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The fate of pathogens during bank filtration with emphasis on hydrological extremes and the application of microbial data in ecological groundwater monitoring

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不忘初心，方得始终

Never forget why you started, and your mission can be accomplished.

Contents

Abstract

Bank filtration is a well-proven and reliable approach to generate raw water for drinking water production, especially where local groundwater resources are insufficient or of low quality. As a way of managed aquifer recharge, bank filtration, a process of guiding surface water through bank sediment to a production well, improves water quality and quantity. During bank filtration, inorganic compounds, organic contaminants, and pathogenic microorganisms and viruses are reduced in concentration and/or mass to varying degrees by hydrodynamic (e.g., convective-dispersive transfer, dilution), mechanical (e.g., straining, settling, filtering), biological (e.g., biodegradation), and physico-chemical processes (e.g., adsorption, precipitation). Pathogenic viruses are one important health concern during bank filtration and in drinking water production because they are characterized by small colloid size, high resistance, high persistence, and low infectious dose compared to other pathogens. In addition, short travel times, elevated source concentration, and other pressures brought by more frequent climate extremes may pose a serious threat to the efficiency of bank filtration in attenuating pathogenic viruses. Because the concentration of pathogenic viruses in the natural aquatic environment is generally below the routine detection limit, this risk of infection has been ignored by humans until cases of large-scale epidemic infections have emerged. Attenuation of pathogens is one of the several essential ecosystem services provided by bank sediments and shallow aquifers. Furthermore, although groundwater is the priority drinking water resource, it is threatened by many factors including intensive agriculture, urbanization, increasing industrial activities, and extreme climate conditions. To better identify and explore the possible reasons for the disturbances and outliers in a group of local groundwaters, it is a pressing need to develop a universally applicable tool for the assessment of the groundwater microbiological-ecological status.

With regard to these challenges, this thesis, first of all, provides a comprehensive evaluation of individual processes involved in the attenuation of allochthonous pathogenic viruses during bank filtration, with a particular focus on the fate of sediment adsorbed viruses and extreme hydrological events. Field experiments were conducted using a sediment column for the simulation of bank filtration at the German Environment Agency's field station in Berlin-Marienfelde. Quantitative polymerase chain reaction (qPCR) and plaque assay were used in combination to quantify total and infectious allochthonous viruses (bacteriophage MS2) in sediment pore water and adsorbed to the sediment matrix, thereby targeting individual attenuation processes. We found that at an input virus concentration of 1.84×10^8 gene copies

mL^{-1} total MS2 was reduced by 0.8 log units, while the fraction of infectious MS2 was reduced by 1.7 log units after 80 cm of sediment passage. An even more efficient removal (3.0 log_{10}) of infectious MS2 was obtained at a lower MS2 load (1.9×10^6 plaque forming units mL^{-1}). As a general observation, the proportion of infectious MS2 phages to total MS2 particle mass constantly declined over time and with sediment depth. A pulse of deionized water simulating a heavy rain event caused a pronounced remobilization of phages formerly adsorbed to the sediment matrix, especially with the fraction of infectious MS2. Therefore, attention should be paid to possible secondary contamination caused by hydrological extremes, e.g., heavy rain, infiltrating into virus-contaminated bank sediment.

Subsequent to this first study, the thesis comprehensively investigated the dynamics of adenovirus, fecal bacteria, coliphages, other biological indicators, and water physico-chemical parameters at a riverbank filtration waterwork in Düsseldorf Flehe, Germany. Different methods were employed to monitor individual biological parameters, i.e., total adenovirus was quantified by qPCR, active fecal bacteria (*E. coli* and coliforms) was enumerated by the Colilert-18 method, *C. perfringens* was quantified by membrane method, infectious coliphages were detected by plaque assay, total prokaryotic cells and total viral-like particles were counted by flow cytometry, microbial activity was determined via ATP measurement. We found that during >72 m of passage through the river bank, i.e., from river channel to the production well, the coliforms were reduced most efficient (>3.6 log_{10}), followed by *E. coli* (>2.9 log_{10}), somatic coliphage (>2.5 log_{10}), *C. perfringens* (>2.3 log_{10}), F+ coliphage (>1.6 log_{10}), total viral-like particles (1.6 log_{10}), and total prokaryotic cells (1.4 log_{10}). Adenovirus was removed in the range of 1.6 and 3.1 log units during the transport through >32 m of bank sediment, i.e., from the river channel to the first row of monitoring well, and was not anymore detected in further distant wells. The relatively highest removal of most biological indicators was achieved at high river level conditions, which were characterized by elevated concentrations of pathogens in the surface water. At low river levels, coliforms and *C. perfringens* were occasionally presented in the raw water at the production well. Through statistical correlation analysis, we found that adenovirus correlated with different biological indicators and physical-chemical parameters at different river levels during bank filtration. In conclusion, a combination of *E. coli*, coliforms, and somatic coliphages may best predict the performance of bank filtration in terms of adenovirus removal.

Finally, this thesis reports the development of a universal tool for the monitoring of the microbiological-ecological water quality based on the measurement of prokaryotic cell counts,

microbial activity, and bioavailable organic carbon present in the groundwater. Data for prokaryotic cell counts and microbial activity are easy to acquire by means of flow cytometry and a simple bio-luminescence assay. In addition, if needed, the optional variable assimilable organic carbon can be quantified indirectly via a batch bacterial growth assay. Data from individual criteria are later merged to an index by means of an effective multivariate metric (i.e., Mahalanobis distance). With the established approach, disturbances can be identified with wide-spread types of groundwater threats, such as organic contamination, surface water intrusion, and agricultural land use. Additionally, this ecological tool's high sensitivity and robustness are shown by comparison with separate univariate analyses of the individual criteria. It is shown that groundwaters of different aquifer types (porous, karst, fissured) in different regions of Germany have an individual microbiological-ecological signature. We concluded that in the future, ecological criteria should be considered in groundwater quality monitoring, in addition to physical-chemical and quantitative parameters.

Zusammenfassung

Uferfiltration ist ein etabliertes und verlässliches Verfahren zur Anreicherung von Rohwasser für die Trinkwasserproduktion, vor allem in Gebieten wo locale Grundwasservorkommen nicht ausreichen oder eine unzureichende Grundwasserqualität vorliegt. Bei dieser gezielten Grundwasseranreicherung wird Oberflächenwasser durch Ufersedimente und dem oberflächennahen Aquifer einem Förderbrunnen zugeführt und während dieser Passage in seiner Qualität verbessert. Es kommt bei der Uferfiltration durch verschiedene Prozesse zu einer Reduktion in Konzentration und Masse von anorganischen und organischen Schadstoffen als auch von pathogenen Mikroorganismen und Viren. Beteiligte Mechanismen sind die hydrodynamische Durchmischung (Verdünnung), mechanische Filtration (z.B. ‚Straining = Stranden', ‚Settling = Absetzen'), Adsorption und Ausfällungen, und der biologische Abbau. Eine wichtige Schadstoffklasse aus Sicht der Trinkwasserproduktion und der menschlichen Gesundheit bilden die pathogenen Viren, da sie als sehr kleine und robuste Partikel mit einer niedrigen Infektionsdosis ein besonderes Risiko für einen weiten Transport und langes Überdauern im Grundwasser darstellen. Zudem stellt die zunehmende Häufigkeit von hydrologischen Extremereignissen im Zuge des Klimawandels mit einer erhöhten Virenlast im Oberflächenwasser und schnelleren Fließgeschwindigkeiten im Untergrund eine Gefahr für die Reinigungseffizient bei der Uferfiltration dar. Da jedoch die Nachweisgrenze für humanpathogene Viren sehr hoch ist, oft über den typischen Konzentrationen der Viren in Oberflächengewässern, wurde diese mögliche Gefahr lange Zeit ignoriert. Verschiedene epidemische Ausbrüche von Krankheiten die sich auf viren-verunreinigtes Wasser zurückführen liessen zeigen ein deutliches Bild. Ohne Zweifel, ist die Reduktion schädlicher Inhaltsstoffe, wie etwa pathogene Viren, eine essentielle Ökosystemleistung der Ufersedimente und oberflächennahen Grundwassersysteme. Experten sind sich einig, dass Ökosysteme solche Leistungen nur umfassend erbringen können wenn sie in einem guten ökologischen Zustand sind. Es bedarf daher geeigneter Indikatoren und Kriterien um den ökologischen Zustand von solchen Ökosystemen zu evaluieren, da die Einflüsse sehr vielfältig sind und aus den unterschiedlichsten Bereichen kommen, z.B. aus der Urbanisierung, Landwirtschaft, Industrie und Klimaextremen. Universell einsatzbare Bewertungsansätze sind demzufolge dringendst gefordert.

