting for infectious agents. To address the need for direct measurements of attached viruses, the use of replicate columns terminated sequentially over the experiment with the addition of elution techniques suggested by Hofmann, Grösbacher and Griebler (2016), were used to examine the temporal variation of viruses retained in columns. This also allowed for investigation of a claim that has generated widespread indirect interpretations on the inactivation of attached viruses, based on the results of an early modelling prediction by Schijven *et al.*, (1999).

4 Methods & Materials

4.1 Experimental Framework

4.1.1 Experimental design

A schematic layout for all sediment column experiments is shown in Figure 1a, while Figure 1b shows adjustments made to enclose the environment for oxygen control. Four experiments were conducted to investigate deviations in virus transport and survival as a result of changes in the following variables: (Exp.1) flow velocity, (Exp.2) viral load, (Exp.3) dissolved oxygen (DO) concentration, and (Exp.4) sediment bioactivity, with multiple conditions tested for each variable (see Section 4.1.7). All experiments were performed in a 12°C incubator, with eight replicate sediment-filled columns run in parallel under complete saturation, connected to a reservoir and infiltrated for a duration of two-weeks by a multi-channel peristaltic pump. All experiments experienced two water phases (i.e., pulse injection), an initial infiltration period using virus-tracer inoculated water (Phase 1) followed by a desorption period using virus-free water (Phase 2). Both sediment columns and reservoirs were stored inside the same incubator for the duration of the experiment where the darkness and controlled temperature mimicked aquifer conditions. This was also expected to alleviate temperature and UV dependent inactivation of viruses (John and Rose, 2005). Experimental conditions and column properties are summarised in Table 1. The porous medium was aquifer sand sampled from Düsseldorf-Flehe waterworks in Germany. The grain size fraction of medium sand (0.25–0.5 mm) was used. The sediment was visibly uniform with a low content of fine-grained fractions or organic carbon. The sample was selected for particular interest in relating results back to the RBF site of the same location. Sediment was stored under saturated conditions in tap water (pH 7-8.3) and mixed to ensure homogeneity prior to packing all columns.

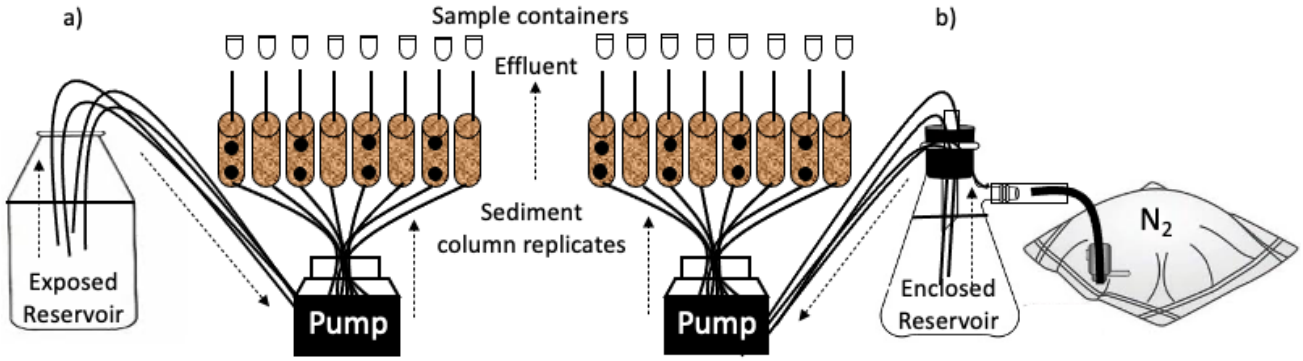


Figure 1. (a) General experimental design for all oxic column experiments. (b) Adjustments made to control oxygen for experiments performed under altered oxygen conditions. Black dots show oxygen sensitive foil spots.

Columns were made of glass with a length and diameter of 10.7cm and 1.3cm, respectively. In order to maintain saturation, water was pumped continuously through the columns from bottom to top. Volumetric flow rate was adjusted to 3 mL min^{-1}, corresponding to a water flux of 1 m d^{-1} calculated from the experimental Darcy velocity (q). This was similar to the aquifer mean flow velocity and set for all experiments, except those which had reduced flow velocity as a primary variable. Both ends were sealed by a mesh wire screen held in place with rubber stoppers which were punctured by a steel capillary for flowthrough capacity. The screen was used to distribute water flow evenly over the

entrance/exit area and limit clogging of capillaries. Columns were packed incrementally under saturated conditions, filled free of headspace with new sediment for each experiment and taking care to achieve dense compaction and a lack of trapped air bubbles. Packed columns had a total porosity of 39%. Steel capillaries were used to connect the reservoir to the columns, interspersed by a set of flexible Teflon tubing set through the pump and at capillary joints. Previous studies have demonstrated that the choice of glass columns and Teflon tubing avoids pronounced viral adsorption or inactivation in the experimental setup (Jin *et al.*, 1997). After packing, columns were flushed overnight in the incubator to establish a steady-state flow and homogenous background conditions prior to each experiment. Background samples confirmed no viruses were present before conducting experiments. The mobile water volume (V_m) was estimated by the following equation, $V_m = M_w - M_d$. Total porosity was estimated by the following equation, $\theta = 1 - (\rho_b/\rho_d)$. The typical mineral particle density (ρ_d) of sand is listed in Table 1. Bulk density (ρ_b) was estimated by the following equation, $\rho_b = (M_d/V_t)$.

Table 1. Summary of experimental conditions and column properties. Reference condition (Rf.)

Experiment		Experimental conditions				Sand & Column properties			
		Temp.	Darcy velocity		Infiltration time	Particle density	ρ_d	2.66	g cm^3
		°C	m d^{-1}	(q) cm min^{-1}	(t_{inf}) min	Bulk density	ρ_b	1.63	g cm^3
1a	Standard flow velocity (Rf)	12	1	0.069	232	Length	x	10.66	cm
1b	Slow flow velocity	12	0.5	0.047	464	Diameter	$\emptyset$	1.34	cm
2a	Low viral load	12	1	0.069	232	Area	A	1.37	cm²
2b	Medium viral load	12	1	0.069	232	Dry weight	M_d	27.58	g
3a	Anoxic condition	12	1	0.069	232	Wet weight	M_w	35.19	g
3b	Hypoxic condition	12	1	0.069	232	Total volume	V_t	16.92	mL
4a	Sterile condition	12	1	0.069	232	Mobile volume	V_m	7.61	mL
4b	Highly bioactive condition	12	1	0.069	232	Total porosity	θ	39	%

4.1.2 **Reservoir preparation and sampling**

All experiments used tap water as a model for groundwater, except those which had a specific variable of sediment bioactivity where water sampled from the Danube Channel in Vienna was used instead. Reservoirs were inoculated with the virus and tracer 1 h prior to each experiment. The virus-tracer solution entered columns simultaneously. Viruses were suspended at a standard viral load of C_0 = ~10^7 plaque forming units per mL for all experiments, except those which had a specific variable of viral load. Virus-tracer inoculations were diluted in reservoirs that were set to the conditions of the column experiment. The tracer stock solution was given time to reach room temperature before inoculation in order to support dilution, however some tracer residue was never fully ejected from the pipette tip. Aside from the virus-tracer inoculation, the water composition and all other experimental parameters were kept the same during both phases. Water and sediment samples were collected manually. The water sampling regime was calculated to account for a fast transport (every 16 min) and plateau period (every 32 min) during Phase 1. Followed by a desorption period (every 64 min until 12 h after inoculation, at 25 h, 1 week and 2 weeks) making up Phase 2. The sediment sampling regime sacrificed two column replicates at the beginning of Phase 2 and again at each subsequent sampling interval. Virus inactivation was also monitored throughout the experiment by sampling the reservoir at inoculation to estimate the input concentration (C_0) at the beginning of Phase 2 and each subsequent sampling interval. All samples were stored at 4°C immediately after

collection and assayed within 3-5 days. Following assays, all samples were stored at -20°C and selected water samples were analysed by molecular techniques within 3 months.

4.1.3 **Conservative tracer**

Fluorescein was added to each reservoir at 2.2 mg L^{-1} and analysed in the column effluent using a BioSystems Modulus Microplate Fluorometer. Briefly, 50 µL of all water samples, for all columns and time intervals on the first day of the experiment were transferred to a 96-well plate under protection from light within 2 h of the final sample. Relative Fluorophore Units (RFU) were converted to fluorescein concentration values by means of a standard curve. The standard curve was prepared by a 10-fold serial dilution of the tracer stock solution with tap water. A new standard curve was prepared for every plate with wells filled in triplicate. Experimental values were means across all columns (eight replicates), per time interval.

4.1.4 **Virus stock production**

Bacteriophage MS2 was used as a model virus with *E. coli 5965* as the host. Phage propagation was prepared with a working culture of Lysogeny broth (LB) using *E. coli* at exponential growth phase, incubated together overnight. A value of 0.3-0.4 optical density at 650nm wavelength (OD_{650}) was calculated for the host bacterium strain to ensure phage inoculations were 10-fold less for optimal replication. Phage purification involved centrifugation (4000 rpm, 4°C, 20 min) and filtration (0.22 µm Millipore filter) to eliminate bacterial cells. The purified phage suspension was precipitated overnight at 4°C with a 1:4 solution to suspension of polyethylene glycol 8000 (20% PEG 8000, 2.5 M NaCl). The precipitated solution was centrifuged again (4000 rpm, 4°C for 30 min) and the virus pellet resuspended in 10-20 mL of filtered tap water (0.22 µm Millipore filter). Viral stock was maintained in tap water to ensure equilibration of the virus to the reservoir solution and to avoid changes in water chemistry when inoculating. Working cultures of *E. coli* were only used in exponential growth phase. New bacterial and viral stocks were prepared for each experiment. Bacterial working cultures were prepared by inoculating LB liquid medium with a colony from an LB plate. Virus inoculations were carried over from previous stock solutions. Incubation of all solutions were overnight at 37°C on a shaker set to 160 rpm. Viral stocks were stored at 4°C, considered favourable for long-term survival (John and Rose, 2005).

4.1.5 **Virus detection methods**

4.1.5.1 Virus assay

Active MS2 concentrations were detected by the double-agar layer method and counted as plaque forming units (pfu). Host working culture (0.5 mL) and phage sample (0.5mL sample or dilution) were combined to form a bacterium-sample solution which was vortexed in a 1:5 solution to molten LB Agar top layer (25 g mL^{-1} LB and 7.5 g L^{-1} Agar) maintained at 40°C. The mixed solution-top layer was poured onto a solid medium bottom layer of 6 mL LB Agar in a Petri dish. After plates had solidified, they were incubated at 37°C for 12 h. Enumeration was achieved by counting virus plaques visible in the host bacterium. Plaques are directly correlated with the number of viable virus particles and can be back-calculated to the original sample (pfu mL^{-1}). When necessary, virus samples were serially diluted (1:10) in a phosphate buffered saline solution (PBS; 0.137 M NaCl, 0.01 M Na_2HPO_4, 0.0027

M KCl and pH 7.4) to obtain between 10 and 200 pfu per plate. Detection was limited to 10 pfu mL^{-1} and only plates in the countable range were accepted for quantification. Sediment samples were pre-treated to re-suspend absorbed viruses from the sediment matrix. Absorbed viruses were extracted from each column by taking 1 mL of homogenised wet sediment sample using a sterile syringe (1 mL = 2.1 g wet weight). The sediment sample was vortexed in 2 mL of a 3% beef extract solution, proven to effectively detach viruses adsorbed to different surfaces (Jin *et al.*, 1997; You *et al.*, 2003). Sediment-virus suspensions were diluted in PBS to obtain the countable range and analysed by the plaque assay method. Effluent samples were plated from four columns chosen randomly per time interval and reservoir samples were taken directly from the reservoir water. Sediment samples were plated for both columns sacrificed at each sampling interval. All samples were plated in duplicate and mean values were calculated for columns at each time interval. All plates were incubated overnight at 37°C. Duplicates with anomalous values were retested the following day.

4.1.5.2 Viral RNA

The concentration of total viral particles in water samples were determined by quantitative real-time polymerase chain reaction (qPCR). All conditions were analysed, except the experiment performed using the lowest viral load which had values too low to be accurately detected (< 10^2-10^4 particles mL^{-1}). Eight time-intervals were selected across the experimental duration, half from Phase 1 on the incline of the peak and half in Phase 2 on the decline. Samples in Phase 2 were only tested up to the Week 1 interval. Week 2 was investigated in early extractions but demonstrated insufficient results at or below detection limits. Samples were extracted from two columns of the same time interval, as duplicates. Only columns with values within the mean range were chosen for extraction, outliers were avoided. Samples were also extracted from the reservoir at the start of the experiment to obtain the value for the input concentration (C_0) of total viral particle in all conditions.

Viral RNA was extracted from 200 µL of water sample using the Qaigen Allprep PowerViral DNA/RNA Kit according to the manufacturer's instructions. Viral RNA was converted to cDNA by using 2 µL of the 50 µL RNA extract and the Finnzymes DyNAmo cDNA Synthesis Kit according to the manufacturer's instructions. Viral genomes were enumerated by taking 2 µL of the cDNA solution processed with the Stratagene Brilliant II QPCR Master Mix and Reference Dye according to the manufacturer's instructions. The following MS2 primer sequences were included, forward primer MS2-TM3-F (GGCTGCTCGCGGATACCC) and reverse primer MS2-TM3-R (TGAGGGAATGTGGGAACCG). The qPCR assays were performed in a Light-Cycler480 System following a Three Step Cycle protocol for 40 cycles (DNA polymerase activation at 95°C for 10 min, denaturation at 95°C for 15 s, annealing at 50°C for 30 s, polymerisation at 72°C for 30 s). In addition to the recommended quality assurance samples for cDNA synthesis and qPCR analyses, RNase free water was used as a negative control alongside two internal positive controls of known high and low concentration RNA samples. The LightCycler software automatically determined the cycle threshold point of each qPCR reaction. Detection of one copy of viral genome corresponded to one phage particle and was calculated to determine RNA genome copies mL^{-1} of the original sample. Detection limits ranged between ~10^2-10^4 RNA genome copies. The software calculated mean viral copy numbers by use of a standard curve. New standard curves were made per cycle. Data were only

obtained from standard curves with an efficiency above 85% and error below 0.02. All samples were added to the 96-well plate in triplicate and the of mean viral copy numbers between well triplicates and column duplicates was used.

4.1.6 **Monitoring parameters**

Four columns per experiment were fitted with two spots of oxygen sensitive foil, attached to the inside of the glass column by epoxy glue, at a distance of 2 cm from the entrance of either end. The spots allowed for non-invasive measurements of DO concentrations inside columns at selected time interval. A PreSens OXY-4 Minisensor device was used to read the DO concentrations from outside the column with a detection limit of 0.01 mg L^{-1}. Oxygen monitoring was conducted for all experiments, except those which had a specific variable exploring variations of DO concentration which were monitored more frequently and with more sensitive detection methods. Two oxygen values were taken per column, with values averaged. Mean values did not vary between the two sensors of one column by more than 1 mg L^{-1}. Water chemistry was monitored for both inflow and outflow of all experiments, taken at each sediment sampling interval. Ion chromatography was used to measure concentrations of sodium, potassium, calcium, magnesium, chloride, sulfate and nitrate, while photometry was used to measure concentrations of ammonium and phosphate. Standard international guidelines for the examination of water for chemical substances were used, including ISO-14911, ISO-7150-1, ISO-10304-1, and ISO-6878.

4.1.7 **Experimental conditions and treatment processes**

4.1.7.1 Variations in flow velocity

The first experiment (Exp.1) investigated variations in flow velocity, with two experimental conditions of a standard flow velocity (Exp.1a) set to 1 m d^{-1} and a slow flow velocity (Exp.1b) set to 0.5 m d^{-1}. These were tested by having discrete pumps for each set of columns. Both conditions used the same experimental reservoir, inoculated with the standard viral input concentration ($C_0 = 10^7$pfu mL^{-1}). All other conditions were the same. Since Exp.1a had the same flow velocity and viral load as all other experiments, this experiment became the Reference Condition (Rf.) The Reference Condition (Exp.1a) was expected to produce results on virus transport and survival in the experimental setup prior to any altered variables (i.e., standard experimental conditions; standard flow velocity, high viral load, an oxygenated environment, and with background sediment bioactivity typical for an oligotrophic aquifer with these conditions). As such these results acted as the baseline, comparable for changes to a null hypothesis.

4.1.7.2 Variations in viral load

The second experiment (Exp.2) explored variations in viral load using discrete reservoirs per condition. One condition of a low viral load (Exp.2a) was tested by inoculating the reservoir with a viral input concentration $C_0 = 10^4$ pfu mL^{-1} and another condition of medium viral load (Exp.2b) was tested by inoculating the reservoir with a viral input concentration $C_0 = 10^5$ pfu mL^{-1}. All other conditions were the same.

4.1.7.3 Variations in dissolved oxygen concentration

The third experiment (Exp.3) investigated variations of DO concentration. The first condition was performed in the absence of oxygen (anoxic, Exp.3a) and the second performed using low DO concentrations (Exp.3b) to simulate a hypoxic environment (< 2 mg L^{-1}). These conditions were established by altering the experimental setup to protect the flow-through environment from exposure to atmospheric oxygen according to Figure 1b. To eliminate oxygen from the reservoir, the water for both treatments was heated in a bath to 80°C then purged with high purity nitrogen gas (99.999% N_2) and sealed with butyl rubber stoppers. The reservoir of Exp.3b performed under hypoxic conditions underwent no further treatment. To further eliminate residual oxygen for the anoxic treatment of Exp.3a, 25 mg L^{-1} of sodium dithionite ($Na_2S_2O_4$) was added to the reservoir as a reducing agent. All tubing material was tested to avoid an influx of oxygen and reservoirs were changed on a daily basis to ensure that targeted DO concentrations were maintained throughout the experiment. Three-way valves were connected between reservoirs and sediment columns to maintain water-pressure and DO conditions during changeovers. Reservoirs were supplemented with an artificial nitrogen atmosphere by a gas filled plastic bag that was used to equalize the oxygen-free pressure gradient that built up as pumps drew solution from the reservoir (Figure 1b).

Sediment columns were conditioned overnight with their respective reservoir solutions. Dissolved oxygen concentrations were monitored daily throughout the experiment by means of the PreSens. For Exp.3a performed under anoxic conditions, DO concentrations below the limit of detection were additionally confirmed using Resazurin ($C_{12}H_7NO_4$), a redox sensitive dye indicating an oxygen-free solution, on subsampled column effluent and reservoir samples. The inoculations were added to the reservoirs after the conditions had been established and the solution had cooled to the temperature of the incubator. To avoid introducing oxygen, low volumes of concentrated stock solutions (virus, tracer, and reducing agent) were introduced using syringes flushed with nitrogen gas and injected by piercing needles through the rubber stoppers. Sample effluent exited the columns into atmospheric oxygen and were stored under ambient conditions, only passage from the reservoir through the sediment columns was affected by adjustments of DO concentration. Virus inactivation in the experimental solutions was investigated in a preliminary study, using a hypoxic and anoxic batch experiment with samples taken periodically over 2 days. The preliminary study demonstrated the need to inoculate Exp.3a performed under anoxic conditions with a viral input concentration of C_0 = 10^8 pfu mL^{-1} 2 h before the experiment to compensate for an early die-off period in order to reach the standard input concentration before starting the experiment. However, unstable survivability in the preliminary study demonstrated the need to inoculate Exp.3b performed under hypoxic conditions with a viral input concentration of C_0 = 10^9 pfu mL^{-1} 30 h prior to the experiment, where viral input concentration was monitored periodically to confirm the standard input concentration had been reached before starting the experiment.

4.1.7.4 Variations in bioactivity

The fourth experiment (Exp.4) explored variations of sediment bioactivity. The first condition was performed in the absence of bioactivity (Exp.4a) and the second was performed in the presence of nutrient-stimulated bioactivity (Ex.4b). Both experiments used filtered Danube Channel water (pH

7.45-7.90) instead of tap water as the influent. The water was collected at the Spittelauer Brücke in Vienna, Austria during September/October 2020. Collected water was allowed to settle for two days in 10 L PVC containers before being filtered through a 0.22 µm Millipore filter to remove particulate matter and microorganisms. Water was not collected on or 2-3 days following a rain event to avoid contamination and high-particulate content. The chemical compositional of the solution was expected to support the growth of autochthonous microbes in the sediment, primarily for the condition run in the presence of stimulated bioactivity (Exp.4b).

To establish the absence of sedimentary bioactivity for Exp.4a, filtered Danube Channel water was autoclaved at 121°C and 0.105MPa for 20min. Sterilised water was used throughout the experiment. Prior to packing, the sediment was autoclaved by the same method in a glass beaker saturated by sterilised water. The sediment sterilisation was repeated three times in-between stirring to achieve homogeneity. After sterilisation treatments, Adenosine Triphosphate (ATP) concentration measurements confirmed the absence of bioactivity for the influent solution and sediment (not shown). The stimulated sediment bioactivity of Exp.4b was achieved by stimulating microbes attached to the sediment inside the column using a preconditioning nutrient treatment for a period of 10 days prior to the start of the experiment. Ten columns were used in the preconditioning, nine were treated with a Danube Channel water reservoir amended with acetate and glucose (50 mg L^{-1}) as simple and readily bioavailable carbon sources. The tenth column acted as a control with unamended Danube Channel water. The treatment was also applied as an influent solution for the entire duration of the experiment. Adenosine Triphosphate (ATP) concentrations were measured daily from the column effluent for the preconditioning and experimental phases, and from the reservoir water which was monitored to prevent contamination. The control column and a replicate of the preconditioned columns were sacrificed at the end of the treatment period to confirm that ATP concentrations from sediment of the nutrient treated column were four-times the values of the control column (not shown). All sediment samples were assessed for ATP concentration during the experiment. Column sediments also displayed a visible change in texture when sampled prior to starting and during the experiment, indicating the presence of exopolysaccharides and biofilm formation.

Adenosine Triphosphate drives metabolic activity and levels are indicative of greater active biomass. An ATP bioluminescence assay was used to measure concentrations. The Promega BacTiter-Glo Microbial Cell Viability Reagent, based on the luciferase reaction, was chosen because of its high sensitivity. Total and external ATP concentrations were measured from water samples to calculate internal ATP. External ATP was extracted by centrifuging 2mL of water sample at 20,000 rmp for 30 min at 4°C. For total ATP 180 µL of each water sample were transferred in triplicate to a 96-well plate, while for external ATP the same volume of supernatant was transferred. Water samples and the reagent were equilibrated to 38°C on a Thermomixer for 3 min prior to incubation. After the allotted time, 20 µL of reagent was added to each sample well and the contents incubated on the Thermomixer at 600 rmp for 1 min at 38°C. Luminescence was recorded using a GloMax Luminometer with an integration time of 0.3 s. Data were recorded as Relative Light Units (RLU) and converted to ATP by means of a standard curve. Standard curves were prepared by a 10-fold serial dilution of the

ATP stock solution in values of nM mixed with ATP-free water. A new standard curve was prepared for every plate. Adenosine Triphosphate-free water was also measured as a blank to monitor contamination and subtract background RLUs from all values.

Total ATP concentration was measured from sediment samples by vortexing 200 mg of sediment with 100 µL of PBS solution. Sediment-PBS tubes and reagent were equilibrated to 30°C on a Thermomixer for 3 min prior to incubation. After the allotted time, 100 µL of reagent was added to each sample, samples were vortexed, and the contents incubated on the Thermomixer at 600 rmp for 1 min at 30°C. Luminescence was recorded immediately by the same method above using 200 µL of the suspension to a 96-well plate. Autoclaved sediment stored in PBS overnight was flushed with new PBS and used to prepare a background measure and standard curve. To prepare the standard curve 200 mg of autoclaved sediment to 100 µL of each 10-fold ATP stock dilution was allowed to sit at room temperature for 1 h. The total ATP concentration was then measured using the same method as other sediment samples.

4.2 Analytical framework

All virus concentration data, except reservoir monitoring results, were normalized with respect to the input concentration (C/C_0). Soluble nonreactive tracers should behave in the same manner as water; therefore, measurements of the conservative tracer were considered a measure of the hydrodynamic conditions in the column system.

4.2.1 Virus inactivation

Inactivation rates of free viruses in solution were determined via the decline of virus concentrations from the reservoir over the duration of each experiment. Inactivation rates of viruses attached to sediment surfaces were determined via the decline in virus concentration eluted from sediment columns over the duration of each experiment. Since the infiltration time was considerably short, decay of the viral input concentration during infiltration was assumed to be negligible. Decline of viruses over the experimental duration is exponential with time, assumed to follow a first order decay model. Virus survival was characterised by the slopes calculated for each condition, expressing the corresponding concentration decrease in Log_{10} units of pfu per day. The inverse of these calculated decay rates was used to estimate T_{90} values, the predicted number of days to achieve a 1 Log_{10} (90%) reduction using Eq.1.

$$T_{90} = \frac{-t}{Log_{10}C} \qquad \textit{Equation 1}$$

The relationship between inactive viruses and infectious viruses (pfu) was investigated as a percentage of the total viral particles present (qPCR) in the effluent using Eq.2. Values of active viruses as a percentage of the total were presented.

$$Inactive\% = \frac{C/C_{0_{genomes}} - C/C_{0_{pfu}}}{C/C_{0_{genomes}}} \times 100\% \qquad \textit{Equation 2}$$

4.2.2 Mass recovery

The total amount of tracer and virus mass that was returned during each experiment was analysed by numerical integration of the concentration curves (C). Mass recovery of free active viruses and

total viral particles from effluent samples (M_{rw}) was calculated according to Eq.3 and presented as a percentage of the total mass injected (M_0).

$$M_{rw} = Q \int_0^t C(t)dt \qquad \textit{Equation 3}$$

Equation 3 assumes complete mixing of the substance with the aqueous phase, negligible dispersion effects, and an expectation that the entire substance will exit the column system completely as a function of time (t) and discharge (Q). The solution to Eq.3 can be acquired by simplifying to Eq.4.

$$M_{rw} = Q \int_0^{t_f} C(t)dt \approx \sum_{i=1}^{n} \Delta t_i \, C_i Q \qquad \textit{Equation 4}$$

To calculate viruses adsorbed in a column after terminating at each sediment sampling interval, the mass recovery of attached active viruses (M_{rs}) was calculated using Eq.5. This value was not subject to a function of discharge. Instead, integration was relative to the dry weight of sediment in the column (M_d) adjusted to account for porosity (θ) and bulk density (ρ_b). This estimation oversimplified complexities of the porous medium, also wet sediment samples held carryover of free active viruses in porewater, but any apparent errors did not lead to changes in the interpretation of results.

$$M_{rs} = \frac{M_d}{(\rho_b/\theta)} \int_0^t C(t)dt \qquad \textit{Equation 5}$$

4.2.3 **Hydrodynamics**

Longitudinal dispersion (D_L) is a measure of the degree that a substance mass spreads along the flow path, calculated using Eq.6. Where x is the length of the column, $\Delta\, t_{1/2}$ is the amount of time during which concentration is greater than half of the peak concentration, and t_{peak} is the time to the peak concentration (Harvey and Garabedlan, 1991). Use of the peak arrival time assumes results are independent of the standard deviation in mean residence time and velocity, instead the measured time travel variance of the breakthrough curve includes the injection distance and setback due to sinuosity of the flow path.

$$D_L = \frac{x(\Delta t_{1/2} \,/\, t_{peak})^2}{16ln2} \qquad \textit{Equation 6}$$

The hydraulic residence time **(**$H\tau_{res}$) describes the hydraulic flow for water through the column, giving insight into the entire volume of mobile water. Eq.7 is calculated by dividing the volumetric flow rate (Q) by the mean volume of mobile water (V_m).

$$H\tau_{res} = \frac{V_m}{Q} \qquad \textit{Equation 7}$$

4.2.4 **Statistical analysis**

All experiments present breakthrough curves in two matrices. The first conventional breakthrough curve measured free-floating viruses sampled from the effluent. This was demonstrated using concentrations of active viruses and total viral particles. The second breakthrough curve is a novel approach measuring the change in concentration of attached active viruses sampled from a temporal variation of sediment. Breakthrough curves of free active viruses and total viral particles expressed attenuation rates relative to the infiltration period (Phase 1) and desorption period (Phase 2), while attached active viruses were only measured during the desorption period (Phase 2) representing adsorption of Phase 1. Statistical comparisons of the mean removal rate for free active viruses measured from the transport experiments were identified by applying a one-way ANOVA on Log-

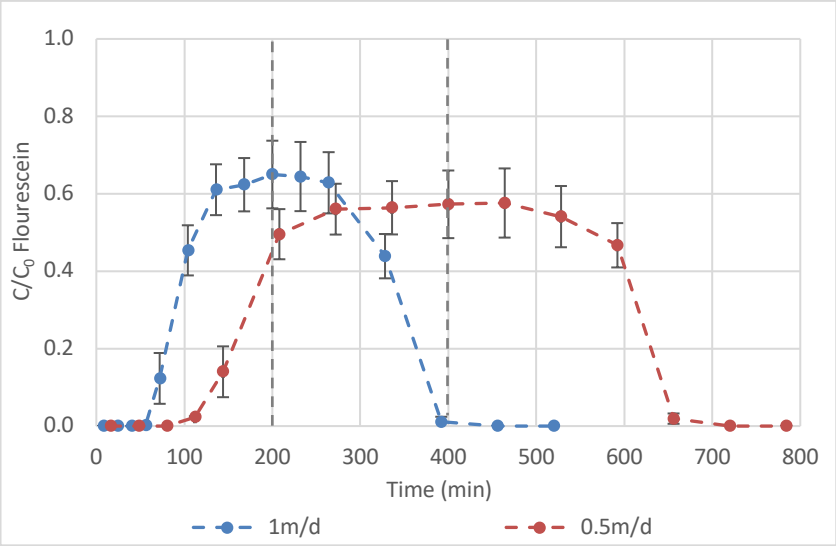
transformed values. The mean removal rate of free active viruses was estimated from the relative Log reduction in viral concentration achieved per unit of time, based on the entire breakthrough curve and normalized to the total amount injected (C/C_0). Null hypotheses (H0) were expressed as no significant difference (1) between the mean removal rate of free active viruses in one condition to that of the reference condition (Rf.) and (2) between related conditions (e.g., Exp.1a and Exp.1b).

The use of non-linear regression facilitated comparison between the rate of attenuation under various conditions relative to the expressed mechanism. The decline in active virus concentrations sampled from the reservoir expressed a single decimal reduction time, interpreted as the inactivation rate of free active viruses. The H0 was expressed as no significant difference (3) between the regression slope and zero, which is more accurately no significant difference in free active virus concentrations over the duration of the experiment (paired t-test). Schijven *et al.* (1999) established during modelling that the intercept from the height of the tail in the breakthrough curve of free active viruses is determined by the value of the detachment rate, while the slope of the tail is determined by the value of the inactivation rate of attached viruses. The second part of this claim is to the authors knowledge, yet to be confirmed by measuring the change in concentration of attached active viruses with time. The slope of the breakthrough curve from the attached active viruses during the desorption period (Phase 2) was interpreted as a more accurate and direct measure of the rate of inactivation for attached viruses (i.e., surface inactivation). This was investigated by comparing the measured rate of inactivation for attached viruses to the slope of the tail from the breakthrough curve of free active viruses, which was interpreted by Schijven *et al.* (1999) as a way to predict the rate inactivation occurring at the surface. Null hypotheses (H0) were expressed as no significant difference (4) between the regression slope from the breakthrough curve of the attached active viruses (i.e., measured rate) and the slope of the tail from the breakthrough curve of free active viruses (i.e., predicted rate) (unpaired t-test). The relationship within and between experiments was also explored with H0 expressed as no significant difference between the regression slopes of the measured and predicted rates of inactivation for attached viruses; (5) and zero or no significant difference in the rate of change over the duration of the experiment (paired t-test); (6) to the rate of inactivation for attached active viruses measured and predicted from the reference condition (Rf.) (paired t-test); and (7) to the rate of inactivation for attached active viruses measured and predicted between related conditions (e.g., Exp.1a and Exp.1b) (paired t-test).

5 Results

5.1 Conservative tracer

Breakthrough curves for the tracer of experiments tested with a flow velocity of 1m d^{-1} (n = 7) and 0.5m d^{-1} (n = 1) are plotted in Figure 2. All replicates columns, across all conditions, showed similar tracer results indicating that flow properties in the columns were stable and hydrodynamic parameters were similar under all experimental conditions. Longitudinal dispersion values for the tracers were similar under all conditions indicating that the porous medium was homogenous across experiments. Small differences in the peak concentrations of each tracer curve (see error bars) can be attributed to inherent heterogeneity in porewater volume and dilution of the injected tracer plume. Plateaus indicate that tracers reached an equal concentration exiting the sediment columns before the influent was switched to a tracer-free solution. It is expected that the relative concentration (C/C_0) would equal 1 to be thought of as being in equilibrium, however uncertainties in tracer recovery cannot support this conclusion particularly because of the low percentage mass recoveries (e.g., PR = 63% for the 1m d^{-1} flow velocity, PR = 53% for the 0.5m d^{-1} flow velocity). Hydraulic residence time of the tracers was used as t_0 to transform the experimental duration into units of relative time (t/t_0). To avoid confusion in the literature surrounding the terminology of pore volume, this study represents further virus breakthrough curves on a scale of Log relative time (Log t/t_0), assuming this is a measure of the time to displace one cumulative injected mass.



	$H\tau_{res}$ min	PR %	q cm min^{-1}	D_L cm^2 min^{-1}
Standard flow velocity (1m d^{-1})	80	63 ±8	0.069	0.757 ±0.04
Slow flow velocity (0.5m d^{-1})	160	53	0.035	0.579

Figure 2. Fluorescein tracer breakthrough curves and estimated properties. Values normalised to the tracer input concentration (C_0). Dotted lines indicate transition to tracer-free influent. Error bars represent standard deviation of all experiments with flow velocity of 1m d^{-1} (Exp.; 1a, 2a, 2b, 3a, 3b, 4a, 4b) and standard deviation of all columns in Exp.1b with flow velocity of 0.5m d^{-1}. Calculated values; hydraulic residence time ($H\tau_{res}$), input flow velocity (q), and longitudinal dispersion (D_L).

5.2 Virus inactivation

Inactivation and/or decay rates of free and attached viruses under the conditions of various experiments are presented in Table 2 and Figure 3. Non-linear regression was sufficient to explain all

inactivation and/or decay profiles by around 90% or more. The inactivation rate of free viruses in the anoxic reservoir solution (Exp.3a) was slowest, calculated to take 5.5 days to reach a 1 Log_{10} reduction (T_{90}) with a regression coefficient of 97%. In contrast, the hypoxic reservoir solution (Exp.3b) showed the fastest inactivation of free viruses, calculated as 1 day to reach a 1 Log_{10} reduction (T_{90}). Although, the probability of the regression was lower (R^2 87%) despite taking additional data points to increase accuracy. Since this inactivation profile was expected to affect the breakthrough curve of free active viruses recovered in Exp.3b performed under hypoxic conditions, and thus comparability to the results of Exp.3a performed under anoxic conditions, a correction was made. The breakthrough curve of free active viruses in Exp.3b was normalised to a linear interpolation of the inactivation rate for free viruses (C/C*). Figure 3a shows that experiments performed using low (Exp.2a) and medium (Exp.2b) viral loads have steep inactivation rates with high probability (R^2 92% and 100%, respectively). Since their rates are similar it is expected that graphical representations of free active virus breakthrough curves are visually comparable, thus no further adjustments were made to account for inactivation. However, it is possible the recovery data of free active viruses in these experiments exhibit some inherent inactivation in solution.