Im Zusammenhang mit den zuvor angeführten Herausforderungen fokusierte sich diese Promotionsarbeit auf die unterschiedlichen Prozesse bei der Uferfiltration die zu einer

Reduktion von Viren beitragen. Im Besonderen, und damit im Unterschied zu bereits durchgeführten Studien, berücksichtigen diese neuen Arbeiten nicht nur im Porenwasser suspendierte Virenpartikel, sondern betrachten auch die ans Sediment adsorbierte Fraktion. Weiters wird neben den ‚aktiven' (infektiösen) Viren auch die Gesamtheit aller Virenpartikel betrachtet. Nicht zuletzt fanden besondere hydrologische Ereignisse, wie Fluß-Hochwasser und –Niedrigwasser, besondere Berücksichtigung. Freilandstudien wurden an einer Test-Uferfiltrationsanlage des Umweltbundesamtes in Marienfelde und einem Uferfiltrations-Trinkwasserwerk in Düsseldorf Flehe durchgeführt.

In Marienfelde wurde die Infiltration von unterschiedlichen Virenlasten im Oberflächenwasser durch eine Sedimentsäule (wassergesättigter Lysimeter) verfolgt. In einem weiteren Versuch, wurde durch die Zugabe von deionisiertem Wasser ein Starkregenereignis simuliert um die Mobilisierung zuvor ans Sediment sorbierter Viren zu untersuchen. Als Modellvirus wurde der Bakteriophage MS2 verwendet, der mittels quantitativer PCR (qPCR) in seiner Gesamtabundanz erfasst wurde. Mittels ‚Plaque Assay' (Plattentest) wurde die Anzahl an aktiven Virenpartikeln bestimmt. Untersucht wurden Wasser als auch Sedimentproben. Die Ergebnisse zeigen dass, bei einer hohen Virenlast von $1{,}84 \times 10^8$ MS2 Genkopien mL^{-1} eine Reduktion der Gesamtvirenzahl um 0.8 log-Einheiten und eine Reduktion der aktiven Viren um 1,7 log-Einheiten erreicht wurde nach einer Transportstrecke durchs Sediment von 80 cm. Bei geringeren Virenlasten war die Reduktionseffizienz noch deutlich höher (≤ 3.0 $\log_{10}$). Generell wurde befunden dass der Anteil aktiver MS2 Virenpartikel über die Zeit und mit zunehmender Sedimenttiefe abnahm. Das simulierte Regenereignis führte zu einer ausgeprägten Mobilisierung (Desorption) zuvor adsorbierter Viren, insbesondere von ‚aktiven' Partikeln. Es sollte daher zukünftig ein Augenmerk auf die Belastungshistorie von Sedimenten und hydrologischen Extremereignissen gelegt werden.

Als Fortsetzung der Experimente in Marienfelde wurde am Standort Düsseldorf Flehe Oberflächenwasser aus dem Rhein und Uferfiltrat bzw. Grundwasser aus Messstellen in unterschiedlicher Entfernung zum Fluß und dem Förderbrunnen für das Rohwasser monatlich über einen Zeitraum von 16 Monaten auf Adenoviren und verschiedene Fekalindikatoren (z.B. *E. coli*, Koliforme, *Clostridium perfringens*, Koliphagen) hin untersucht. Erneut kamen verschiedene molekulare Methoden (z.B. digital droplet PCR) und Kultivierungsmethoden zum Einsatz. Die Untersuchungen an diesem realen Wasserwerksstandort zeigen, dass auf einer Fließstrecke von mindestens 70 m zwischen Fluß und Förderbrunnen die Koliformen am effizientesten in ihrer Konzentartion reduziert wurden, i.e. >3,6 $\log_{10}$, gefolgt von *E. coli* (>2,9

log_{10}), somatischen Koliphagen (>2,5 log_{10}), *C. perfringens* (>2,3 log_{10}), F+ Koliphagen (>1,6 log_{10}), der Gesamtzahl an Virenpartikeln (1,6 log_{10}), und der Bakteriengesamtzellzahl (1,4 log_{10}). Adenoviren wurden um 1,6 bis 3,1 log-Einheiten reduziert in einer Ufersedimentpassage von >32 m, danach ware sie nicht mehr nachweisbar. Die größte Reduktion an Viren und Mikroorganismen erfolgte in allen Fällen schon in den ersten Sedimentabschnitten. Interessanterweise, waren die absoluten Rückhalte- und Reduktionseffizienzen (in log-Einheiten oder in %) für beinahe alle Indikatoren am höchsten während der Hochwassersituation, bedingt durch stark erhöhten Konzentrationen im Oberflächenwasser. Während einer langen Niedrigwasserphase und Hitzeperiode kam es gelegentlich zum Durchbruch von Koliformen und *C. perfringens* beim Förderbrunnen. Adenoviren korrelierten in ihrer Verteilung und Reduktion mit unterschiedlichen Fekalindikatoren und kaum mit geläufigen physikalisch-chemischen Messgrößen. Um das Verhalten von Adenoviren während der Uferfiltration bestmöglich zu kontrollieren, emphiehlt sich ein kombiniertes Monitoring von *E. coli*, Koliformen und Koliphagen.

In ihrem letzten Abschnitt berichtet diese Arbeit über ein neues Konzept zur mikrobiologischen-ökologischen Bewertung von Grundwassersystemen. Es basiert auf der simultanen Erfassung der Gesamtbakterienzahl (D für ‚density'), der mikrobiellen Aktivität (A für ‚activity'), und optional dem gelösten organischen Kohlenstoff (C für ‚carbon'). Mittels Durchflußzytometrie und einem Biolumineszenz-Test lassen sich schnell und einfach Messwerte für die oben erwähnten Parameter generieren. Unter Anwendung eines statistischen Verfahrens (Mehalanobis Distanz) können die Messwerte in einen Index (D-A Index oder D-A-C Index) verrechnet werden der sich zur integrativen Bewertung und zum Aufspüren von Kontaminationen und Störungen eignet. Im Rahmen diese Arbeit wurde der Einfluß von (i) organischen Belastungen (Altlast), (ii) Uferfiltration, und (iii) von unterschiedlicher Landnutzung auf die mikrobiologisch-ökologische Grundwasserqualität untersucht. Es wird auch gezeigt, dass Grundwässer aus verschiedenen Regionen in Deutschland, in Abhängigkeit zur Geologie, dem Grundwasserleitertyp, und der Hydrochemie, ihre eigene mikrobiologisch-ökologische Signatur haben. Das neue Bewertungs- und Biomonitoringverfahren soll zukünftig ergänzend zu bisherigen Parametern zum Einsatz kommen um seine Einsetzbarkeit und mögliche Einschränkungen zu validieren.