Table 2. Virus inactivation rates of (a) free-floating viruses and (b) viruses attached to sediment. Inactivation of free-floating viruses in tap water recorded by the reference condition (Rf.). Slopes and correlation coefficients (R^2) are of non-linear regression from Log10 concentration values. Rates in percentage per day and time to reach a 1 Log10 reduction (T90).

		a) Free floating viruses				b) Attached viruses			
	Experiments	Slope Log10 day^{-1}	R^2	Decay rate % day^{-1}	T90 days	Slope Log10 day^{-1}	R^2	Decay rate % day^{-1}	T90 days
1a	Standard flow velocity (Rf)	-0.06	92%	16	2.5	-0.17	97%	39	1.3
1b	Slow flow velocity					-0.16	94%	41	1.2
2a	Low viral load	-0.14	92%	36	1.3	-0.06	93%	15	2.7
2b	Medium viral load	-0.16	100%	41	1.2	-0.07	99%	18	2.2
3a	Anoxic conditions	-0.04	97%	10	5.5	-0.11	95%	28	1.5
3b	Hypoxic conditions	-0.24	87%	59	1.0	-0.18	99%	45	1.2
4a	Sterile conditions	-0.05	91%	12	2.8	-0.14	96%	34	1.4
4b	Stimulated bioactivity	-0.09	87%	22	1.8	-0.23	99%	57	1.0

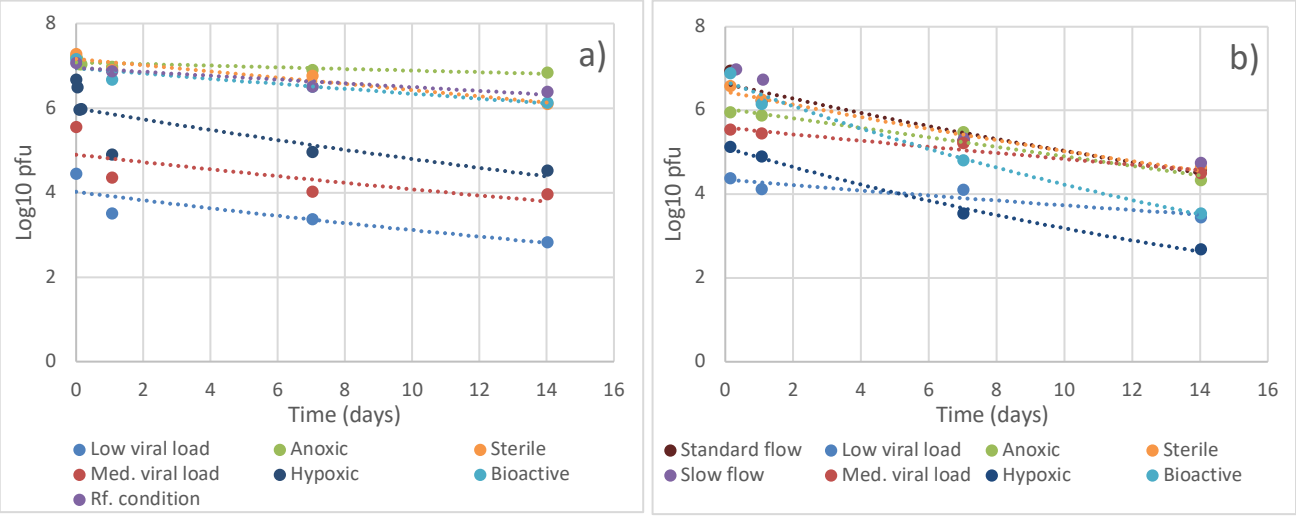


Figure 3. Virus inactivation rates of (a) free-floating viruses and (b) viruses attached to sediment. Trendlines fitted for each experiment. Inactivation of free-floating viruses in tap water recorded by the reference condition (Rf.).

Inactivation profiles of free viruses for all remaining experiments, including those performed with variations of flow velocity (Exp.1a standard flow (Rf.), Exp.1b slow flow); those performed with variations of sediment bioactivity (Exp.4a in the absence of bioactivity, Exp.4b stimulated bioactivity); and the anoxic reservoir solution (Exp.3a), showed no significant difference from zero. This indicated that inactivation of free viruses in these conditions did not significantly change in concentration for the duration of the experiment. Although, virus inactivation in the reservoirs of both the experiments performed with variations of flow velocity (Exp.1) and those performed with variations of sediment bioactivity (Exp.4) do not accurately represent inactivation of free viruses in their respective conditions. For example, Exp.1a using a standard flow velocity (Rf.) and Exp.1b using a slow flow velocity shared a reservoir, however since no reservoirs had the capacity to regulate any flow through activity, thus results were more accurately a measure of the inactivation rate for free viruses using the standard viral load in tap water. Additionally, the reservoirs of experiments performed with variations of bioactivity (Exp.4) were more accurately a measure of inactivation relative to the solution chemistry of the autoclaved Danube Channel water (Exp.4a) and the Danube Channel water with a nutrient treatment (Exp.4b). Since results show little change between these experiments and the results of the reference condition (Rf.), the effect of Danube Channel water had little to no impact on the inactivation rate of free viruses.

For Exp.1a using a standard flow velocity (Rf.) and Exp.1b using a slow flow velocity, the values of inactivation for attached viruses were two times faster than inactivation of free viruses using a standard viral load in tap water. Since the rate of inactivation for free viruses in Exp.1 does not change significantly over the duration of the experiment (i.e., no significant difference from zero), the difference in inactivation rate to attached viruses may simply be related to the presence of hydraulic loading and shear flow that only occurred inside the columns. Thus, the inactivation rate of attached viruses could be a minimum of two times faster than free viruses of all experiments using a standard viral load in tap water. In comparing Exp.1a using a standard flow velocity (Rf.) to Exp.1b using a slow flow velocity, the calculated times to reach a 1 Log_{10} reduction (T_{90}) of attached viruses were almost identical suggesting that a difference in velocity inside the columns of each experiment does not appear to affect the inactivation rate of attached viruses.

Viruses attached on the sediment surface in the experiment performed in the presence of stimulated bioactivity (Exp.4b) inactivated twice as fast as free viruses in the Danube Channel water with a nutrient treatment, calculated to take 1 day to reach a 1 Log_{10} reduction (T_{90}) (R^2 99%) and faster than the inactivation rate of attached viruses in all other experiments. In the experiment performed in the absence of bioactivity (Exp.4a), values of inactivation for attached viruses are also two times faster than values measured for free viruses in the autoclaved Danube Channel water. In contrast, experiments performed using low (Exp.2a) and medium (Exp.2b) viral loads show values of inactivation for attached viruses that are two times slower than inactivation of medium and low viral loads in tap water. In the experiment performed in the absence of oxygen (Exp.3a), values of inactivation for attached active viruses are four times faster than values measured for free viruses in the anoxic tap water solution. However, a comparison to the rate of inactivation for free viruses in the anoxic reservoir solution is difficult because this value was likely overestimated since the

concentration does not change significantly over the duration of the experiment. Conversely, the experiment performed under hypoxic conditions (Exp.3b), shows steep values of inactivation for attached and free viruses that are comparable between the hypoxic sediment and hypoxic tap water solution.

5.3 Virus breakthrough curves

Figure 4 shows breakthrough curves of relative concentrations (C/C_0) measured from various experiments, performed under different environmental conditions. These include free active viruses and total viral particles measured in sediment porewater from effluent, active viruses attached to the sediment, and the conservative tracer. Values are presented on a logarithmic scale as a function of relative time (t/t_0). Table 3 presents the mass recovery percentages of free active viruses, attached active viruses, and total viral particles. Most noticeably, all breakthrough curves for viruses measured in effluent were consistent with the tracer curves, suggesting a lack of retardation. Exp.1b performed using a slow flow velocity was an exception, where the breakthrough curves sampled from effluent showed some minor retardation in relation to the tracer (see Figure 4, Exp.1b). All free viruses first arrive in the effluent at a relative time of 0.5 t/t_0, except in Exp.3a performed in the absence of oxygen where free active viruses across all replicate columns arrived at a relative time of 0.3 t/t_0. None of the experimental conditions resulted in a complete removal of free active viruses from the effluent by the end of sampling period, thus free viruses continued to be released after the conservative tracer was absent in the effluent (> 4.8 t/t_0). No experimental conditions resulted in a complete removal of attached active viruses from the sediment by the end of sampling period either, thus attached viruses continued to remain active on the surface of sediments. Reaching equal concentrations between influent to effluent ($C/C_0 = 1$) indicates that no more adsorption sites were available to occupy. However, values of all breakthrough curves for free viruses, in all experiments, were below 1 C/C_0 suggesting that adsorption sites were not a limiting factor throughout the duration of the experiment.

In comparing the mean removal rate of free active viruses, experiments performed using the standard flow velocity (Exp.1a) and slow flow velocity (Exp.1b) showed no significant difference to each other ($p = 0.72$). This indicated that a decrease in flow velocity, from 1 m d^{-1} (Exp.1a) to 0.5 m d^{-1} (Exp.1b), had no measurable impact on the mean removal of free active viruses in the effluent. The mass recovery percentage of free viruses from Exp.1a performed using the standard flow velocity (Reference condition, Rf.) was calculated to 66% of active viruses compared to the input concentration and 76% of the total viral particles recovered. This indicated that a large proportion of the total viral particles were still in the active fraction. Similar results were measured from Exp.1b performed using a slow flow velocity, with 58% of free active viruses and 68% of total viral particles recovered from the effluent. Peak concentrations of free active viruses reached almost complete breakthrough, with relative values of 0.92 and 0.90 C/C_0 recorded for Exp.1a performed using a standard flow velocity (Rf.) and Exp.1b using a slow flow velocity, respectively. The time to peak concentrations (3.2 t/t_0) for free active viruses was the same for both experiments, suggesting that a decrease in flow velocity had no measurable impact on arrival of the peak viral concentrations.

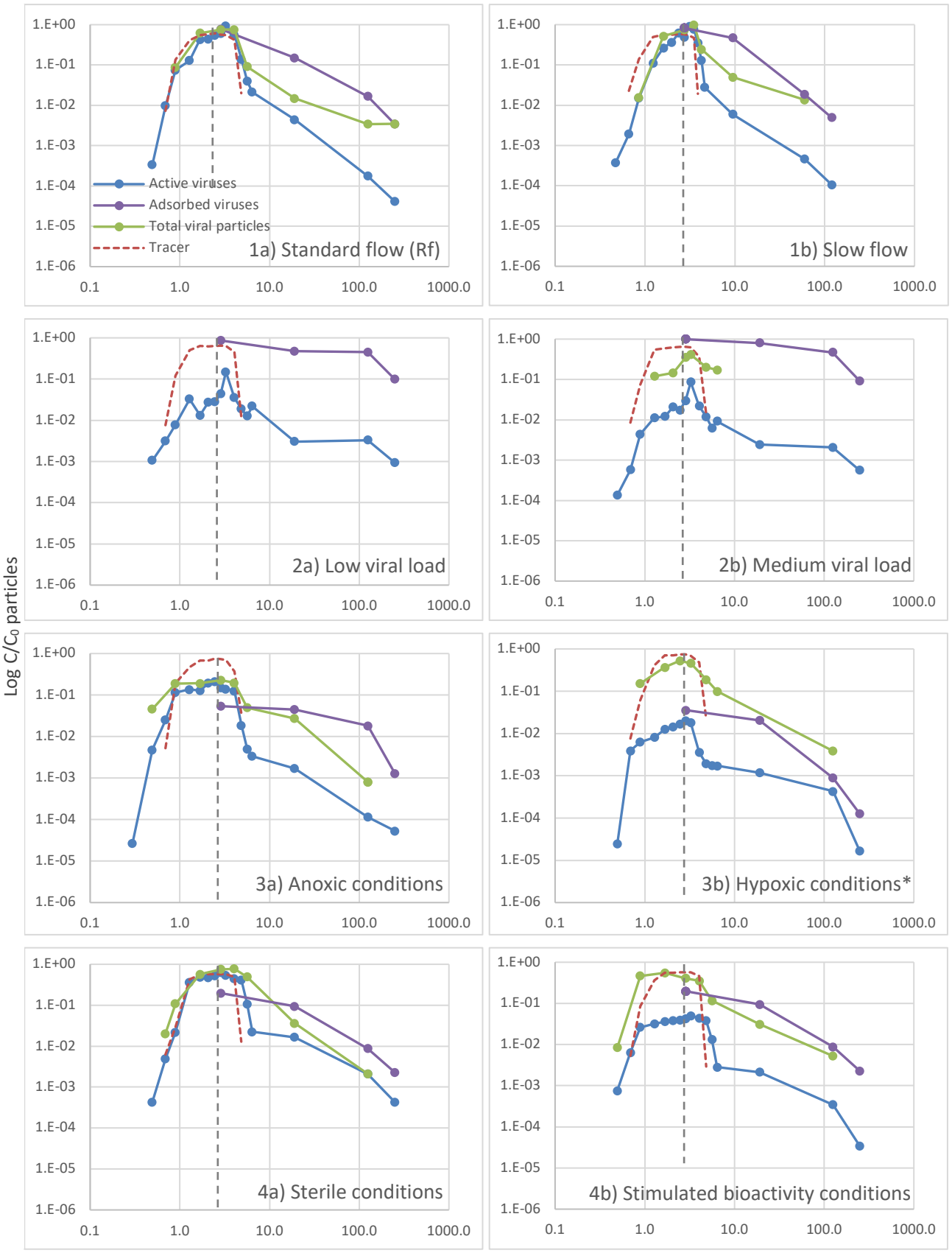


Figure 4. Breakthrough curves for virus and tracer values normalised to the input concentration (C_0). Active viruses in solution and on sediment (pfu/mL), total viral particles (genome copies/mL), and fluorescent tracer (concentration/mL), collectively particles/mL. Both axes on Log-scale. Dotted lines indicate transition to virus-free influent. Reference condition (Rf.). Hypoxic condition uses C/C for active viruses in solution.*

In Figure 4, Exp.1a (Rf.) and Exp.1b show breakthrough curves of free viruses, both active and total concentrations, that match until the onset of tailing indicating that the desorbed fraction of free viruses were largely inactivated. Attached active virus concentrations for experiments performed using the standard flow velocity (Exp.1a, Rf.) and slow flow velocity (Exp.1b) peaked at relative concentration of 0.77 and 0.82 C/C_0 respectively, both of which decreased by 2 Log units (99%) over the duration of the experiment. The mass recovery percentages of attached active viruses accounted for 36% of the input concentration from Exp.1a using a standard flow velocity (Rf.) and 25% from Exp.1b using a slow flow velocity.

No significant difference was calculated between the mean removal rate of free active viruses for experiments that were performed using a low (Exp.2a) and medium (Exp.2b) viral load ($p = 0.64$). This indicated that an increase in viral load, from 10^4 (Exp.2a) to 10^5 (Exp.2b) pfu mL^{-1}, had no measurable impact on the mean removal of free active viruses in the effluent. Recovery from the effluent was calculated to 24% of free active viruses from Exp.2a performed using a low viral load and 15% of the medium viral load from Exp.2b. Both experimental conditions showed a significant difference in the mean removal rate of free active viruses compared to the experiment that supplied a high viral load at the same flow velocity (Rf.) (Exp.2a $p = 0.01$, Exp.2b = < 0.001). This indicated that a decrease in viral load from 10^7 pfu mL^{-1} (Rf.) to 10^4 or 10^5 pfu mL^{-1} (Exp.2a and Exp.2b, respectively) had a significant increase on the mean removal of free active viruses in the effluent. For Exp.2b performed using a medium viral load, the detection of total viral particles measured from the effluent was focused on samples collected near the peak of breakthrough curve for free active viruses (Figure 4, Exp.2b) but could not be accurately measured either side due to concentration limits (qPCR LOD < 10^2-10^4 particles mL^{-1}). The trend showed that total viral particles were consistently 1 Log unit (90%) higher than free active viruses, suggesting that the recovery of free viruses from the peak of the breakthrough curve were largely inactivated viruses. The time to reach the peak concentration of free viruses, both active and total, from experiments that used a low (Exp.2a) and medium (Exp.2b) viral load, occurred at the same relative time (3.2 t/t_0) as the experiment that used a high viral load at the same flow velocity (Rf.). This suggested that a change in viral load did not result in a measurable difference of the arrival of peak concentrations in effluent. Peak concentrations for free active viruses reached a relative value of 0.12 C/C_0 from Exp.2a using a low viral load and 0.09 C/C_0 for Exp.2b using a medium viral load. In contrast, the peak concentrations of attached active viruses reached relative values of 0.86 C/C_0 and 0.99 C/C_0 from the application of low (Exp.2a) and medium (Exp.2b) viral loads, respectively. Mass recovery percentages of the attached active viruses accounted for 72% of the low viral load from Exp.2a and 90% of the medium viral load from Exp.2b. Both experiments showed a 1 Log unit (90%) decrease in concentrations of attached active viruses over the duration of the experiment.

The mass recovery percentage of free active viruses from Exp.3a performed in the absence of oxygen was 20% of the input concentration, with 31% of total viral particles recovered from the effluent. This indicated that a large proportion of the total viral particles were still in the active fraction. In Figure 4 Exp.3a, the breakthrough curves show that free active viruses and total viral particles matched until the onset of tailing, where inactivated viruses preferably desorb under anoxic

conditions. Figure 4, Exp.3a also shows that the free virus breakthrough curves of both active and total concentrations, plateaued from a relative time of 0.89 t/t_0, where relative values under anoxic conditions did not measure above 0.2 C/C_0 (1 Log unit, or 90% < 1.0 C/C_0). The mass recovery percentage of free active viruses for Exp.3b performed under hypoxic conditions returned the lowest recovery of all the experiments, calculated to 4% of the input concentration. Although, 42% of the total viral particles were recovered in effluent, indicating that a much larger proportion of the inactive viruses can be recovered under hypoxic conditions (Exp.3b). In Figure 4 Exp.3b, the breakthrough curve of free active viruses reached a plateau from a relative time of 0.69 t/t_0 and a relative value no higher than 0.02 C/C_0, which was consistently 2 Log units (99%) lower than the concentration of total viral particles. In contrast, the total viral particles reach a peak concentration at a relative time of 2.5 t/t_0 with a relative value of 0.5 C/C_0. In comparing the mean removal rate of free active viruses, experiments performed under anoxic conditions (Exp.3a) and hypoxic conditions (Exp.3b) showed a significant difference from each other ($p = < 0.001$). This indicated that the experiment performed under low concentrations of DO (Exp.3b) had a significant increase in the mean removal of free active viruses in effluent compared to the experiment performed in the absence of oxygen (Exp.3a). Both experiments also showed a significant difference in the mean removal rate of free active viruses compared to the experiment that was performed under oxic conditions with the same viral load and flow velocity (Rf.) (Exp.3a anoxic $p = 0.01$, Exp.3b hypoxic = 0.01). This indicated that a decrease in DO concentration from oxic (Rf.) to either hypoxic (Exp.3b) or anoxic (Exp.3a) conditions, had a significant increase on the mean removal of free active viruses in the effluent. Attached active viruses in experiments performed under anoxic conditions (Exp.3a) and hypoxic conditions (Exp.3b) reached a peak concentration of 0.05 and 0.03 C/C_0, respectively, with both concentrations decreasing by 2 Log units (99%) over the duration of the experiment. The mass recovery percentages of attached active viruses in the experiments performed under anoxic conditions (Exp.3a) and hypoxic conditions (Exp.3b) accounted for 7% and 4% of the input concentration, respectively.