List of Scientific Communications

Scientific Communications Related to the Dissertation

Peer-reviewed publications

1. **Wang, H.**, Kaletta, J., Kaschuba, S., Klitzke, S., Chorus, I., Griebler, C. Attachment, re-mobilization, and inactivation of bacteriophage MS2 during bank filtration following simulation of a high virus load and an extreme rain event. *Journal of Contaminant Hydrology*. 2022, 103960.
2. **Wang, H.**, Knabe, D., Engelhardt, I., Droste, B., Rohns, H., Stumpp, C., Ho, J., Griebler, C. Dynamics of pathogens and fecal indicators during riverbank filtration in times of high and low river levels. *Water Research*. 2021, 117961.
3. Fillinger, L., Hug, K., Trimbach, A. **Wang, H.**, Kellermann, C., Meyer, A., Bendinger, B., and Griebler, C. The D-A-(C) index: a practical approach towards the microbiological-ecological monitoring of groundwater ecosystems. *Water Research*. 2019, 163, 114902.

Conference contributions

1. **Wang, H.**, Griebler, C. Spatio-temporal dynamics in natural attenuation of pathogens during river bank filtration. Poster presentation at 17th International Symposium on Microbial Ecology (ISME 17), Leipzig, Germany, August 12-17, 2018.
2. **Wang, H.**, Knabe, D., Engelhardt, I., Droste, B., Rohns, H., Stumpp, C., Ho, J., Griebler, C. Dynamics of pathogens and fecal indicators during riverbank filtration in times of high and low river levels. Presentation at EGU22 (European Geosciences Union), Wien, Austria, April 3-8, 2022

Other Scientific Communications Not Related to the Dissertation

Peer-reviewed publications

1. Marozava, S., Meyer, A.H., Pérez-de-Mora, A., Gharasoo, M., Zhuo, L., **Wang, H.**, Cirpka, O.A., Meckenstock, R.U., Elsner, M. Mass transfer limitation during slow anaerobic biodegradation of 2-methylnaphthalene. Environmental Science & Technology. 2019, 53, 9481-9490.

List of Figures

1. Introduction

1.1 An introduction to bank filtration

The production of safe drinking water in sufficient quantity and quality relies on a sustainable source of water in high quality and a set of technical treatment methods. Groundwater where available in sufficient amounts is the preferred source of drinking water globally because it is generally of higher quality than surface water (Carrard *et al.*, 2019; Völker and Borchardt, 2019). Where groundwater resources are under threat from overexploitation and pollution, surface water is alternatively used. However, surface waters are often polluted by chemicals and pathogens via raw and treated sewage inputs, land-based runoff, and open defecation (Boehm *et al.*, 2019). Between 54% and 98% of the American surface water bodies assessed by Boehm *et al.* (2019) were contaminated with pathogens. Bank filtration of surface water is considered a reliable treatment technology for the production of high-quality raw water and subsequent drinking water production. It exploits natural biological, mechanical, and physical processes to purify the surface water, as explained in detail in Section 1.2. In bank filtration, surface water is guided through bank sediment and a shallow aquifer to a pumping well, i.e., production well. During the underground passage, suspended and dissolved contaminants, including pathogenic bacteria and viruses, are reduced in numbers and inactivated to varying degrees.

The approach of bank filtration has been used for more than 200 years. The earliest known bank filtration system was started since 1810 in Scotland by the Glasgow Water Works consisting of a perforated collector pipe (Maliva, 2019). After its successful operation, the technique was adopted by many other cities in the UK and eventually spread throughout Europe, e.g., to cities in the Netherlands, Belgium, Sweden, France, and Austria (Sprenger *et al.*, 2017). The advantages of bank filtration have also been recognized and applied in Germany, and today 300 more water suppliers make use of this technology. In the city of Düsseldorf, Germany, riverbank filtration has been used for more than 150 years in the production of drinking water (Schubert, 2002a). Approximately 75% of the drinking water in Berlin, Germany, is produced by bank filtration (Schmidt *et al.*, 2003). Bank filtration and infiltration, another method of water pretreatment using underground passage, were used by 5% and 17% of all drinking water sources in Europe (2013) and Germany (2010), respectively (*Figure 1*). Currently, the

production of drinking water via bank filtration is not only widespread in Europe, but also South America, North America, and Asia (Gillefalk *et al.*, 2018).

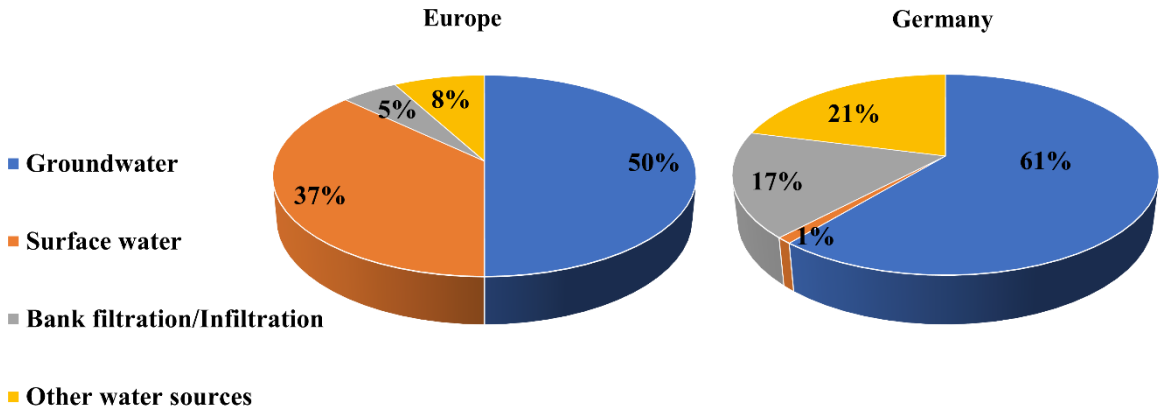

Figure 1 Sources of drinking water in Europe and Germany (adapted from Völker and Borchardt, 2019 and Trinkwassertalsperren et al., 2018).

Depending on the type of surface water, bank filtration can be further subdivided into riverbank filtration, lake bank filtration, and canal bank filtration, with riverbank filtration being the most commonly used approach (Dillon *et al.*, 2019). Historically, vertical and horizontal collector wells have been used primarily for riverbank filtration worldwide due to site conditions and the choice of the waterwork (Hunt *et al.*, 2006; Ray *et al.*, 2002). The horizontal collector well is sunk into the ground with one circular central collection caisson and the connected horizontal lateral well screens pushed out into unconsolidated aquifer deposits, while the vertical well is drilled directly downward into aquifers (*Figure 2)*. However, a combination of the application of vertical wells and horizontal collector wells can also be used in bank filtration systems (Hunt *et al.*, 2006). Nowadays, the siphon tube concept, which uses a pump to withdraw water from the manifold connected to one or more vertical collector wells, has been a popular technique in German waterworks, e.g., the Düsseldorf Flehe waterworks (one of our studies), due to its low operation and maintenance costs at a high rate of water production (Hunt *et al.*, 2006).

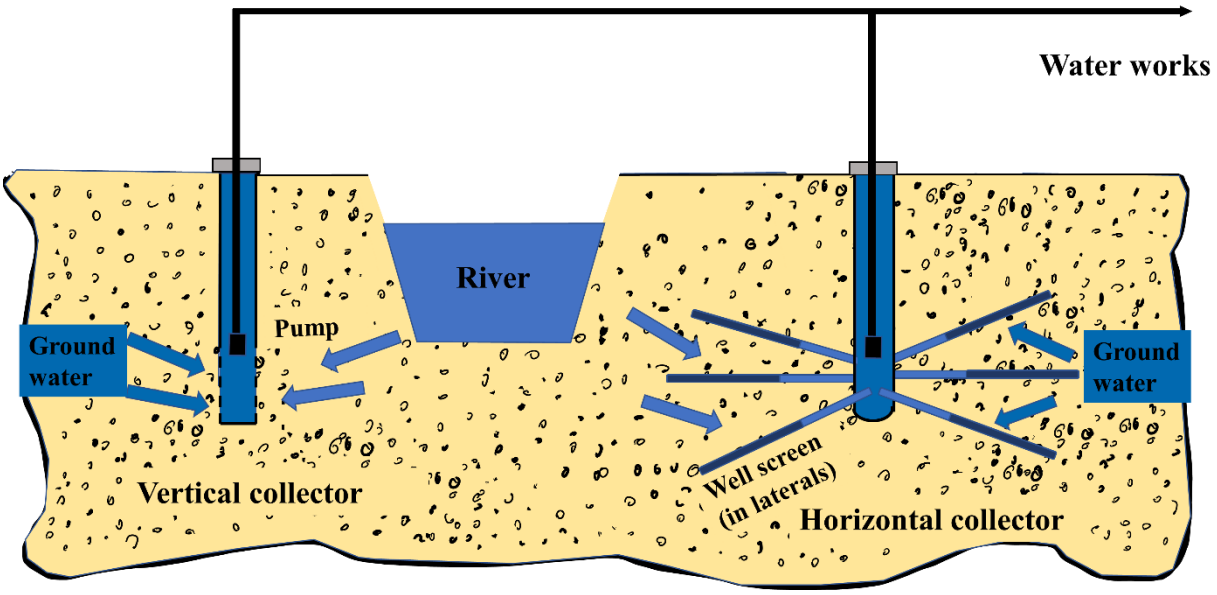

Figure 2 Schematic diagram of riverbank filtration with a vertical and horizontal collector well.

1.2 Attenuation processes during bank filtration

Contaminants contained in surface water are attenuated during underground sediment passage due to a combination of hydrodynamic, mechanical, biological, and physio-chemical processes (Doussan *et al.*, 1998).

1.2.1 Hydrodynamic processes

Hydrodynamic processes in bank filtration include convective-dispersive transport and dilution (Doussan *et al.*, 1998).

Convective-dispersive transport of temporal contaminated compounds is governed by many factors, such as water velocity, bank sediment permeability, and diffusion rate. The contaminated compounds keep spreading over a larger area with less concentration as time goes on (Mustafa *et al.*, 2014). Consequently, few temporal concentration variations are found in the aquifer.

Dilution takes place when the recharged surface water mixes with the groundwater in the aquifer and the production well. The mixing proportion of waters depends on the extraction rate and surface water flow. If the water transported through the bank sediments and the groundwater have different chemical characteristics, the water in the production well reflects

the degree of mixing. For example, agricultural activities can contaminate groundwater to a high concentration of nitrate, while the surface water filtered through the bank sediments generally has a much lower nitrate concentration. In consequence, the raw water, being a mixture of bank filtrate and contaminated groundwater, possesses a better water quality than the groundwater alone. With pathogens, it often is the other way round, because surface waters are more often contaminated with bacteria and viruses of fecal origin. In addition, high surface water levels during flood events may cause excessive inflow of surface water into the shallow aquifer, reducing or even eliminating the positive effect of dilution.

1.2.2 Mechanical processes

Mechanical processes mainly contribute to improve the water quality by removing suspended material including fine sediments, particulate matter (e.g., microorganisms and viruses), and substances adsorbed to the particles, especially in the first few meters of the river bank passage (Doussan *et al.*, 1998; Jaramillo, 2011). Although the mechanical process is often described as the most important attenuation process during bank filtration, the potential clogging of the porous media is a weakness. Besides filtering, straining and settling (also termed as "sedimentation in pores") are two main processes involved in mechanical attenuation as shown in *Figure 3*.

Straining refers to the mechanical removing process of the particles that are small enough to enter the porous media but sieved by smaller pore space (McDowell-Boyer *et al.*, 1986). It depends on the size of pore throats of bank filter media and the size of the suspended particles (Schijven *et al.*, 2002). Straining occurs when the ratio of the colloid and median grain diameter is >0.5% (Bradford *et al.*, 2004). And if this ratio is >10%, particles cannot penetrate anymore through porous media, i.e., they are attenuated by filtering (McDowell-Boyer *et al.*, 1986). Straining is considered one of the main processes for attenuating bacteria during bank filtration (McDowell-Boyer *et al.*, 1986).

Settling is also recognized as sedimentation in pores. The velocity of settling depends on the mass density difference between the particle and the fluid, gravity, and particle size (Jaramillo, 2011). A low water flow velocity increases the chance for settling to occur (Schijven *et al.*, 2002).

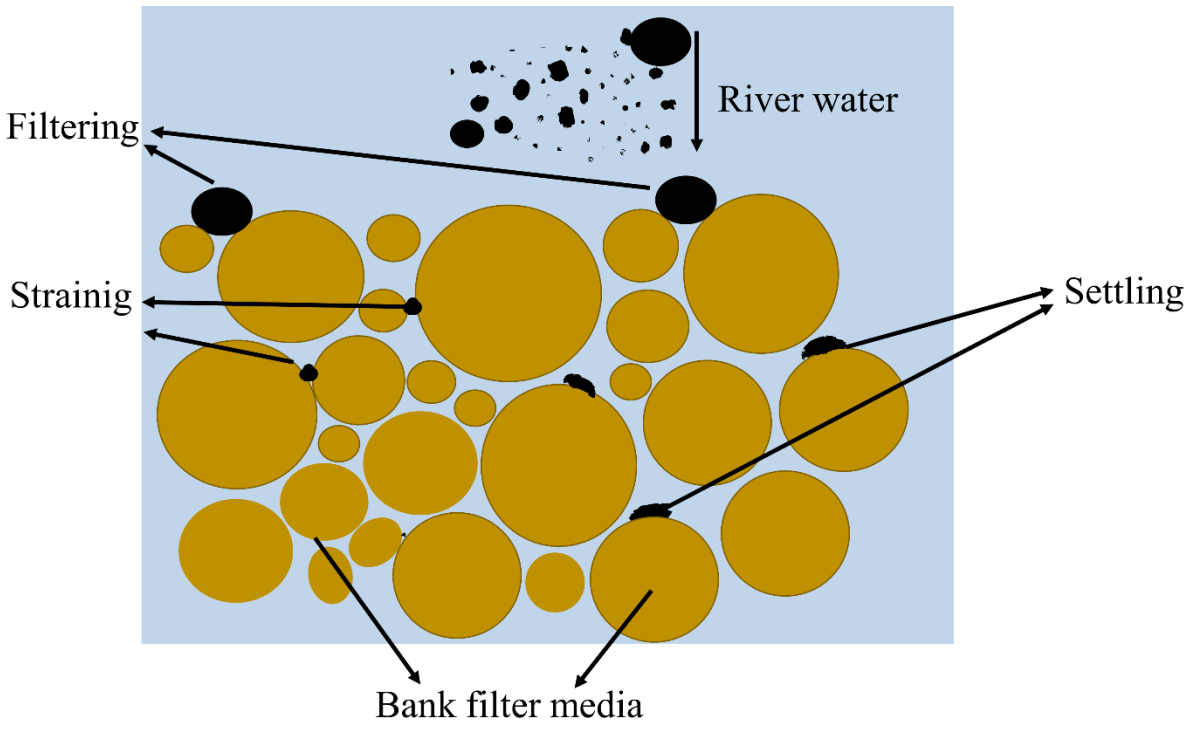

Figure 3 Schematic diagram illustrating mechanical processes during bank filtration

1.2.3 Biological processes

Biological attenuation refers to the biological mediated removal processes of organic compounds by the autochthonous microbes in the riverbed, bank sediments, and the aquifer (Derx *et al.*, 2013). Organic compounds including hydrocarbon and anthropogenic chemicals deriving from the river are partly transformed and in best case mineralized by microorganisms. During this process, the redox condition may change as oxygen is consumed in aerobic conditions. Jaramillo (2011) indicates that biological processes are the key to determining the final quality of the bank filtrate and raw water.