In Exp.4a performed in the absence of bioactivity, the mean removal rate of free active viruses showed no significant difference to the experiment performed using background sediment bioactivity but the same viral load and flow velocity (Rf.) ($p = 0.84$). This indicated that a decrease in the presence of microbes from background sediment bioactivity (Rf.) to sterile conditions (Exp.4a) had no measurable impact on the mean removal of free active viruses. The mass recovery percentage of free active viruses for the experiment performed under sterile conditions (Exp.4a) was 85% of the input concentration, with 78% of total viral particles recovered from the effluent, thus a large proportion of the total viral particles were still in the active fraction. In Figure 4 Exp.4a, the breakthrough curves show free active viruses and total viral particles that match so closely over the breakthrough period that it is not possible to determine a relationship to the recovery of inactive viruses. Figure 4 Exp.4a also shows that free virus breakthrough curves in both active and total concentrations, plateaued from a relative time of 1.68 t/t_0, where the relative values under sterile conditions measured around 0.4-0.5 C/C_0. In contrast, the mass recovery percentage of free active viruses for Exp.4b performed in the presence of stimulated bioactivity returned a low recovery percentage of 9% of the input concentration. Although, 63% of the total viral particles were

recovered in effluent, indicating that a larger proportion of inactivated viruses can detected under stimulated bioactivity conditions. In Figure 4 Exp.4b, the breakthrough curve shows that free active viruses reached a plateau from a relative time of 0.89 t/t_0 with a relative concentration no higher than 0.03-0.05 C/C_0, which is consistently 1 Log unit (90%) lower than the concentration of total viral particles. However, the total viral particles reach a peak concentration of 0.5 C/C_0 at a relative time of 1.6 t/t_0, earlier than all other breakthrough curves. This suggests that inactive viruses arrive in the effluent in the presence of stimulated bioactivity (Exp.4b), in higher abundance earlier than all other experimental conditions. In comparing the mean removal rate of free active viruses, experiments performed in the absence of bioactivity (Exp.4a) and the presence of stimulated bioactivity (Exp.4b) were significantly different from each other ($p = <0.001$), with the experiment performed in the presence of stimulated bioactivity (Exp.4b) also showing a significant difference from the experiment performed using background sediment bioactivity but the same viral load and flow velocity (Rf.) ($p = 0.01$). This indicated that an increase in the activity of microbes from background sediment bioactivity (Rf.) or from sterile conditions (Exp.4a) to stimulated bioactivity had a significant increase on the mean removal of free active viruses in the effluent. Attached active viruses in Exp.4a performed in the absence of bioactivity reached a peak concentration of 0.19 C/C_0, while Exp.4b performed in the presence of stimulated bioactivity reached a peak concentration of 0.55 C/C_0. Mass recovery percentages of the attached active viruses accounted for 12% of the input concentration from the experiment performed in the absence of bioactivity (Exp.4a) and 25% from the experiment performed under stimulated bioactivity conditions (Exp.4b). The concentration of attached active viruses from both experiments decreased around 1-2 Log units (90-99%) over the total duration.

*Table 3. Mass recovery of active viruses measured in effluent (M*rw*) and adsorbed to sediment (M*rs*), and total viral particles in effluent (M*rw*). Combined active viruses show the total percentage of active viruses calculated from those recovered in effluent and adsorbed to sediment (i.e., theoretical value). Degree of elimination shows the calculated percentage of viruses not recovered, between the combined active viruses and total viral particles.*

	Mass recovery percentages	Active viruses		Combined active viruses %	Total viral particles	Degree of elimination
	Experiments	M_{rw} %	M_{rs} %	$(M_{rw} + M_{rs})$	M_{rw} %	%
1a	Standard flow velocity (Rf.)	66	36	102	76	0 – 24
1b	Slow flow velocity	58	25	83	68	17 – 32
2a	Low viral load	24	72	96	-	4
2b	Medium viral load	15	90	105	24	0 – 76
3a	Anoxic conditions	20	7	27	31	69 – 73
3b	Hypoxic conditions	4	4	8	42	58 – 92
4a	Sterile conditions	85	12	97	78	3 – 22
4b	Stimulated bioactivity	9	25	34	63	37 – 66

5.4 **Combined virus recovery and elimination**

Figure 5a presents the distribution of mass recovery percentages for free active viruses and total viral particles during Phase 1 of the infiltration period and Phase 2 of the desorption period for each experiment. Figure 5b presents the mass recovery percentage for attached active viruses during Phase 2 of each experiment. All values from Figures 5a and 5b are presented in Table 3. During Phase 1, the recovery percentage of free active viruses accounted for those breaking through during infiltration of the virus-tracer solution. They are essentially fast transporting viruses that experience little adsorption. Since inactivation of free active viruses did not occur during the Phase 1, those not recovered in the effluent during that same period could only be due to reversible adsorption detected

by sampling of effluent from Phase 2, or irreversible adsorption detected by the sampling of sediment during Phase 2. Phase 2 is characterised by the changeover to virus-free influent. Therefore, the percentage recovery of free active viruses during this period accounted for active viruses that were reversibly adsorbed in the column during Phase 1 and maintained infectivity following detachment into the effluent of Phase 2. A small percentage of free active viruses held in the pore volume carried over from the infiltration of Phase 1 were washed through directly after the changeover to virus-free influent. However, free active viruses measured in the preceding residence time interval calculated an average contribution of < 2% to the overall recovery percentage of Phase 2. Active viruses not recovered in the effluent during Phase 2 could be due to inactivation of free or attached viruses detected by the value of total viral particles from Phase 2, or irreversible adsorption detected by the sampling of sediment during Phase 2. Attached active viruses presented in Figure 5b were irreversibly adsorbed during both phases and maintained infectivity after attachment to the sediment. Table 3 presents the combined recovery percentage of both free and attached active viruses, which is a theoretical value of all active viruses recovered from each experiment.

The lack of inactivation that occurred during the infiltration period meant that the recovery of total viral particles in Phase 1 accounted for all free active viruses recovered in that same period, plus any inactive viral particles already present in the reservoir. For total viral particles recovered during Phase 2, the fractions consist of all free active viruses recovered in that same period (i.e., desorbed from reversible adsorption that occurred during Phase 1), any viruses that experienced inactivation in solution during Phase 2, or inactivation of irreversibly attached viruses on the surface of sediments during the cumulative time of both phases, plus any inactive viral particles that were already present in the reservoir. Total viral particles that were not recovered during Phase 2 could be due to irreversible adsorption of active viruses, accounted for by the sampling of sediment from Phase 2, or decay of inactive viral particles beyond detection levels (i.e., complete elimination). Table 3 presents the degree of elimination, which is a theoretical value calculating the percentage of viruses not recovered between the value of combined active viruses (i.e., free and attached) and total viral particles when compared to the input concentration (i.e., 100%). While reversible and irreversible adsorption is well established in the literature for active viruses, these mechanisms are not commonly applied to free viral particles like MS2 RNA and/or inactive viruses, thus the action of adsorption-desorption of these fractions was excluded from conclusions.

In Figure 5a, the general trend shows that total viral particles in the effluent calculated a higher mass recovery percentage than free active viruses. The only exception is Exp.4a performed in the absence of bioactivity (Figure 5a), where more free active viruses were recovered in the effluent. Such a small difference was likely due to variations in the detection limit between active viruses (plaque assay LOD < 10 pfu mL^{-1}) and total viral particles (qPCR LOD < 10^2-10^4 particles mL^{-1}). Across all experimental conditions, the mass recovery percentage of total viral particles were more frequently detected during Phase 2, likely to accumulate in the effluent of the desorption period due to the combined effects of active virus desorption, inactivation in solution, inactivation on the sediment surface followed by desorption of inactive viruses, and flowthrough of inactive viruses and viral particles from the reservoir.

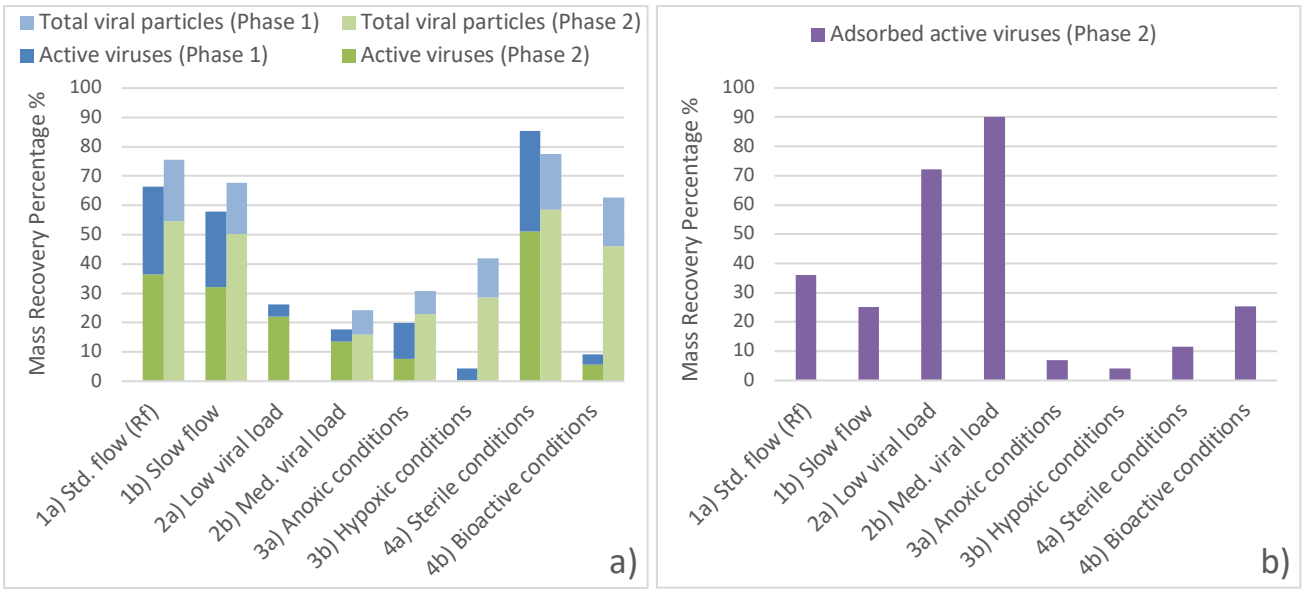


Figure 5. (a) Combined mass recovery of active viruses (dark shaded) and total viral particles (light shaded) in solution. Split between mass recovered during the infiltration period (Phase 1, blue) and desorption period (Phase 2, green). (b) Mass recovery of adsorbed active viruses (Phase 2 only). Standard (Std.) flow velocity. Reference condition (Rf.).

For Exp.1a performed using a standard flow velocity (Rf.), free active viruses are recovered in roughly equal fractions from Phase 1 and Phase 2 (Figure 5a). Attached active viruses (Figure 5b) bring the combined recovery percentage of active viruses to for Exp.1a (Rf.) to 102.4%. This suggested that, theoretically, all active viruses can be recovered with the addition of the adsorbed fraction. The trend in recovery of free active viruses calculated in roughly equal distributions between Phase 1 and Phase 2, does not differ in Exp.1b performed using a slower flow velocity (Figure 5a). With the addition of attached viruses, the combined recovery percentage of active viruses reached a total of 82.9%. While the trend of fluid phases does not differ from Exp.1a performed under standard flow velocity (Rf.), attached active viruses recovered under a slower flow velocity seen in Figure 5.b (Exp.1b) adsorbed in lesser amounts than what was possible from Exp.1a. A degree of 17-32% elimination can be calculated for Exp.1b performed using a slower flow velocity, likely due to inactivation of attached viruses.

In experiments performed using a low (Exp.2a) or medium (Exp.2b) viral load, the fraction of active virus recovery shifted towards values concentrated in the sediment-attached portion (Figure 5b). For Exp.2a performed using a low viral load and Exp.2b using a medium viral load, the combined recovery percentage of active viruses reached a total of 96.3% and 104.9%, respectively. This suggests that these experiments show little to no measured degree of elimination. In Figure 5a, the percentage of free active viruses shows a low overall recovery for both experiments performed using a low (Exp.2a) and medium (Exp.2b) viral load, with a considerably low percentage calculated from Phase 1 and the bulk fraction recovered by desorption during Phase 2. Limited breakthrough of fast transporting, free active viruses from Phase 1 (Figure 5a) suggests that much of these viruses become adsorbed early in the experiment during the infiltration period to result in the high percentage of attached active viruses (Figure 5b). The high percentage of active viruses recovered from the sediment (Figure 5b) and overall recovery of free active viruses in solution indicates that low to medium viral loads promote irreversible attachment and do not promote inactivation or desorption of attached viruses.

Although, attached active viruses may have continued to inactive on the surface of sediments or released into solution had sampling continued.

Figure 5b shows that the recovery of attached active viruses has little contribution to the combined percentage of active viruses from both experiments performed under anoxic conditions (Exp.3a) and hypoxic conditions (Exp.3b). In Exp.3a performed in the absence of oxygen, the low recovery percentage of free active viruses (Figure 5a) was calculated in roughly equal fractions during Phase 1 and Phase 2. With the addition of the sediment attached fraction, the combined recovery of active viruses reached a total of 26.9%. However, this is still less than half of the mass input concentration, suggesting a degree of 69-73% elimination. In Figure 5a, Exp.3b performed under hypoxic conditions shows almost all active viruses recovered in solution are calculated during Phase 1, with 0.4% detected during Phase 2. This suggests that of the few free active viruses recovered under hypoxic conditions (Exp.3b), the majority are recovered by fast transport, experiencing little adsorption. The lack of free active viruses recovered during Phase 2 indicated that few of the attached active viruses were detached (i.e., they experienced irreversible adsorption) or detached in an activated state (i.e., they experienced inactivation on the sediment surface). The mechanism of inactivation at the sediment surface is supported by the large fraction of total viral particles recovered during Phase 2 (Figure 5a), where almost no active viruses are recovered in effluent during that same period. Even with the addition of the sediment-attached fraction, combined active viruses (8.4%) equal less than a quarter of the total viral particles in effluent confirming that the from all viruses detected the larger fraction is inactive. Results suggests a degree of between 58-92% elimination in Exp.3b performed under hypoxic conditions.

For Exp.4a performed in the absence of bioactivity, the addition of the sediment-attached fraction (Figure 5b) brings the combined recovery percentage of active viruses to 96.9%. Although almost all active viruses were already recovered in effluent, supported by the highest percentage of free active viruses breaking through during Phase 1 (Figure 5a). This indicated for Exp.4a performed in the absence of bioactivity, that free active viruses were adding to the effluent recovery from an early stage of the experiment. However, the recovery of free active viruses from Phase 1 are still outweighed by the percentage of those recovered during Phase 2. This is unusual since the proportion of attached active viruses was so low (Figure 5b) that it is difficult to interpret where this large recovery of free active viruses during Phase 2 comes from, if not the remobilisation of active viruses that were adsorbed during Phase 1. For Exp.4b performed the presence of stimulated bioactivity, the addition of attached viruses brings the combined recovery percentage of active viruses from 9% in solution to 33.8% in total, indicating that sediment largely shields active viruses. Despite this, the recovery percentage of total viral particles are concentrated in Phase 2 (Figure 5a), particularly when compared to the low recovery of free active viruses during the same period. This indicates that of the attached active viruses, few were detached (i.e., they experienced irreversible adsorption) or detached in an activated state (i.e., they experienced inactivation on the sediment surface). Results suggest a degree of between 37-66% elimination in Exp.4b performed under stimulated bioactivity conditions.

5.5 Virus infectivity and persistence

When comparing the absolute mass values (i.e., concentrations not made relative) of free active viruses and total viral particles inoculated into the reservoir, active viruses account for 0.05-1.8% of the total viral particles prior to experiments (not shown). In effect, more that 98% of all viruses were of the inactive fraction before entering columns and following experiments the fraction was closer to 99%, although these values are not uncommon (e.g., Lodder *et al.*, 2013). Figure 6 presents a comparison of relative values (C/C_0) for active viruses and total viral particles measured from the effluent, detailing the persistence of virus infectivity over the duration of the experiments.

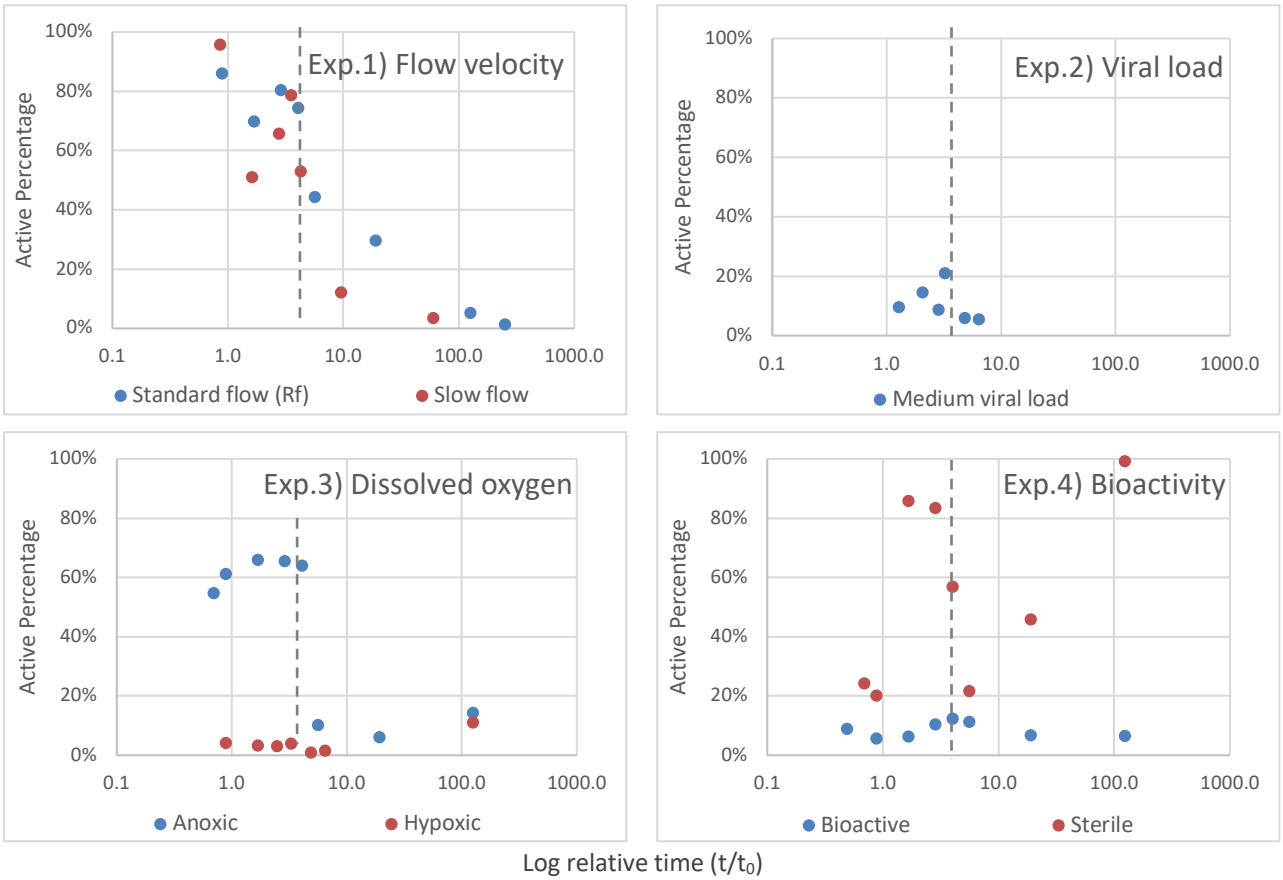


Figure 6. Fraction of active viruses as a percentage of the total viral particles measured in effluent. Total viral particles measured in particles/mL and active viruses measured in pfu/mL. Dotted lines indicate the transition to virus-free influent.

Under both experiments performed using the standard flow velocity (Exp.1a, Rf.) and slow flow velocity (Exp.1b), the percentage of active viruses showed an average of 49 ±34% and 51 ±34%, respectively. High standard deviations indicate that values were not stable over the duration of the experiment. As seen from Figure 6 Exp.1, active viruses begin both experiments at high percentages of those detected in the effluent (85-95%) and decline linearly over the duration of the experiment to low concentrations (1-3%) at the end. This indicates that active viruses make up a large proportion of viruses in the effluent early on, but by the changeover to virus-free influent inactivation returns more inactive viruses in the effluent outweighing active viruses and making up the bulk of those in the effluent by late stages of the experiment. In Exp.2b performed using a medium viral load, the percentage of active viruses shows an average of 11 ±6%, indicating little variation over the duration of the experiment with a peak in the middle at 20% and the lowest value of 5% measured at the end.

This suggests that active viruses that made up a small proportion of viruses in the effluent throughout the entire experiment, outweighed by inactive viruses in effluent.

Exp.3a performed in the absence of oxygen shows an average of 43 ±27% of active viruses, where the high standard deviation comes from a stark separation seen in Figure 6 Exp.3a. The results show a high concentration of active viruses in the effluent during the infiltration period of Phase 1 (62 ±5%) that drops down to an average of 10 ±4% calculated in the effluent during the desorption period of Phase 2. This indicates that active viruses make up just over half of viruses detected in the effluent during Phase 1, but soon after the changeover to virus-free influent results show that the effluent returns a majority of inactive viruses. In contrast, Exp.3b performed under hypoxic conditions (4 ±3%) shows little variation over the duration of the experiment, with active viruses consistently outweighed by inactive viruses in the effluent. In Exp.4b performed in the presence of stimulated bioactivity, the percentage of active viruses were calculated to an average of 9 ±3% reaching a peak concentration in the middle of the experiment at 12% and ending the experiment at 6% of those detected in the effluent, thus active viruses make up a small proportion of viruses in the effluent throughout the experiment. While Exp.4b performed in the absence of bioactivity shows no clear separation, where active viruses begin the experiment at a concentration of 20% but an average percentage of active viruses (55 ±32%) shows high standard deviation and no obvious trends. This suggested an irregularity of active virus concentrations detected in the effluent over the duration of the experiment, with inactive viral particles outweighing active viruses in the effluent at the beginning of the experiment and again straight after the changeover to virus-free influent.

5.6 Detachment and inactivation rate of attached viruses

Viruses may be both reversibly and irreversibly adsorbed to sediment. Reversibly adsorbed viruses may be subject to equilibrium and kinetic adsorption mechanisms. Equilibrium adsorption, equal rates of attachment and detachment, was ignored due to its lack of applicability to results (e.g., Figure 4 shows no strong retardation of active viruses relative to the conservative tracer; and models with equilibrium adsorption equations are known to be inappropriate for breakthrough curves with a strong tailing effect also seen in Figure 4). In kinetic attachment-detachment, the two processes involve a mass transfer from one bulk matrix to another and in reverse (e.g., free-floating, to adsorbed, then desorbed). Active viruses recovered in effluent during the desorption period of Phase 2 accounted for active viruses that were reversibly attached and maintained infectivity after detachment. Those recovered as part of the slope from the tail of the breakthrough curve of free active viruses represent the maximum number of detached viruses from the sediment during Phase 2. This has traditionally been interpreted as a predicted rate of inactivation for viruses attached to sediment surfaces (i.e., surface inactivation) (Schijven *et al.* 1999). The slope of the breakthrough curve from the adsorbed active viruses measured during Phase 2 represent active viruses that have become irreversibly adsorbed. Irreversibly adsorbed active viruses can be considered a more accurate measure of the rate of inactivation specific to the processes occurring at the sediment surface. The statistical relationship between the predicted inactivation rate of attached viruses (i.e., the slope of the tail from the breakthrough curve of the free active viruses) and the measured inactivation rate of attached viruses (i.e., the slope of the breakthrough curve from the attached

active viruses) is summarised in Table 4. Both slopes are presented in Figure 7, adapted from Figure 4. The rate of active virus detachment predicted from the intercept of the height of the tail from the breakthrough curve of free active viruses (Schijven *et al.* 1999) is also presented in Table 4.

Table 4. Statistical relationship between the (a) predicted inactivation rate of attached viruses (i.e., the slope of the tail from the breakthrough curve of the active free-floating viruses) and (b) the measured inactivation rate of attached viruses (i.e., the slope of the breakthrough curve of the adsorbed active viruses). Detachment rate (K_{det}) predicted from the intercept of the height of the tail from the breakthrough curve of free-floating active viruses. Slope results from non-linear regression of C/C_0 values, also regression coefficients. Statistical comparisons of the predicted and measured inactivation rate of attached viruses from each experiment were made; to zero as no significant rate of change over the experimental duration; to the reference condition (Rf); between related conditions (Rel); and between predicted and measured slopes (Slopes). Significant differences were bolded.

Inactivation rate of attached viruses		K_{det}	a) Predicted					b) Measured					
		day^{-1}	Slope	R^2	Zero	Rf.	Rel.	Slope	R^2	Zero	Rf.	Rel.	Slopes
1a	Standard flow (Rf)	0.020	-1.60	100%	**<0.001**	-	0.60	-1.17	97%	**<0.001**	-	0.40	0.39
1b	Slow flow	0.019	-1.63	99%	**<0.001**	0.60		-1.41	96%	**<0.001**	0.40		0.40
2a	Low viral load	0.009	-0.68	89%	**<0.001**	**0.01**	0.86	-0.38	89%	0.10	**0.03**	0.87	0.40
2b	Medium viral load	0.011	-0.62	83%	**<0.001**	**<0.001**		-0.45	89%	0.21	**0.05**		0.59
3a	Anoxic conditions	0.003	-1.19	98%	**<0.001**	**0.02**	0.60	-0.95	89%	0.12	0.69	0.48	0.65
3b	Hypoxic conditions	0.002	-1.08	74%	**<0.001**	0.09		-1.45	95%	**0.02**	0.55		0.57
4a	Sterile conditions	0.022	-1.06	93%	**<0.001**	**0.04**	0.88	-0.99	88%	**0.01**	0.50	0.14	0.82
4b	Stimulated bioactivity	0.003	-1.12	87%	**<0.001**	0.14		-1.63	99%	**0.01**	0.10		0.32

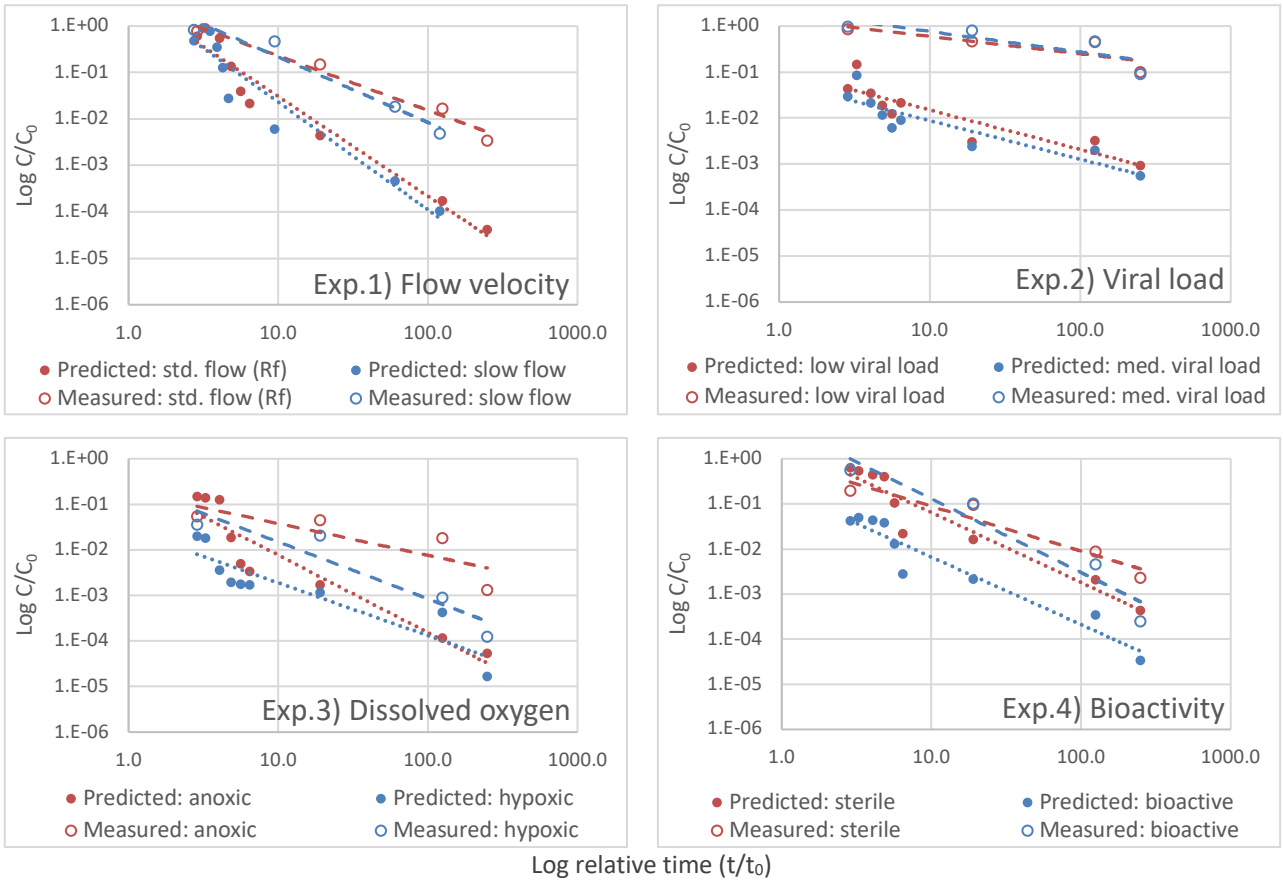


Figure 7. Graphical relationship between the predicted and measured inactivation rates of attached viruses (adapted from Figure 4). Trendlines fitted for each experiment; dotted lines reflect the solid colour data points; dashed lines reflect the unfilled data points. Reference condition (Rf).

In comparing the predicted and measured inactivation rates of attached viruses, no significant differences among all conditions (Table 4 Slopes) confirmed assumptions made by modelling that the inactivation of attached viruses can be predicted from the slope of the tail from the breakthrough

curve of the free active viruses (Schijven *et al.*, 1999). From experiments performed using the standard flow velocity (Exp.1a, Rf.) and slow flow velocity (Exp.1b), the measured inactivation rates of attached viruses are not significantly different from each other ($p = 0.40$), confirmed by the predicted rate of surface inactivation ($p = 0.60$). Exp.1a using the standard flow velocity and Exp.1b using a slow flow velocity show no significant differences between the predicted detachment rate (K_{det}). These results suggest that a decrease in flow velocity, from does not alter the rates of inactivation for attached viruses or kinetic detachment processes.

The measured inactivation rates of attached viruses under experiments performed with low (Exp.2a) and medium (Exp.2b) viral loads show no significant difference to each other ($p = 0.87$), confirmed by the predicted rate of surface inactivation ($p = 0.86$). In contrast, the measured inactivation rates of attached viruses for Exp.2a performed using a low viral load and Exp.2b using medium viral load are significantly different from the experiment performed using a high viral load (Rf.) (Exp.2a $p = 0.03$, Exp.2b $p = 0.05$), also seen by the predicted rate of surface inactivation (Exp.2a and Exp.2b $p = < 0.01$). Results suggest that the inactivation rate of attached viruses is similar under low (Exp.2a) and medium (Exp.2b) viral loads but is increased at high viral loads (Rf.), concluded by both predicted and measured methods. It is likely to expect that the inactivation rate of attached viruses decreases over the duration of the experiment (i.e., show a significant difference from zero). However, the measured inactivation rates of attached viruses in both experiments performed using a low (Exp.2a) and medium (Exp.2b) viral load showed a weak but not significant difference from zero (Exp.2a low viral load $p = 0.10$, Exp.2b medium viral load $p = 0.21$). Thus, the inactivation rate of attached viruses under these conditions is so slow over the experimental duration that it does not change significantly, although this would not be concluded had this result been determined from the predicted rate of inactivation for attached viruses (Table 4a). The detachment rate for both experiments performed using a low (Exp.2a) and medium (Exp.2b) viral load was half the value of the experiment performed using a high viral load (Rf.) (Table 4 K_{det}), suggesting that active viruses in viral loads below the medium concentration of 10^5 pfu mL^{-1} (Exp.2b) have decreased rates of detachment.

The measured inactivation rates of attached viruses in experiments performed under anoxic (Exp.3a) and hypoxic (Exp.3b) conditions do not differ significantly from each other ($p = 0.48$) confirmed by the predicted rate of surface inactivation ($p = 0.60$). The measured inactivation rates of attached viruses for both experiments also shows no significant difference from the experiment performed under oxic conditions (Rf.) (Exp.3a anoxic conditions $p = 0.69$, Exp.3b hypoxic conditions $p = 0.55$), although this would not have been concluded from the predicted rate of surface inactivation (Table 4a). The measured inactivation rate of attached viruses from Exp.3a performed in the absence of oxygen, shows a weak difference from zero ($p = 0.12$), also not concluded from the predicted rate of surface inactivation (Table 4a). These results indicate that experiments performed under various DO concentrations, whether anoxic (Exp.3a), hypoxic (Exp.3b), or oxic (Rf.), have little impact on the measured inactivation rate of attached viruses. Although, for Exp.3a performed in the absence of oxygen the measured inactivation rate of attached viruses is so slow that it does not change significantly over the experimental duration. Analysing from the predicted rate of surface inactivation alone (Table 4a) would result in different conclusions. The detachment rate for both experiments

performed under anoxic conditions (Exp.3a) and hypoxic conditions (Exp.3b), are within the range of each other but less than half of the rates observed under most other oxic conditions (Table 4 K_{det}), suggesting that under anoxic (Exp.3a) to hypoxic (Exp.3b) conditions detachment of active viruses is low.

In Exp.4a performed in the absence of bioactivity, the measured inactivation rate of attached viruses shows no significant difference to the experiment performed using background sediment bioactivity (Rf.) ($p = 0.50$), which is not confirmed by the predicted rate of surface inactivation (Table 4a). In Exp.4b performed in the presence of stimulated bioactivity, the measured inactivation rates of attached viruses showed a weak though not significant difference to the experiment performed using background sediment bioactivity (Rf.) ($p = 0.10$), confirmed by the predicted rate of surface inactivation ($p = 0.14$). Both experiments performed in the absence of bioactivity (Exp.4a) and in the presence of stimulated bioactivity (Exp.4b) showed a weak though not significant difference from each other ($p = 0.14$), also not concluded from the predicted rate of surface inactivation (Table 4a). These results suggest that the measured inactivation rates of attached viruses were not affected by the absence of bioactivity (Exp.4a) but were increased by the presence of stimulated bioactivity (Exp.4b), which could not be concluded from predicted rates alone. In addition, the detachment rate in Exp.4b performed in the presence of stimulated bioactivity, is as low as both the experiments performed under anoxic (Exp.3a) and hypoxic (Exp.3b) conditions. This is also much lower than in Exp.4a performed in the absence of bioactivity, which is in the range of most other oxic conditions, suggesting that the decreased detachment rate of Exp.4b is also linked to the presence of stimulated bioactivity and/or DO depletion.