1.2.4 Physico-chemical processes

Physico-chemical processes are critical to the efficiency of bank filtration in removing contaminants. It includes sorption, precipitation, complexation, flocculation/coagulation, and abiotic redox reactions (Doussan *et al.*, 1998). All of these processes generally contribute to improving the water quality by affecting the concentration and behavior of metals, organic and inorganic compounds, as well as microbes and viruses.

1.3 The fate of contaminants during bank filtration

As receiving waters, surface waters (e.g., rivers) can be polluted by organic or inorganic contaminants, pathogenic microorganisms, and viruses from point or non-point sources, including industrial discharges, domestic discharges, urban stormwater, construction sites, and waste dumping (Abdul Aziz *et al.*, 2020; Karakurt *et al.*, 2019). Ray (2011) pointed out bank filtration can remove some dissolved contaminants that are difficult to remove from single treatment technology, because it applies a combination of attenuation processes including straining, colloidal filtration, sorption, and microbial degradation.

1.3.1 Inorganic contaminants

Contamination of surface waters with inorganic contaminants like heavy metals, nitrate, and sulfate may be caused by human activities such as mining, road salting, wastewater discharge, and agriculture (Tufenkji *et al*., 2002). Extensive studies on the attenuation of metals (Bourg and Bertin, 1993; Von Gunten *et al.*, 1991) and other inorganic compounds (Bourg *et al.*, 1989; Doussan *et al.*, 1998; Von Gunten and Kull, 1986) during bank filtration have been conducted. Factors controlling the fate of inorganic pollutants include immobilization due to precipitation, sorption, redox reactions, as well as biotransformation, and complexation with organic matter (Tufenkji *et al.*, 2002). The individual processes were generalized in Jacobs *et al.* (1988). Precipitation and adsorption can decrease the concentration of metals in solution, while dissolution and desorption increase their concentration. In addition, oxide-forming metals (e.g., Mn or Fe) and any associated trace metals can be mobilized when the redox potential changes and microbially mediated reductive dissolution of metal oxyhydroxides occurs. Furthermore, metals such as Cu, Cd, Zn, and Pb, can be mobilized along with organic matter degradation because they are frequently contained in organic matter.

1.3.2 Organic contaminants

Organic contaminants, a complex mixture of particulate and dissolved compounds, including odorous compounds, oil sub-products, pesticides, and pharmaceuticals, are key factors deciding water quality (Jaramillo, 2011; Tufenkji *et al.*, 2002). The efficiency of bank filtration has repeatedly been demonstrated in reducing these organic compounds, such as pesticides (Dragon *et al.*, 2019; Nagy-Kovács *et al.*, 2018), aromatic hydrocarbons (Abdelrady *et al.*, 2019; F.Jüttner, 1999), and organic matter in general (Weiss *et al.*, 2003; Kuehn and Mueller, 2000;

Schubert, 2002b; Glorian *et al.*, 2018). The fate of organic contaminants during bank filtration depends on many factors including compound hydrophobicity, aquifer organic carbon content, sorption to inorganic minerals, biodegradability, microbial activity, infiltration rate, and groundwater dilution(Tufenkji *et al.*, 2002). Tufenkji *et al.* (2002) suggested that organic contaminants with a small molecular weight or with high hydrophilic fraction may be transported further during bank filtration. Furthermore, the concentration of organic contaminants decreases during bank filtrate with enhanced retention time leading to a significant improvement in water quality. However, some organic compounds are difficult to remove by bank filtration, such as certain pesticides, pharmaceuticals, and halogenated organic compounds due to their high persistence (Jaramillo, 2011; Kuehn and Mueller, 2000; Ray *et al.*, 2002).

1.3.3 Pathogenic microorganisms and viruses

A particular concern is the hygienic quality of bank filtrate and drinking water. In this context, recent studies tackled the reduction of pathogens during bank filtration, such as protozoan pathogens (e.g., *Giardia lamblia, Cryptosporidium parvum;* Irmscher and Teermann, 2002; Weiss *et al.*, 2005; Weiss *et al.*, 2003), selected pathogenic bacteria (e.g., *Clostridium, Pseudomonas aeruginosa*; Nagy-Kovács *et al.*, 2019; Weiss *et al.*, 2003;), indicators of fecal contamination (e.g., coliforms, *E. coli*; Ren *et al.*, 2019; Schubert, 2002b; Weiss *et al.*, 2005) and viral surrogates (e.g. coliphages;; Kvitsand *et al.*, 2017; Sprenger *et al.*, 2014; Weiss *et al.*, 2005). Microbial pathogens are attenuated in infiltrating surface water during bank filtration mainly by inactivation, adsorption to sediments, straining, and sedimentation (Jaramillo, 2011; Schijven *et al.*, 2002). These processes depend mainly on the type of microorganisms, the nature of the soil, and the environment (Jaramillo, 2011; Yates *et al.*, 1987). Straining, settling, and physical filtration are the main processes for attenuating bacteria (Doussan *et al.*, 1998; Schijven *et al.*, 2002). Although viruses are generally less affected by these processes due to their small size, they can be removed by filtration and/or sedimentation as well in particular when they are aggregated or associated with larger, settleable solids (Yates *et al.*, 1987; Verbyla and Mihelcic, 2015). However, according to Schijven *et al.* (2002), this may only apply to a small fraction of viruses, as a considerable fraction are freely suspended or adsorbed to particulate solids less than 0.3 µm in size. The major mechanisms of virus migration underground, i.e., inactivation and adsorption, will be described in detail in Section 1.4.

1.4 Vulnerability of bank filtration to pathogenic viruses

Human enteric viruses, which are almost exclusively excreted by humans, are a major cause of waterborne diseases. They enter the aquatic system either through sewage or surface runoff from agricultural lands where manure has been applied (*Figure 4*) (Fong and Lipp, 2005; Grabow, 1996; Melnick et al., 1980; Ye *et al.*, 2012). Exposure routes for human enteric viruses are diverse such as ingestion, inhalation, and dermal absorption (*Figure 4*) (Patz *et al.*, 2001). For example, people may be infected by drinking contaminated water, eating food contaminated with pathogens, or during recreational activities such as swimming. Infection with enteric viruses not only causes mortality and morbidity worldwide, but also increases the spectrum and incidence of many water-related diseases, including gastroenteritis, meningitis, paralysis, respiratory disease, diarrhea, eye infections, and hepatitis (Albinana-Gimenez *et al.*, 2009; Wyn-Jones and Sellwood, 2001). Groups of human enteric viruses currently under study include the families *Picornaviridae* (e.g., enteroviruses), *Adenoviridae* (e.g., adenoviruses), *Caliciviridae* (e.g., noroviruses), and *Reoviridae* (e.g., rotaviruses) (Fong and Lipp, 2005).