5.7 Solution chemistry

The chemical composition of influent reservoir water and effluent porewater solutions from the various column experiments is presented in Table 5. Parameters measured from the effluent show little variation, neither by comparison to the influent, throughout the experiments (standard deviation < 2 mg L^{-1}, not shown), nor between experiments. However, there is a clear distinction between the composition of the influents; tap water and Danube Channel water. Notable differences include elevated background values of sodium, chloride, sulphate, phosphate, and reactive nitrogen species in Danube Channel water, compared to tap water (Table 5 Reservoir influent).

The concentration of DO shows a low standard deviation in values measured within and between various experiments. In experiments where DO was the primary variable, concentrations were consistent throughout the experiment where the anoxic condition of Exp.3a was maintained at 0 mg L^{-1} and the hypoxic condition of Exp.3b had a standard deviation of ±0.2 mg L^{-1}. Of note are the DO concentrations measured in the experiments testing variations of bioactivity (Table 5 Exp.4 DO), where a comparatively higher DO concentration was measured in the experiment performed in the absence of bioactivity (Exp.4a) and a low DO concentration was measured from the experiment performed under stimulated bioactivity (Exp.4b). These effects are a reflection of the stimulated bioactivity which consumed oxygen when present and was not available to consume oxygen when absent (i.e., sterile). Representing the typical background bioactivity of an oligotrophic aquifer, values of ATP concentration are shown for the sediment used in all experiments prior to any amendments

(Table 5 Reservoir influent s-ATP). Also, typical ATP concentration values for tap water and Danube Channel water as influent solutions prior to any amendments (Table 5 Reservoir influent w-ATP). Adenosine Triphosphate concentrations demonstrated the establishment of experiments testing variations in bioactivity (Exp.4) by a clear decrease (Exp.4a, sterile condition) or increase (Exp.4b stimulated bioactivity) of ATP in both the sediment and Danube Channel water (Table 5 Exp.4). For the experiment tested in the presence of stimulated bioactivity (Exp.4b), ATP results show that activity is largely concentrated on the sediment surface (Table 5 Exp.4b s-ATP).

Table 5. Composition of various influent and effluent solutions. Outflow chemistry averaged over the experimental duration; standard deviation not shown. Dissolved oxygen (DO) and Adenosine triphosphate (ATP) averaged across the experimental duration. Concentrations of ATP measured in water samples (w-ATP) presented as internal ATP values, concentration per L^{-1}. Concentrations of ATP measured in sediment samples (s-ATP) presented as total ATP values, concentration per g^{-1}. Values of ATP that were measured once but apply to the matrices used in other experiments shown in brackets. Danube Channel water (DCW). Absent values (-) not measured.

		Reservoir influent		Experimental effluent (Tap)					(DCW)	
				Flow Velocity	Viral Load		Dissolved Oxygen		Bioactivity	
				Exp.1a & 1b	Exp.2a	Exp.2b	Exp.3a	Exp.3b	Exp.4a	Exp.4b
Parameters		Tap	DCW		Low	Medium	Anoxic	Hypoxic	Sterile	Bioactive
Na+	mg L^{-1}	2.1	7.8	1.7	1.2	1.1	1.5	1.5	8.0	7.6
K+	mg L^{-1}	0.2	1.6	0.3	0.3	0.3	0.3	0.3	1.2	1.5
Ca^{2+}	mg L^{-1}	44.5	40.7	40.3	42.5	41.9	41.2	42.2	40.9	41.1
Mg^{2+}	mg L^{-1}	10.4	9.5	10.2	9.3	9.0	9.5	9.2	9.6	9.6
NH_4^+	mg L^{-1}	0	4.5	0	0	0	0	0	0.2	4.5
Cl-	mg L^{-1}	4.5	14.3	4.7	3.0	2.9	4.6	4.6	14.3	14.1
NO2-	mg L^{-1}	0	0.9	0	0	0	0	0	0	1.2
NO3-	mg L^{-1}	6.9	4.9	6.4	5.0	5.2	5.2	5.2	3.5	4.2
SO_4^{2-}	mg L^{-1}	18.2	22.9	15.2	12.5	12.3	16.1	16.0	23.1	22.9
PO_4^3	mg L^{-1}	0.005	299	3.2	1.2	5.6	0.1	0.4	231	255
DO	mg L^{-1}	-	-	6.4 ±0.8	6.2 ±0.6	6.4 ±0.9	0	0.8 ±0.2	7.2 ±1.2	0.2 ±1.0
w-ATP	mM	1.3 ±0.3	1.8 ±0.7	(1.3 ±0.3)	(1.3 ±0.3)	(1.3 ±0.3)	(1.3 ±0.3)	(1.3 ±0.3)	0.03 ±0.01	2.8 ±0.2
s-ATP	mM	1.2 ±0.4	-	(1.2 ±0.4)	(1.2 ±0.4)	(1.2 ±0.4)	(1.2 ±0.4)	(1.2 ±0.4)	0.09 ±0.03	52.2 ±4.2

Due to the clear decreased recovery rate of viruses in experiments performed under variations of DO (Exp.3), it seems possible that the second order decrease in DO concentration shown in the experiment performed under stimulated bioactivity (Exp.4b) may have contributed to the low recovery results. To confirm whether it was a contributing factor further analysis between the two experiments that resulted in low DO concentrations; Exp.3b the hypoxic condition; and Exp.4b the stimulated bioactivity condition, were investigated. When comparing the mean removal rate of free active viruses recovered in the effluent, Exp.3b performed under hypoxic conditions (4% recovery of free active viruses) showed a significant difference ($p = < 0.001$) to Exp.4b performed under stimulated bioactivity conditions (9% recovery of free active viruses). This indicates that free active viruses are decreased further by induced hypoxic conditions with typical background bioactivity (Exp.3b), rather than second order hypoxic conditions created from the stimulation of bioactivity (Exp.4b). This may be because, as previously shown, attached active viruses experienced some preservation on bioactively stimulated sediment, more of which were available to desorb into effluent than the few attached active viruses from the experiment performed under hypoxic conditions (Exp.3b). However, when comparing the measured inactivation rate of attached viruses there was a weak correlation ($p = 0.74$), confirmed by the predicted rate of surface inactivation ($p = 0.95$) and the detachment rate is almost exactly the same, suggesting that inactivation and kinetic detachment processes occurring at the sediment surface may be affected by low concentrations of DO.

6 Discussion

6.1 Standard experimental conditions

Experiment 1a was also known as the reference condition (Rf.) because it set the expectation for how MS2 phage transport and survival behaviour occurred in the sediment columns under standard experimental conditions including, standard flow velocity (1m d^{-1}), standard viral load (C_0 10^7pfu mL^{-1}), oxic conditions, and a background of typical sediment bioactivity for an oligotrophic aquifer with these conditions. The inactivation rate of free viruses measured from the reservoir solution was negligible for the duration of the experiment. Although, this was more accurately a measure of inactivation in the virus-tracer tap water and not representative of inactivation for free viruses in the porewater of the experiment, since there was no flow activity in the reservoir. Inactivation of MS2 in solution under 4°C within the pH range of tap water has been reported at 0.028-0.15 Log_{10} day^{-1} (Schijven *et al.*, 1999). Results of inactivation rates for free viruses across all experiments were within the range of these values (Section 5.2, Figure 3a). Findings from the novel sediment sampling method used in this study also confirmed the validity of interpreting the rate of inactivation for attached viruses from the slope of the tail from the breakthrough curve of free active viruses as predicted by Schijven *et al.*, 1999. Many other researchers have since adopted this approach in their analysis (e.g., Van Der Wielen, Senden and Medema, 2008; Sadeghi *et al.*, 2011; Lodder *et al.*, 2013; Frohnert *et al.*, 2014; Sasidharan *et al.*, 2017). However, the original authors acknowledged notable discrepancies in matching the rising and declining limbs of breakthrough curves with sensitivity analysis using the inactivation rate of attached virus from the results of fluid-phase concentrations alone (Schijven *et al.*, 1999). Schijven *et al.*, (1999) also noted mismatches between predicted and observed results of adsorbed viruses by sampling sediment. Such discrepancies may come from interpreting the rate of inactivation for attached viruses indirectly from the breakthrough curve of free active viruses, rather than developing a unique breakthrough curve of the sediment matrix. Results from the present study demonstrated that exploring the inactivation rate of irreversibly adsorbed viruses by sampling the sediment directly during transport experiments allowed for more accurate conclusions.

It is well established that the inactivation rate of attached viruses generally occurs at a faster rate than in solution (Loveland *et al.*, 1996). In a comparison to the inactivation rate of attached viruses, measured rates were two times faster than free viruses in the reservoir solution (Section 5.2, Figure 3b). The inactivation rates of attached viruses are strongly virus type-dependent, relative to the isoelectric point of the protein capsid (Harvey and Ryan, 2004). Similar inactivation rates have been recorded for MS2, for example model fitting by the evaluated study showed that the inactivation rate of attached viruses was generally three times faster than in solution under sand filtration (Schijven *et al.*, 1999). Ryan *et al.* (2002) explored the inactivation of attached viruses with the use of radiolabelled cellular components, also reporting rates three times greater than in solution measured using ferric oxyhydroxide-coated aquifer sand. By observing a difference in the release of radiolabelled and infective MS2, Ryan *et al.* (2002) concluded that the inactivation of attached viruses disintegrated MS2 particles rather than just rendering them inactive. Despite the current study applying molecular detection methods to enumerate total viral particles, results were not able conclude on the differences between the population of inactive viruses and decayed viral particles.

Harvey and Ryan (2004) have attributed the strong attachment force of ferric oxyhydroxides, which have opposite surface charge to MS2, to irreversibly attach and thus break down viral particles under oxic conditions. While no iron precipitates were visible in the porous medium of this study, it is likely from characteristics of the sample location that they were present.

The small particle size and conservative adsorption capacity of MS2 are reasons why it is regularly chosen to represent a worst case scenario (Schijven and Hassanizadeh, 2000), often making transport appear similar to conservative tracers. For example, Cao, Tsai and Rusch (2010) observed MS2 breakthrough to be similar to bromide in the presence and absence of OM and across a range of ionic strength solutions using high purity silica sand. Figure 4 shows that active viruses and total viral particles conform well to the fluorescent tracer. Quantitatively, all active viruses were recovered with 66% of the input concentration from free active viruses in effluent and 36% maintaining infectivity during attachment. Cao, Tsai and Rusch (2010) demonstrated 100% recovery of MS2 in solution following a virus-free flush of columns filled with high purity silica sand, prompting the conclusion that MS2 is effectively a conservative tracer in freshwater environments. Virus removal is calculated by inactivation and adsorption. With a third of the viral load adsorbed, indications were that the homogenous sediment under standard experimental conditions must have impurities responsible for adsorption. Although, inactivation rates calculated clear inactivation of attached viruses hence an error is presented in the recovery of attached active viruses, where some must have been eliminated though not quantitatively evidenced. Half of the free active viruses recovered in solution arrive by fast transport during the infiltration period, the other half during the desorption period (Section 5.4, Figure 5). Those recovered during fast transport present significant risk, characterised as low adsorbing, fast transporting and infectious. Kvitsand, H. M. L., Ilyas and Østerhus (2015) explored the consequences of fast flowing preferential paths in an oxic aquifer, suggesting that the risk of rapid transport for viable viruses should be explicitly recognised. It would be interesting to investigate if those recovered during the infiltration period are structurally or functionally different from those recovered following the changeover to virus-free influent. Researchers have identified the length of amino acid loops in the protein capsid of MS2 to explain additional steric repulsion measured in attachment efficiency calculations (Penrod, Olson and Grant, 1996). Population dynamics would suggest that capsid assembly occurs at different rates among individuals, where some maturation stages may be just as infectious but less adsorptive than others.

Attachment-detachment of viruses are dictated by electrostatic forces. Hydrophobic interactions were found to be of strong involvement in the adsorption of viruses to sandy mediums (Azadpour-Keeley, Faulkner and Chen, 2003). MS2 is relatively hydrophobic with few polar groups located on its capsid (Xagoraraki, Yin and Svambayev, 2014). As such, hydrophobic attraction, where non-ionic surfaces have a preference to associate with each other, is thought to be one of the main processes involved in MS2 adsorption (Xagoraraki, Yin and Svambayev, 2014). Although MS2 under the pH values of tap water were expected to have repelled from primary minerals making up the sampled sediment. Since no conditions completely remove free active viruses from effluent or attached active viruses eluted from sediment by the end of the sampling period and molecular detection was not applied to sediment samples, it is not possible to conclude how many inactive viruses remain

adsorbed. Free active viruses reach a peak that indicates almost all available adsorption sites were occupied by the changeover to virus-free influent, consistent with a similar peak of attached active viruses (Section 5.3, Figure 4). Thus, under standard experimental conditions, the standard viral load was capable of reaching close to equilibrium between the influent, effluent, and the porous medium in the sediment column (27.6g dry weight). Tong *et al.* (2012) investigated this effect as a jamming limit of viruses on particle surfaces, where adsorption rates level off when the available electrostatically attractive spaces have complete surface coverage. Their results confirmed that deposition kinetics were significantly increased by coating silica surfaces with clay (Tong *et al.*, 2012). While grain size fractions were separated in this study, it is not uncommon for clays to deposit on primary minerals and could not be completely excluded. Further charecterisation of the porous medium would support conclusions of geochemical heterogeneity in aquifer grains.

During breakthrough, total viral particles only diverge from free active viruses during the desorption period, where inactivation returns more inactive viral particles than active viruses. Quantitatively, the recovery of total viral particles equates to 76% of the input concentration, with almost two thirds detected during the desorption period adding support to the conclusion of inactivation for attached viruses where inactive particles must have been preferably released back into solution. Further analyses on the persistence of infectivity with time showed that active viruses begin the experiment at high percentages of the effluent, declining linearly to be outweighed by inactive viruses at the changeover to virus-free influent making up almost all of those detected in the effluent by the end of the experiment (Section 5.5, Figure 6). High proportions of active viruses detected in the effluent during early stages of the experiment suggested that fast transporting viruses were mostly of the active fraction. The linear decline indicates that there is a slow inactivation rate in porewater and preferential detachment of inactive viral particles. This action must not have been related to nucleic acid damage, perhaps interrupting infectivity at the protein level instead, since much of the input concentration could be detected by genome copies using qPCR analysis. Few studies have combined molecular and culture detection techniques in the same transport experiment, fewer sampling into the desorption period. However, Lodder *et al.* (2013) demonstrated almost identical MS2 breakthrough behaviour as a pilot used to calibrate a two-site kinetic transport model for the investigation of disinfection techniques. Prior to any treatments they observed that the breakthrough curve of free active viruses and total viral particles matched during slow sand filtration until the desorption period similar to results seen in Section 5.3, Figure 4. Lodder *et al.* (2013) also measured that infectious MS2 particles attached more to sand than defective particles, supporting assumptions in this study that adsorption is not relevant for non-active viral fractions.

6.2 Decreased flow velocity

Broadly characterised, adsorption is a time dependent effect thus slower flow velocities typically result in higher adsorption. In this study, few changes are observed when slowing the flow velocity from the experiment performed using a standard flow of 1m d^{-1} (Rf.) to Exp.1b performed using a slower flow velocity of 0.5m d^{-1}. Like results from the experiment performed using a standard flow velocity (Rf.), inactivation is twice as fast for attached viruses than free viruses indicating that halving the velocity by this degree, despite the increased residence time, does not significantly affect the

inactivation rate of attached viruses. Behaviour of the breakthrough curves were almost identical to the experiment performed using a standard flow velocity (Rf.) in divergence of total viral particles from free active viruses during the desorption period (Section 5.3, Figure 4) and the profile of infectivity with time (Section 5.5, Figure 6). Quantitatively, this equates to 68% of the total viral particles recovered compared to the input concentration, with no difference in effluent distribution to results from the experiment performed at 1m d^{-1} (Rf.). The mean removal rate of active viruses does not differ either, including similar peaks for active viruses in solution and adsorbed to sediment. Quantitatively, 83% of the active viruses can be recovered, with 58% in solution and 25% that remain infective during attachment. Of the combined recovery, active viruses detach similarly and distribute evenly between effluent periods for both flow velocity experiments.

Results from the experiment performed using a slower flow velocity showed a temporal difference between the arrival of the free virus breakthrough curves and the tracer (Section 5.3, Figure 4). Retardation is typically used to describe the slower release of reactive colloids, where arrival is delayed in the effluent relative to a conservative tracer (Jaramillo, 2012). This would imply that under decreased velocity active viruses during the fast transport period are retarded by the attachment-detachment mechanisms of the sediment. Since fewer active viruses are recovered from the sediment portion at experiments performed using flow velocities of 0.5m d^{-1} than from 1m d^{-1} (Rf.), results suggest there is a measurable degree (17-32%) of elimination related to the adsorbed phase. This decrease is attributed to a combination of colloid filtration mechanisms leading to inactivation and/or decay of attached viruses. Results correlate with modelling by Han (2008) which concluded that colloid deposition was significantly increased with decreasing flow velocity. Mechanisms were attributed to the increased contact efficiency of slower flows, facilitated by reduced fluid drag and Brownian diffusion (Han, 2008). Additional residence time leading to longer exposure to developing biogeochemical conditions which influence survivability, may also have contributed to removal by inactivation on sediment surfaces as suggested by Verbyla and Mihelcic (2015).