The occurrence of human enteric viruses in wastewater and surface water is of particular concern because of their high release from infected individuals (i.e., 10^5 to 10^{11} virus particles per gram of stool; Fong and Lipp, 2014), limited efficacy of removal during water treatment, and low infectious dose (e.g., a single virus particle may cause infection) (Haas *et al.*, 1993; Teunis and Havelaar, 2000; Yezli and Otter, 2011). Furthermore, virus particles are more persistent compared with most pathogenic bacteria in aquatic environments. Survival of more than 100 days in sediment, freshwater, and sewage is not rare for viruses (Fong and Lipp, 2014; Krauss and Griebler, 2011). Moreover, viruses show higher resistance to conventional water treatment processes such as chlorination (Fong and Lipp, 2014; Verbyla and Mihelcic, 2015). Finally, viruses can travel longer distances through the subsurface due to their small size, as physical attenuation processes may be less effective against viruses than bacteria (Betancourt *et al.*, 2014; Bitton and Harvey, 1992). It has been reported that virus particles can be transported through sediments over distances of up to 1600 m and to a depth of 64 m below land surface (Bales *et al.*, 1989; Krauss and Griebler, 2011). Depending on the efficiency of subsurface removal, the use of surface water as a source can result in impaired and variable bank filtrate quality, particularly through contamination with pathogenic viruses (Schijven *et al.*, 2002; Tufenkji *et al.*, 2002). In consequence, a better understanding of the attenuation processes involved in bank filtration, i.e., adsorption and inactivation of viruses, will support sustainable and safer drinking water production.

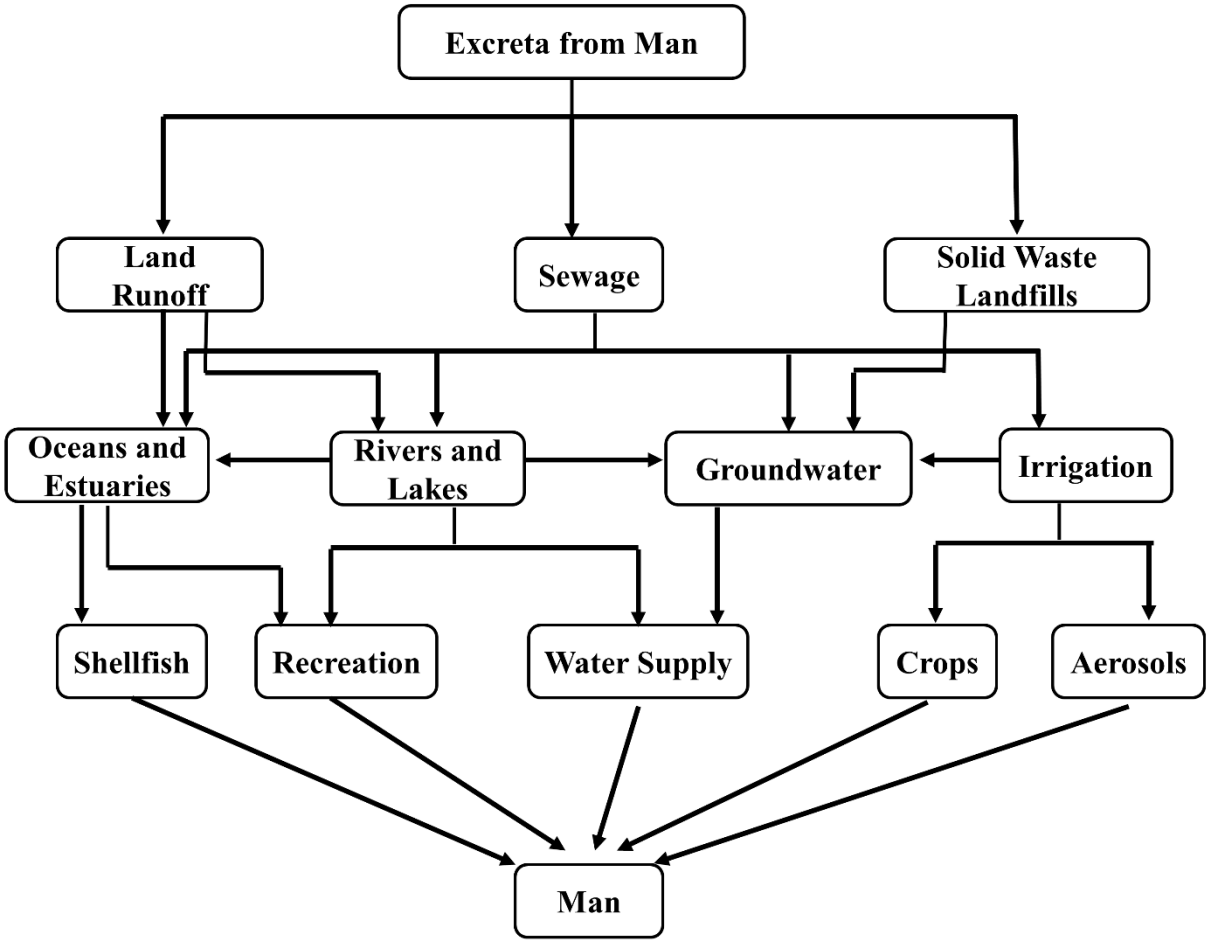

Figure 4 Potential enteric virus transmission (adapted from Melnick et al., 1980)

1.4.1 Adsorption and desorption

The mobility of viruses in the subsurface is determined by advection, dispersion, dilution, and adsorption. The removal efficiency of bank filtration is highly dependent on the adsorption and inactivation rates of viruses (Schijven *et al.*, 2016). Adsorption can be classified as irreversible or reversible adsorption, where irreversible adsorption does not undergo later desorption. Schijven and Hassanizadeh (2000) proposed two different types of reversible adsorption of viruses to solid grains: 1) equilibrium adsorption occurs when adsorption and desorption are fast relative to flow velocity; 2) kinetically limited adsorption that is relative to flow velocity with constant adsorption and desorption rate coefficients. At very low flow velocities, kinetically limited adsorption can be successfully described by equilibrium adsorption. The

reduction in contaminant abundance by adsorption is mainly controlled by kinetic adsorption, while retardation, i.e., delayed transport, is mainly caused by equilibrium adsorption (Bales *et al.*, 1991; Schijven and Hassanizadeh, 2000). Since there is no clear definition available to distinguish between attachment and adsorption, I mainly use the term "adsorption" in this thesis to describe the sorption processes of particles and substances.

The mechanism of how virus particles adsorb to the soil matrix during underground transport can be described and explained by the DLVO (Derjaguin-Landau-Verwey-Overbeek) theory and the colloid filtration theory as will be explained in the following section.

1.4.1.1 DLVO (Derjaguin-Landau-Verwey-Overbeek) Theory

Several factors contribute to the adsorption of viruses and other colloids on sediment particles, including electrostatic attraction and repulsion, van der Waals forces, covalent-ionic interactions, hydrogen bonding, and hydrophobic effects (Bales *et al.*, 1991). The net effect of attractive and repulsive forces on interactions between surfaces is described by the DLVO theory, which combines the potential energies of poorly characterized short-range "non-DLVO" forces (e.g. hydration and steric repulsion), double layer repulsion or attraction, and the London-van der Walls attraction (Ryan and Elimelech, 1996). The van der Waals attraction depends linearly on the value of the Hamaker constant, which is low for organic colloids and tends to be high for inorganic matter so that a strong inherent attraction develops between viruses and inorganic matter (Schijven and Hassanizadeh, 2000). The double layer repulsion or attraction refers to the potential energy that arises from the overlap of diffuse layers (i.e., double layer) to balance the surface charge. A double layer structure is shown in *Figure 5* including two layers. The first layer, called the stern layer, consists of closely bound ions of opposite charge to the surface of viruses and solid due to chemical interactions. The second layer, i.e., diffuse layer, consists of ions attracted to the surface charge via the Coulomb force. Ions in the second layer are diffused more freely around the particles (Fatehah *et al.*, 2015). The both negative surface charge of aquatic solids, except with metal-coated, and viruses at pH 7 to 8, as in many aquatic environments, decide the double layer potential energy is repulsive (Gerba, 1984; Loveland *et al.*, 1996; Schijven and Hassanizadeh, 2000). The potential energy of the double layer is sensitive to variations in the surface potentials of the colloid and the collector, the colloid size, and the ionic strength of the solution. Mobilization of colloids can be achieved by alteration of solution chemistry. Changes in solution chemistry mainly alter the double layer potential energy, while van der Waals attraction is independent of these changes. The non-

DLVO forces may also be affected by changes in solution chemistry, but the knowledge of these forces is not sufficient to assess their impact (Ryan and Elimelech, 1996). Although DLVO theory provides insight into the interaction between virus particles and solid surfaces, such as pH, ionic strength, and colloid size, it has several drawbacks in predicting adsorption and desorption rates, particularly under unfavorable conditions (e.g., in the presence of repulsive energy barriers or in the absence of attractive double-layer interactions) (Schijven and Hassanizadeh, 2000).