6.3 **Lowered viral load**

Experiments testing variations of viral load represent scenarios where the viral load may increase or decrease relative to seasonal occurrence, climatic events, proximity to compromised wastewater infrastructure, or disturbances in hydrological setting. Surrogate bacteriophage are often used in transport experiments at high concentrations in order to maintain detectability. However, in a review of pathogenic viruses, concentrations of 10^4-10^5 copies g^{-1} were frequently detected in dewatered sludge (Xagoraraki, Yin and Svambayev, 2014). Therefore, investigating viral load was tested by reducing the input concentration from a high value (Rf.) by a factor of three Log units for Exp.2a performed using a low viral load (10^4 pfu mL^{-1}) and two Log units for Exp.2b performed using a medium viral load (10^5 pfu mL^{-1}). Analysed separately, both conditions show few differences between each other but demonstrate clear changes from the experiment performed using a high viral load (Rf.). Inactivation rates of medium to low viral loads in the reservoir solution decreased faster than the high viral load (Rf.) (Section 5.2, Figure 3), however no further adjustments were made to the breakthrough curves or the recovery values. As a result, both values were likely to have an inherent factor of inactivation in the porewater solution. The high rates of inactivation for free viruses in

solution may be explained as a dose-response effect, where lower concentrations in a dilute solution effectively limits aggregation behaviour. Galasso and Sharp (1965) showed that isolated viral particles are much easier to destroy than those in groups. Conversely, the inactivation of attached viruses for both low and medium viral load experiments were measured at rates two times slower than inactivation of free viruses in the reservoir solution (Section 5.2, Figure 3).

The mean removal rates of experiments performed using low and medium viral loads do not show any difference between each other, however both experiments did show a significant difference to the experiment performed using a high viral load (Rf.). This is because quantitatively, for experiments performed using low and medium viral loads, the bulk of active viruses were concentrated in the sediment matrix with 72% of the low viral load and 90% of the medium viral load recovered as attached viruses and only 24% and 15% recovered as free viruses from the effluent, respectively (Section 5.4, Figure 5). The maximum recovery of attached active viruses in the experiment performed using a high viral load (Rf.) suggests that the available sorption spaces and their respective jamming limit in the sediment of the column must be higher than the medium viral load. Thus, even with the high adsorption under experiments performed using low and medium viral loads, electrostatically attractive spaces must have been left available by incomplete surface coverage. Of those adsorbed to sediment, results showed that the inactivation rate of attached viruses was so slow that the concentration does not decrease significantly over the experimental duration. This suggests that the ability of viruses to remain active whilst adsorbed might be concentration dependent. The rate of desorption also decreased with viral load, where the desorption rate of active viruses was roughly the same between experiments performed using low and medium viral loads but approximately half of the experiment performed using a high viral load (Rf.).

Mechanisms controlling these differences are likely the result of nanoscale heterogeneities on the grain surface between the experiments performed using a high viral load (Rf.) and medium to low viral loads. For example, it is well established that surfaces coated with dissolved OM exhibit physicochemical properties and hence reactivities that differ substantially from uncoated bare surfaces (Armanious, 2014). It may be that interactions of free viruses and virus-coated sediment surfaces from the experiment performed using a high viral load (Rf.) also had an altered surface charge potential and thus a different electrostatic environment to those experiments using lower viral loads. Bradford and Bettahar (2006) showed that an attachment and detachment model was only consistent with results when a time dependent straining parameter of blocking, by the filling of straining sites, was included. They concluded that straining was only hindered at higher collision frequencies due to repulsive colloid-colloid interactions (Bradford and Bettahar, 2006). The difference between an adsorption site presenting as reversible or irreversible is related to the surface charge potential between the porous medium and virus. Primary minerals should not have had an attractive surface charge to MS2 under pH of the tap water solution, leaving enough impurities under oxic conditions to adsorb almost the entire viral load of 10^7 pfu mL^{-1} during the infiltration period (Rf.) in 27.6g dry weight of the porous medium from each sediment column. For desorption to occur, some of those surfaces must have presented as weakly attractive, to allow adsorbed viruses to become reversibly attached as they did in the experiment performed using a high viral load (Rf.).

With a series of strong and weakly adsorbing sites, the most attractive sites may have preferentially adsorbed viruses outcompeting weakly adsorbing sites under lower viral loads. Zhang *et al.* (2010) found that desorption using MS2 attached to red soil was not time dependent, with the measured difference in virus recovery over a series of reaction times minimal because any possible desorption was completed within the first reaction time. Since impurities may vary in composition, results from experiments performed using low and medium viral loads could be the effect of preferential adsorption to strongly attractive attachment sites and fast detachment of any reversibly adsorbed viruses from weak mineral sites.

Of the free active viruses recovered from the effluent, less than 5% of both experiments using a medium and low viral load arrive during the fast transport period while the rest are recovered during the desorption period (Section 5.4, Figure 5). Despite being such a small concentration, free active viruses recovered by fast-transport during the infiltration period survive the low-aggregation inactivating environment of the reservoir and do not adsorb despite an overabundance of available spaces. Researchers have noted that enteric viruses exhibit an infective dose as low as 10^2 pfu (Pinon and Vialette, 2018). Results from this study suggested that even 5% of the 10^4-10^5 pfu mL^{-1} loads used in these experiments released into a previously unexposed sediment with attachment surfaces available, might still result in breakthrough of between 10^2-10^3 pfu at a fast-transporting rate. Quantitatively, the experiment performed using a medium viral load recovered 24% of the total viral particles suggesting that a large proportion of the total viral particles were still in the active fraction (Section 5.4, Figure 5). As such, any notable inactivation of free viruses in the porewater solution disintegrated viral genomes beyond detection levels. Regardless, the overall recovery of free active viruses in the porewater solution was low as evidenced by the persistence of infectivity with time, where active viruses remain around 10% of those detected in effluent throughout the duration of the experiment (Section 5.5, Figure 6). This result supports the theory of surface preservation, where the high recovery of attached active viruses were able to remain active throughout the experiment.

6.4 Anoxic conditions

Experiments performed using variations in DO concentration represent scenarios where viruses transport through natural subsurface gradients of oxic to hypoxic and anoxic conditions, typically driven by microbial oxygen consumption. Inactivation rates of free viruses in the reservoir solution varied widely between Exp.3a performed under anoxic conditions and Exp.3b performed under hypoxic conditions. The longest survival rate of free viruses was measured in the anoxic reservoir solution, but it is likely an overestimate since little change in the concentration of free active viruses was actually detected (Section 5.2, Figure 3). Regardless, researchers support a conclusion of prolonged virus survival in anoxic conditions citing the absence of reactive oxygen species that can decay capsid proteins (Frohnert *et al.*, 2014). Inactivation of attached viruses in the experiments performed under anoxic and hypoxic conditions occur faster than either inactivation rates of free viruses measured from their respective reservoir solutions (Section 5.2, Figure 3). However, results suggest that experiments performed under various DO concentrations, whether anoxic, hypoxic, or oxic (Rf.), have little impact on the measured inactivation rate of attached viruses (Section 5.6, Figure 7). Although, in the absence of oxygen the measured inactivation rate of attached viruses was so

slow that it did not change significantly over the duration of the experiment, which is suggestive of surface preservation under anoxic conditions.

Free active viruses recovered in the effluent of the experiment performed in the absence of oxygen arrived earlier than all other breakthrough curves (Section 5.3, Figure 4). While preferential transport was not formally quantified, the significance was detected across eight replicate columns measured in effluent earlier than six other experiments performed using the same flow velocity, each with eight replicate columns. Dissolved oxygen concentrations have a significant effect on the valence state of trace metals. In anoxic conditions metal-bearing impurities that would otherwise expose positively charged surfaces capable of adsorbing negatively charged viruses like MS2, are solubilised. While viruses were not significantly enhanced by preferential transport, the movement promoted through sediment columns might reflect the lack of precipitated metal oxides known to adsorb and also likely to disrupt the passage of free active viruses under oxic conditions. Furthermore, free active viruses reach an early plateau that was around 1 Log unit less than the input concentration (Section 5.3, Figure 4) suggesting that the jamming limit, or available attachment spaces, had decreased from the standard input concentration that reached a relative value close to 1 C/C_0 in the effluent of the experiment performed under oxic conditions (Rf.). Van Der Wielen, Senden and Medema (2008) report reduced collision efficiencies under anoxic conditions when compared to oxic conditions, which was used as evidence for the changes in mineral dissolution states and their relationship to adsorption.

Findings often show higher recovery of bacteriophage in experiments performed under anoxic conditions, when compared to the recovery under oxic conditions (Schijven and Hassanizadeh, 2000; Van Der Wielen, Senden and Medema, 2008; Frohnert *et al.*, 2014). This appears to be in contradiction with the outcomes of this study, although such results are notably virus dependent. For example, Van Der Wielen, Senden and Medema (2008) investigated removal in a sandy anoxic aquifer demonstrating generally slower inactivation and adsorption under anoxic conditions for MS2 and ΦX174. However, the inactivation rate of MS2, while lower than other published oxic conditions, was much higher than ΦX174 (Van Der Wielen, Senden and Medema, 2008). Frohnert *et al.* (2014) compared the transport of MS2 to ΦX174 under oxic and anoxic conditions using a range of ionic strength solutions in sand columns. MS2 results were consistently lower than ΦX174 irrespective of the condition, leading to no conclusions drawn from the experiments performed using MS2 (Frohnert *et al.*, 2014). Quantitatively, in the absence of oxygen only 27% of the free active viruses were recovered, with 20% in effluent and 7% maintaining infectivity during attachment (Section 5.3, Figure 4). The mean removal rate of free active viruses was significantly lower than the experiment performed under oxic conditions (Rf.). This is inconsistent in theory, but other studies demonstrate that drawing conclusions from MS2 as a model phage in experiments performed under decreasing DO concentration is more difficult than with other virus types. Of the free active viruses recovered in effluent, approximately half arrive by fast transport during the infiltration period and half during the desorption period (Section 5.4, Figure 5). The detachment rate of active viruses under both experiments performed under anoxic and hypoxic conditions was less than half the detachment rate taken from the experiment performed under oxic conditions (Rf.) (Section 5.6, Figure 7). Although,

this is likely a reflection of the fact that few attached active viruses were even measured from the adsorbed phase to be detached.

Like the experiment performed under oxic conditions (Rf.), the breakthrough curves of free active viruses and total viral particles diverge during the desorption period where more inactive viruses were returned in the effluent. Although only 31% of the total viral particles can be recovered compared to the input concentration, with around two thirds detected during the desorption period. The results of virus recovery from the breakthrough curves suggest an elimination of around 70% (Section 5.4, Figure 5). Active viruses begin the experiment at more than half of those detected in the effluent and remain at a constant value during the infiltration period but then shift dramatically to a low concentration, largely outweighed by inactive viral particles shortly after the changeover to virus free influent (Section 5.5, Figure 6). Results suggest that by ceasing the virus-tracer infiltration, the recovery of active viruses in solution also decreases indicating a link to the solution chemistry. Results from the experiments performed by Van Der Wielen, Senden and Medema (2008) showed MS2 recovery that was always low, ranging from 5% to less than 1% throughout their experiments. Like the methods of this current report, Van Der Wielen, Senden and Medema (2008) established oxygen-free conditions with the use of sodium dithionite. They suspected the presence of sodium dithionite was detrimental to MS2 and following evaluation concluded that MS2 was sensitive towards the effects of the reducing agent (Van Der Wielen, Senden and Medema, 2008). A preliminary survival study was done for this current report (results not shown). Observations of an early die-off period were followed by a stable population which guided adjusted of the input concentration value and adding a settling period to compensate. Instead, it may be that MS2 is already ineffective as a model for experiments investigating variations in DO concentration concluded by Frohnert *et al.*, (2014), combined with a sensitivity to sodium dithionite (Van Der Wielen, Senden and Medema, 2008). It is also possible that MS2, like other viruses, may have an oxygen preference that should be considered when selecting model phage for decreased DO concentration experiments. For example, indicator selection and analysis may benefit from considerations of work done by Morinet *et al.* (2015) where it was established that infection can vary in response to hypoxia, namely upregulation or downregulation in the rate of reproduction relative to virus type.

6.5 Hypoxic conditions

In Exp.3b performed under hypoxic conditions, inactivation of free viruses in the reservoir solution was considerably fast and comparable to values measured for inactivation of viruses attached on the surface (Section 5.2, Figure 3). Thus, inactivation contributed to removal by mechanisms affecting both the free viruses in the reservoir solution, suggesting steep inactivation of free viruses in the porewater solution, and inactivation of attached viruses on sediment surfaces. In contrast to methods used to establish the experiment performed under anoxic conditions, the hypoxic environment was simulated by nitrogen sparging alone. This means that, while inactivation can be associated with the oxygen preference of MS2, it cannot be linked to use of the reducing agent. Transitional biogeochemistry that occurs between the exposed aerobic sections of aquatic sediments and unexposed oxygen-depleted sections is highly complex. Many factors known to impact virus

transport and survival across these regions, including the decrease of surrounding bioactivity, redox reactions controlling the precipitation and dissolution of attachment sites, and subsequent changes in water chemistry that alter the surface potential of minerals and viruses including pH, ionic strength, and presence of divalent cations (Harvey and Ryan, 2004; Jaramillo, 2012). The experiments performed using reductions in the concentration of DO may not have been able to simulate all these changes in the column space or over the experimental duration, nor were all these parameters monitored. However, the occurrence of background sediment bioactivity typical for an oligotrophic aquifer and the presence of iron oxyhydroxides, which were both established in the experiment performed under oxic conditions (Rf.), were likely to be altered in the columns under experiments performed under decreased DO concentration. Although, neither of these factors contributed to the measure of inactivation for free viruses in solution, since they do not occur in the reservoir. To be sure results were correct, this experiment was repeated on three separate occasions presenting similar low recovery breakthrough curves of free active viruses and similar inactivation rates of free viruses in the reservoir solution (results not shown).

Like the experiment performed under anoxic conditions, free active viruses plateaued at a low concentration over the breakthrough curve (Section 5.3, Figure 4). For the experiment performed under hypoxic conditions, the concentration of free active viruses was detected at 2 Log units less than the input concentration. Quantitatively, this resulted in the lowest measure of active viruses recovered, with just 4% of the input concentration calculated from the effluent and 4% maintaining infectivity during attachment (Section 5.4, Figure 5). Of those recovered in the effluent, most are detected by fast transport during the infiltration period, with < 0.5% detected during the desorption period. Thus, few if any of the attached active viruses were detached (i.e., they experienced irreversible adsorption) or detached in an activated state (i.e., they experienced inactivation on the sediment surface). Evidence for the significant inactivation that occurred can be observed in the breakthrough curves of total viral particles, consistently measured at concentrations 2 Log units higher than free active viruses throughout the experiment (Section 5.3, Figure 4). Results showed that free active viruses contributed only 5-10% of those detected in effluent during the entire experimental period (Section 5.5, Figure 6). Quantitatively, this equates to 42% of the total viral particles recovered compared to the input concentration and, like the anoxic condition, around two thirds are detected during the desorption period (Section 5.4, Figure 5) supporting conclusions of attached virus inactivation on the sediment surface. Recovery suggests that the experiment performed under hypoxic conditions had a degree of between ~60-90% elimination (Section 5.4, Figure 5).

The steep inactivation rates measured in the reservoir solution can only be explained by the unstable nature of MS2 in environments with low DO concentrations. However, Heffron, McDermid and Mayer (2019) identified the environment induced by low DO conditions as one which could apply to the mechanisms occurring inside the columns, specifically inactivation of free viruses in the porewater solution and attached to sediment surfaces. Their study explored iron based water disinfection techniques, observing that slower oxidation rates in sediment columns maintained by low pH and DO concentration (< 2 mg L^{-1}) were the most promising for inactivation of MS2 (Heffron,

McDermid and Mayer, 2019). The mechanism was described by dose-limited disinfection, where better contact between viruses and reactive partially hydrolysed ferrous iron facilitated more rapid inactivation compared oxic and anoxic conditions (Heffron, McDermid and Mayer, 2019). The effect of precipitate size in the experiment of this study may also be a contributing factor, attributed to the relatively high ionic strength of tap water due to dissolved cations like calcium, magnesium, sodium and the presence of anions like sulphate and chloride, also present in groundwater. Van Der Wielen, Senden and Medema (2008) have suggested an increase in the length of protection zones for known anoxic abstraction wells to account for the increased transport and survival. However, results from this study indicate that it is possible viruses transporting from oxic to anoxic regions may be further reduced by additional mechanisms in the transitional hypoxic zones.

6.6 **Stimulated bioactivity**

Biological process and community composition in aquatic sediments are generally dictated by available nutrients. Experiments performed using variations of bioactivity represent scenarios on either side of the surface water-groundwater interface, where bioactivity concentrates on the side of unfiltered nutrients, light, DO and warmer temperatures to decrease beyond the interface into the aquifer as these parameters filter (Jaramillo, 2012). Unfortunately, measurements of inactivation in the reservoir solution for free viruses was not representative of Exp.4b performed in the presence of stimulated bioactivity, since the reservoir solution had low measurable bioactivity (Section 5.7, Table 5). Inactivation of free viruses was more accurately relative to the solution chemistry of the Danube Channel water with a nutrient treatment, which had little to no impact on virus survival over the experimental duration (Section 5.2, Figure 3). The inactivation rate of attached viruses occurred two times faster than inactivation of free viruses in the reservoir solution and appears to inactivate more rapidly than all other experimental conditions. This is supported by the measurements of ATP, which showed that bioactivity was heavily concentrated on the sediment surface with markedly less than in the influent or effluent solutions (Section 5.7, Table 5). Concentrations of ATP from sediment were measured by total values including both intracellular and extracellular ATP, thus the sediment concentration demonstrates bioactive microbes on grain surfaces and ATP-bearing biproducts. The nutrient treatment developed observable biofilm formation within the columns, however hydraulic conductivity was maintained thus bio-clogging did not occur.

Many factors have been proposed for the attenuation of viruses by the presence of bioactivity. Although, based on community composition and microbial density, the mechanisms could be the result of simultaneous factors. Free active viruses showed a significant difference compared to the mean removal rate measured from the experiment performed with typical background bioactivity (Rf.). Quantitatively, combined recovery returns 34% of the active viruses, although only 9% of these were recovered in the effluent solution with a clear plateau that was 2 Log units less than the input concentration (Section 5.3, Figure 4). Of those recovered in the effluent, 3% are detected by fast transport during the infiltration period with the rest recovered during the desorption period (Section 5.4, Figure 5). Despite the steep rate of inactivation for attached viruses, the bulk of active viruses (25%) were still recovered from the sediment matrix, indicating that sediment largely shielded active viruses. Adhesion is a possible viral resistance mechanism in bioactive environments, where organic

particles shield a virus from antiviral agents or the ability of virucidal disinfectants to reach the virus (Pinon and Vialette, 2018). However, such adhesion behaviour would have to be partial to the elution method used to enumerate attached active viruses in this current study. It is also possible that viruses removed by adsorption to mineral impurities, as opposed to those removed by adhesion to biofilm, were more easily eluted thus recovering a fraction of the viruses selectively attached to minerals. Irreversible attachment was concluded as one of the dominant factors in MS2 removal by Ramazanpour Esfahani *et al.* (2020), where biofilm formation was developed in sand columns using injection of treated wastewater. Selective attachment would also indicate that the presence of biofilms did not completely obstruct mineral attachment surfaces.