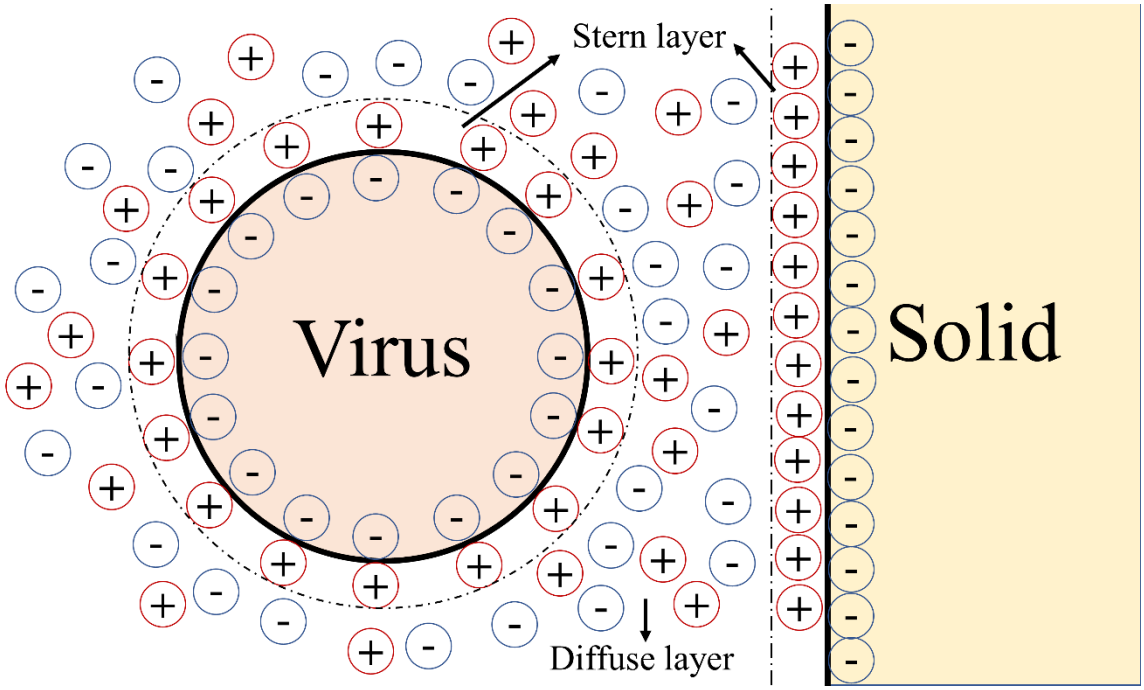

Figure 5 Schematically description of double layers formation around a virus particle (adapted from Fatehah et al., 2015).

1.4.1.2 Colloid filtration theory

To maintain and restore drinking water supplies, understanding the processes involved in the transport of microorganism and viruses through saturated porous media is an important goal (Nelson and Ginn, 2005). Colloid filtration theory has been suggested as the most promising method for predicting microorganism and viruses transport in porous media (Schijven *et al.*, 2002). A two-step process in the deposition of viruses on porous media grains is described by colloid filtration theory: mass transfer to the surface and virus-surface interactions (Nelon and Ginn, 2005; Schijven and Hassanizadeh, 2000).

First, a suspended particle can enter the vicinity of a particle (collector) by either interception, sedimentation, or diffusion (*Figure 6*) (Yao *et al.*, 1971). Different mechanisms determine interception, sedimentation, and diffusion, respectively. Interception describes the transport process in which a suspended particle comes in contact with the collector along the streamline of the flow because of its size. In contrast to interception, sedimentation and diffusion occur mainly due to gravity and random bombardment of suspended particles, respectively. Since the transport of viruses in contact with the collector occurs mainly by Brownian diffusion, the effects of interception and gravitation are usually negligible (Schijven and Hassanizadeh, 2000). The fraction of collisions between suspended particles and the collector is described by the collision efficiency.

Second, the particle attaches to a solid surface. The portion of particles that remains attached to the collector after colliding with the solid grains is described by the sticking efficiency (Martin *et al.*, 1992; Schijven and Hassanizadeh, 2000). Sticking efficiency reflects the net effect of repulsive and attractive forces between particles and collectors and therefore depends on the type of virus and soil, pH, organic carbon content, and ionic strength (Schijven and Hassanizadeh, 2000). The coefficient of adsorption rate is related to collision efficiency and sticking efficiency. Furthermore, colloid filtration theory predicts that virus removal during underground transport is inversely proportional to flow velocity (Yao *et al.*, 1971; Schijven and Hassanizadeh, 2000).

However, there are also limitations in the application of colloid filtration theory. One example is that the theoretical values of the sticking efficiency considerably underestimate experimental values, resulting in an inaccurate coefficient of attachment rate (Schijven and Hassanizadeh, 2000). In comparison to DLVO theory, experimentally determined sticking efficiencies are sensitive to the zeta potentials of particles and collectors and the ionic strength of the solution and more independent of colloid particle size (Schijven and Hassanizadeh, 2000).

Hydrophobic effects may also play an important role in the adsorption of viruses to soil particles (Gerba, 1984). When both the virus surface and the soil particle surface are negatively charged at high pH, hydrophobic interactions may be the major factor in maintaining virus adsorption (Bales *et al.*, 1991; Gerba, 1984; Schijven and Hassanizadeh, 2000). However, Loveland *et al.* (1996) argued that the phenomena of increasing virus adsorption may be attributed to a decreased double-layer repulsion rather than hydrophobic expulsion.