In contrast, the breakthrough curve of the total viral particles diverged from active viruses by 1 Log unit throughout the entire experimental duration, with the peak arriving earlier in solution than all other breakthrough curves (Section 5.3, Figure 4). Quantitatively, this equates to 63% of total viral particles recovered compared to the input concentration, with three quarters detected during the desorption period compared to the low number of active viruses (6%) recovered in solution during that same time (Section 5.4, Figure 5). This indicated that, while active viruses can be preserved on sediment surfaces, inactive viral particles outweighed active viruses throughout the experiment. Results suggest that the inactivation rate of attached viruses was linked to the presence of stimulated bioactivity. For example, the inactivation rate of attached viruses measured in the experiments performed in the absence of bioactivity and performed using typical background bioactivity (Rf.) showed a difference to the same rate of change measured from the experiment performed in the presence of stimulated bioactivity (Section 5.6, Figure 7). Mechanisms of inactivation occurring on sediment surfaces may be due to the production of proteolytic enzymes destroying the viral capsid (Azadpour-Keeley, Faulkner and Chen, 2003) while irreversible attachment could be attributed to viral adhesion, enmeshment and/or sedimentation (Liu, Liu and Tay, 2004; Templeton, Andrews and Hofmann, 2008). More accurate insight would be gleamed from applying molecular detection techniques to the sediment matrix to quantify the number of inactive viruses recovered from adsorption/adhesion. Results suggest a degree of between 37-66% elimination in the presence of bioactivity. Additional analysis could confirm if viruses are just inactivated and/or irreversibly attached on the surface of sediments, or if mechanisms causing elimination including genomes decayed beyond detection levels and/or consumed by grazing protozoa or predatory flagellates (Deng *et al.*, 2014) are a contributing factor.

The detachment rate of active viruses was low, measured in the range of the experiments performed under hypoxic and anoxic conditions and much lower than the experiment performed under conditions of typical background bioactivity (Rf) and the experiment performed in the absence of bioactivity (Section 5.6, Figure 7). Since the presence of stimulated bioactivity led to a rapid decrease in DO concentrations, any mechanisms that occurred in the experiment performed under hypoxic conditions could also have existed under the experiment performed in the presence of stimulated bioactivity. For example, the previous suggestion citing dose-limited iron-based disinfection (Heffron, McDermid and Mayer, 2019) might also have impacted virus survival in the columns of the experiment performed in the presence of stimulated bioactivity, however viral resistance by

adhesion could negate some of these effects. In comparing results, the outcomes showed that free active viruses decreased further by induced hypoxic conditions with typical background bioactivity as in the experiment performed under hypoxic conditions, rather than hypoxic conditions created from the stimulation of bioactivity (Section 5.7, Table 5). This may be because, as previously explained, attached active viruses experienced some preservation on bioactively stimulated sediment, creating an infectious reservoir. This meant more active viruses were available to desorb into effluent than the few attached active viruses from the experiment performed under hypoxic conditions. However, in a comparison of the inactivation rates for attached viruses, the weak correlation between the experiments performed under hypoxic conditions and with stimulated bioactivity and almost exactly the same detachment rate, both suggested that inactivation and kinetic detachment processes occurring at the sediment surface may be affected by low concentrations of DO (Section 5.7, Table 5). Although this would be better confirmed by the application of molecular detection techniques to sediment samples.

6.7 Eliminated bioactivity

Groundwater aquifers are often characterised as low activity environments hence their use as clean drinking water resources. Results showed a significant difference in the mean removal rate of free active viruses between the experiment performed in the absence of bioactivity (Exp.4a) and the experiment performed in the presence of stimulated bioactivity (Exp.4b). The solution chemistry of Danube Channel water, used in both experiments, showed elevated levels of sodium, chloride, sulphate, phosphate, and reactive nitrogen species. The presence of divalent cations like calcium and magnesium are thought to increase attachment by binding with the carboxyl functional groups in the protein capsid or with surface hydroxyls and cation exchange sites on minerals, thus reducing surface charge densities and decreasing double layer repulsion (Schijven and Hassanizadeh, 2000; Harvey and Ryan, 2004). However, monovalent cations like the elevated sodium in the Danube Channel water is typically abundant in groundwater and will likely not bind to surface functional groups. Although Harvey and Ryan (2004) suggested that monovalent cations may influence the double layer thickness between grains and viruses, possibly increasing attachment by a small degree. The abundance of monovalent anions in solution such as chloride, sulphate and phosphate are likely a reflection of dissolved organic matter. The adsorption of dissolved organic matter is favourable on positively charged mineral surfaces like the impurities identified earlier, known to block virus attachment or to reduce and even reverse the surface charge of mineral surfaces (Harvey and Ryan, 2004), which would also be unfavourable for attachment of negatively charged MS2. All these effects are dependent on pH values which were not monitored during the experiments, although tap water pH was at 7-8.3 and Danube Channel water was 7.45-7.90. Reactive nitrogen species like ammonium are known for their virucidal activity (Bosch, Pintó and Abad, 2006), however they are most effective at slightly acidic levels and the concentrations in Danube Channel water were so low that they likely had little effect on virus survival.

While it is possible that many of the solution chemistry mechanisms were in place in the experiment performed under stimulated bioactivity, viruses may have been shielded from such effects by the presence of bioactivity/biofilms thus observing virus transport and survival in the absence of

bioactivity (Exp.4a) reduced conclusive variables. Free active viruses in the experiment performed in the absence of bioactivity showed no significant difference in mean removal rates from the experiment performed under typical background bioactivity conditions (Rf.) (Section 5.3, Figure 4). Quantitatively, the combined recovery shows that most if not all active viruses are returned, however the bulk were heavily concentrated in the effluent at 85% of the input concentration (Section 5.4, Figure 5). In the breakthrough curves, the peak of the free active viruses also occurred earlier in the effluent than all other conditions, while those that maintain infectivity during attachment account for only 12% of the input concentration suggesting that the elevated sodium from the Danube Channel water did little to enhance adsorption (Section 5.3, Figure 4). The attachment-inhibiting behaviour from the abundance of chloride, sulphate and particularly high phosphate concentrations in Danube Channel water may have been more dominant under the experiment performed using absent bioactivity conditions and could explain the low recovery from sediment. Researchers have established that dissolved OM competes with viruses for hydrophobic attachment sites (Farkas, Varsani and Pang, 2014). However, Bales *et al.* (1991) established that this effect was strongly virus-dependent, with dissolved OM found to promote hydrophobic interactions between grain surfaces and relatively hydrophobic viruses like MS2, not seen for PRD1. In fact, Zhang *et al.* (2010) even showed that soil sterilisation increased dissolved organic carbon concentrations which then resulted in more MS2 removal by adsorption, not seen for ΦX174. Since low adsorption capacity occurs in this study, it may be that the separated sand fraction of the sampled sediment simply had no organic carbon to dissolve.

Of the free active viruses recovered in solution, one-third arrive by fast transport during the infiltration period and the other two-thirds were recovered during the desorption period. This is unusual since the adsorbed portion was so low that it is hard to interpret where this large recovery comes during the desorption period, if not the remobilisation of adsorbed viruses during infiltration. Unless this condition had an early release effect of free active viruses in the effluent that was not captured by the sediment-attached mass recovery which only sampled from Phase 2 onwards. Zhang *et al.* (2010) demonstrated that any possible MS2 desorption in their experiments occurred within the first reaction time of 20 minutes. Results of this study lumped recovery times together by the changeover to virus-free influent, however attachment and detachment could also have occurred before the changeover. Quantitatively, 78% of total viral particles were recovered compared to the input concentration suggesting that a large proportion of the total viral particles detected in effluent were still in the active fraction, measured by around two thirds during the desorption period. (Section 5.4, Figure 5). However, results from analysing the persistence of infectivity with time showed an irregular pattern where active viruses fluctuated in concentration between 20-80% of those detected in the effluent over the entire experiment (Section 5.5, Figure 6). This would suggest that active viruses exited the columns in irregular bunched concentrations, since it occurs across both phases this might be an aggregating effect of the sterilised Danube Channel water solution. Generally, survival is expected to be prolonged in sterile solutions. The rate of inactivation for free viruses in the sterile Danube Channel water reservoir solution was comparable to the inactivation rate of free viruses from the reservoir of the reference condition (Section 5.2, Figure 3). The reservoir of the reference condition used tap water and had low measurable bioactivity, indicating that sterilised

Danube Channel water did not impact survival. While the inactivation of attached viruses, like the experiment performed under typical background bioactivity (Rf.), was two times faster than in solution with no significant difference between the measured inactivation rates of attached viruses (Section 5.6, Figure 7). These conditions also share the same detachment rate. All these factors reduce the likelihood of sterile Danube Channel water affecting the inactivation of viruses in the columns. Perhaps the most likely outcome is that concentration shifts are simply showing the range of variation between detection limits of both molecular and assay methods, particularly if humic acids were present in the Danube Channel water since they are known to interfere with molecular extractions.

7 Summary & Conclusion

Objectives of this study were to explore the mechanisms of inactivation, attenuation and elimination, using uniquely combined detection methods, under experimental conditions that simulated common subsurface gradients. Various conditions included Exp.1a performed using a standard flow velocity (1 m d^{-1}), a standard viral load (C_0 10^7 pfu mL^{-1}), under oxic conditions, and a background of typical sediment bioactivity (Rf.); Exp.1b using a slow flow velocity (0.5 m d^{-1}); Exp.2a performed using a low viral load (C_0 10^4 pfu mL^{-1}); Exp.2b using a medium viral load (C_0 10^5 pfu mL^{-1}); Exp.3a performed under anoxic conditions; Exp.3b under hypoxic conditions (< 2 mg L^{-1} DO concentration); Exp.4a performed in the absence of bioactivity; and finally, Exp.4b in the presence of nutrient stimulated bioactivity. Current transport and survival mechanisms obtained through other sediment column studies are frequently limited to interpretations based on the profile of free-floating active viruses. This study demonstrated that a more sensitive measure of removal mechanisms can be illuminated by differentiating between discrete fractions of free active viruses, attached active viruses, and inactive viral particles. These distinctions provided insight into the behaviour of viruses during passage through aquatic sediments, such as the recovery of free active viruses by fast transport during Phase 1 (i.e., the infiltration period); of free active viruses by reversible adsorption during Phase 2 (i.e., the desorption period); of attached active viruses by irreversible adsorption during Phase 2 (i.e., the desorption period); and the recovery of inactive viral particles throughout the experiments.

Results from the experiment performed using a standard flow velocity demonstrated typical inactivation rates between free and attached viruses, a slow decline in the concentration of active viruses compared to inactive viral particles and helped to establish the maximum concentration limit for adsorbed viruses from the sediment column under standard experimental conditions (Rf.). By slowing the flow velocity, the sediment-attached fraction showed a small degree of elimination that was due to inactivation of adsorbed viruses driven by features that characterise slow velocity environments. By decreasing the viral load, the recovery of active viruses was largely concentrated in the sediment matrix. Attached viruses experienced low rates of inactivation suggesting that that processes on the surface of sediments may be concentration dependent and points to mechanisms of preferential adsorption-desorption relative to strong and weak attachment sites. In experiments performed under anoxic and hypoxic conditions, the main mechanism of removal was elimination. This is in contrast to studies which observe high recovery in the absence of oxygen compared to oxic conditions. However, like other studies, results suggests that MS2 is particularly unsuitable for investigating the effects of varying DO concentration. Under hypoxic conditions, findings point to additional mechanisms of inactivation and elimination that are specific to the transitional state of low DO concentrations. In the experiment performed under stimulated bioactivity, the addition of active viruses adsorbed to the sediment matrix contributed to recovery three times more than the low concentration in solution. However, the rate of inactivation for attached viruses was also a dominant feature, leading to high recovery of inactive viral particles in solution. Thus, mechanisms point to sediment bioactivity as a driver of viral inactivation as opposed to elimination. In contrast,

the absence of bioactivity lead to high recovery of free active viruses and extremely limited removal by adsorption.

Inactivation of free viruses was characterised in the reservoir solution using batch experiments and from the effluent by combining results obtained from the application of qPCR analyses recovering total viral particles and plaque assays detecting infectious viral agents. As in many previous studies, inactivation of free viruses when sampling solution from parallel batch experiments would suggest that the inactivation of free viruses in the sediment porewater was negligible in the timescale of the experiment. However, the combination of viral detection methods applied to the effluent demonstrated a changing population of both free active viruses and inactive viral particles recovered from the sediment porewater during transport experiments. This differentiation challenges the conventional use of approximating inactivation in solution from batch experiments to transport experiments. Results suggest that a more precise representation of the persistence of viral infectivity with time, relative to mechanisms occurring inside the porewater of sediment columns, can be obtained by combining identifying the active and inactive viral fractions in effluent.

In recent reviews, researchers point to the investigation of retained microbes in sediment column studies as a more sensitive measure of attenuation behaviour. In this study, the use of replicate columns to investigate the temporal variation of retained viruses provided the opportunity to reliably account for the changing concentration of adsorbed viruses with time. By using a virus pulse and extending analysis into the desorption period; the recovery of irreversibly adsorbed (i.e., retained) viruses, conventionally thought to be eliminated from the aquatic system, could be distinguished from reversible adsorption where active viruses could continue to desorb into solution, versus true elimination where viruses decayed beyond detection levels. Such conclusions demonstrated the difference between environmental conditions that could be destructive to viruses, versus conditions which simply inactivated viruses, or worse preserved them in sediment as an infectious reservoir. Further analysis using the application of qPCR on sediment samples could provide additional insight into the destructive or simply inactivating nature of irreversible adsorption under experimental conditions.

It is well recognised that virus inactivation rates are relative to whether they are free-floating or attached to the sediment matrix, although different inactivation rates are rarely provided in transport studies. Researchers that do report inactivation rates for attached viruses tend to infer these values indirectly from the slope of the tail of a breakthrough curve measured by free active viruses, as suggested by the results of an early modelling prediction. The inactivation rate of attached viruses during transport was presented in this study, accompanied by a comparison between the methods of predicted and measured rates. Measured results demonstrated the validity of the prediction method, however outcomes also established that further interpretation can be gained from direct sampling of sediment-attached viruses that indirect predictions could not provide. To facilitate better cross study comparisons and build more robust modelling approaches, inactivation rates of attached viruses should be measured directly.

7.1 Outlook for Riverbank Filtration

The location that sediment was sampled from for this study, Flehe waterworks in Düsseldorf, has been using RBF since 1870. Bank filtrate from this waterworks is the primary source of public drinking water supplied in this region. However, indications are that pathogenic viruses may not be sufficiently removed by RBF technology, particularly as the diversity, frequency and concentration of viral contaminants increases with the growth of populations and agricultural expansion. Viral recovery rates are constrained not only by the setting and design of RBF sites, but also the local environmental and hydrogeological properties. As such, viral risk-based analysis of the boundaries at each RBF site are developed through three main elements: (1) sample monitoring to assess the current risk, (2) transport modelling to predict the potential of risk scenarios, and (3) enhanced operation of filtration technology to avoid future risk. Results and data produced in transport studies like this one are required to inform risk-based analysis, by developing a conceptual understanding of the removal dynamics during water filtration.

Presented conclusions contribute to each of the elements listed above. In the context of sample monitoring, results from this study demonstrate that investigating areas expected to have low to medium viral loading or high sediment bioactivity should include both the fluid and sediment matrices. This is because testing of fluid concentrations alone would lead to considerable underestimation of risk, particularly if mechanisms controlling virus attachment were subject to changing environmental conditions. For accurate transport models to be developed, this study promotes a series of recommendations, alongside a dataset that will be incorporated into an existing predictive model. First, findings show that models should contain two different inactivation rates relative to processes occurring in the solution versus on the surface of sediments, particularly in experiments where reversible/irreversible adsorption is high. Second, results establish that low to medium viral loads, which represent a more realistic concentration relative to natural occurrences in the environment, behave differently to standard viral loads. Thus, modelling should consider accounting for changing viral load as a primary rather than secondary factor. And third in the context of transport studies, researchers should promote an indicator concept that considers the oxygen preference of model bacteriophage. Hopefully this leads to further charecterisation of other phage ‘preferences’ that can be built into a more robust viral indicator selection, making choice easier for water managers seeking to use indicators in the field. The presented results also conform to methods already used by water managers to enhance removal, namely reduced fluid flow during groundwater recharge that leads to increased removal by filtration. However, findings also promote some additional operational considerations, such as the possibility that viruses passing from oxic to anoxic environments through a hypoxic zone may experience significantly increased removal rates by unique mechanisms occurring specifically in this region. Although, if that same hypoxic zone was characterised by high bioactivity, as is often the case, then the action of biofilm formation may provide additional surface preservation to adsorbed viruses. These results should encourage water managers to schedule abstraction and recharge periods during times of high sediment bioactivity such as warmer months or locate abstraction wells in regions that have a flow path passing through areas of high bioactivity.

The continued performance of RBF techniques relies on an empirical understanding of factors that influence the transport and survival of pathogenic microbes through aquatic sediments. This study has presented the use of a novel suite of methods to quantify removal of viruses through dynamic hydrological, physicochemical, and biological gradients. Exploration of these natural transitions by predictive modelling will provide a better understanding of the mechanisms governing virus attenuation. Reliable characterization on the fate of waterborne viruses has significant implications for water managers tasked with accurately assessing and avoiding the risks of groundwater contamination, particularly in light of the current pressure on water resources and the future outlook on water demands.

8 Reference list

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