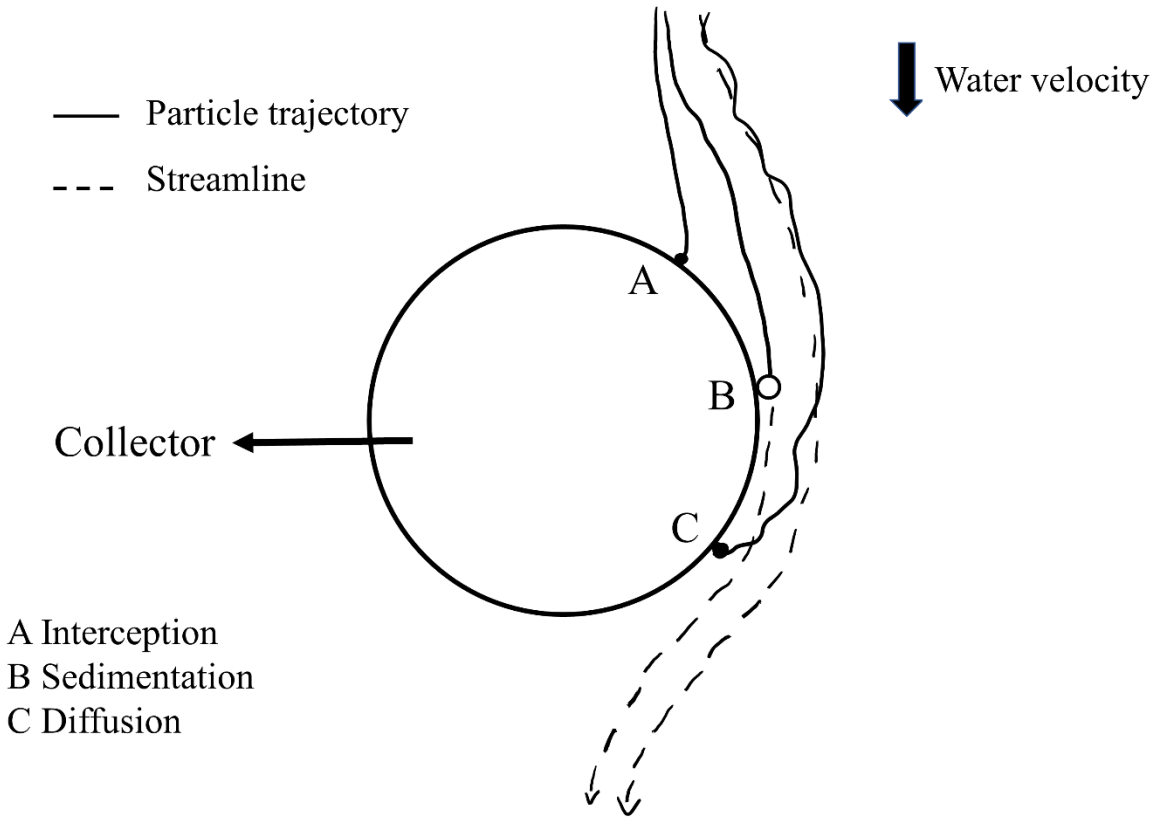

Figure 6 Basic transport mechanism during underground (adapted from Yao et al., 1971).

1.4.1.3 Factors affecting virus adsorption to soil

As explained earlier, the interactions between viruses and collectors are a combination of electrostatic and hydrophobic forces determined by their surface properties (Schijven and Hassanizadeh, 2000). The surface properties depend not only on the type of virus and soil but are also influenced by salts, pH, and the presence of organic matter (Gerba, 1984).

Differences in the electrical charge and hydrophobicity of virus surfaces are assumed to account for the differential adsorption by different viruses (Gerba, 1984; Schijven and Hassanizadeh, 2000). Since the isoelectric point of a virus can vary even from strain to strain, the electrical charge on the surfaces will be correspondingly different under the same conditions (Gerba, 1984). Differences in hydrophobicity are due to amino acid spans of the coat proteins and the different folding of these proteins (Schijven and Hassanizadeh, 2000).

The electrical charge of the soil and mineral surfaces should also be considered to affect the adsorption of the virus to the soil, particle or matrix. The isoelectric point of the soil and mineral surface were summarized in Gerba (1984). Furthermore, clay and other naturally occurring

solids have been found to have a heterogeneous distribution of charges on the surface, so that viruses with different isoelectric points can adsorb on different regions of the same solid.

Many studies have shown that the adsorption of viruses to solids is generally lower at higher pH (Bales *et al.*, 1991; Sadeghi *et al.*, 2011; Schulze-Makuch *et al.*, 2003; Walshe *et al.*, 2010). This can be explained by increased electrostatic repulsion at higher pH, according to DVLO theory (higher maximum and higher primary minimum), which causes a decreased rate of attachment and an increased rate of detachment (Schijven and Hassanizadeh, 2000).

DLVO theory can also explain the influence of ionic strength on the adsorption of the virus on the soil, as the thickness of the double-layer changes (Chu *et al.*, 2000). The double-layer is compressed at high ionic strength, which promotes the distance between virus particles and solid, which can be assumed to result in high adsorption (Gerba, 1984). The same theory can explain why viruses can be eluted from solids by precipitates with low electrical conductivity.

Multivalent cations can increase the adsorption of viruses on solids by forming a salt bridge or by charge reversal (Schijven and Hassanizadeh, 2000). Schijven *et al.* (2000) showed that under conditions of high concentration of multivalent cations, more negatively charged viruses are adsorbed than less negatively charged ones. Furthermore, divalent cations are supposed to promote adsorption more effectively than monovalent cations (Bales *et al.*, 1991).

Organic matter also has a negatively charged surface and can therefore block the adsorption sites of viruses on solids (Gerba, 1984). In addition, hydrophilic organic matter can enhance electrostatic repulsion between viruses and solid surfaces, facilitating onward transport (Zhuang and Jin, 2003). Zhuang and Jin (2003) also point out that the effect of organic matter is mainly due to electrostatic rather than hydrophobic interactions.

1.4.2 Inactivation

Virus inactivation is the loss of the ability to infect host cells over time through the disruption of coat proteins and the degradation of nucleic acids (Gerba, 1984). Previous studies were almost exclusively concerned with the active fraction of virus particles using plaque assays and cell lines for quantification. Therefore, virus inactivation in this part also includes viruses that decay (degradation). As viruses occur in the subsurface as suspended and adsorbed particles, inactivation occurs not only in suspended viruses but also when viruses adsorb to soil or sediment particles.

1.4.2.1 Inactivation of viruses in water

Viruses are composed of labile organic compounds (i.e., mainly proteins and nucleic acids) and thus experience natural decay in water (Harvey and Ryan, 2004). The inactivation of suspended viruses is influenced by both external and internal factors (Schijven and Hassanizadeh, 2000). External factors include environmental factors such as temperature, conductivity, turbidity of the water, and the ability to support bacterial growth (Hurst *et al.*, 1992). Harvey and Ryan (2004) suggested that adverse conditions and inadequate temperature accelerate inactivation through nucleic acid degradation or conformation changes at the host recognition site. Internal factors affecting viral inactivation may include the presence of viral aggregates, loss of genetic material due to the displacement of vertex structure at a non-host surface, or inter-surface forces leading to fracture of the capsid (Harvey and Ryan, 2004; Schijven and Hassanizadeh, 2000). However, virus aggregation is thought to be negligible in most aquatic environments due to low virus concentrations and relatively rapid rates of inactivation (Grant, 1994). Furthermore, the aggregation of viruses reduces the number of active virus particles but improves their resistance to inactivation.

1.4.2.2 Inactivation of viruses during adsorption

Compared to the inactivation of suspended viruses, the inactivation of viruses adsorbed on the soil may occur at a different rate. Ryan *et al.* (2002) proposed a complicated model in which the inactivation rate of viruses may be different during adsorption and desorption. Some adsorption types can accelerate the inactivation, while others inhibit it.

Prolongation of virus survival is generally found along with adsorption to the sediment, especially when organic matter and clay-sized particle content are high (Gerba, 1984; Harvey and Ryan, 2004; Yates *et al.*, 1987). Based on DLVO theory, the interaction of viruses with negatively charged surfaces such as organic matter, clay minerals, and some oxide surfaces (e.g., quartz) is generally weak, so inactivation near the surface of geologic media is either not observed or viruses are sheltered from inactivation by solution (Harvey and Ryan, 2004). However, the inactivation of viruses can be accelerated by adsorption to geological media such as iron and aluminum oxides and other materials that strongly bind viruses (Gerba, 1984; Harvey and Ryan, 2004; Ryan *et al.*,2002). During adsorption, the strong van der Waals interactions between metals and transition metal oxides and viruses cause spontaneous degradation of viruses because the surfaces are oppositely charged (Murray and Laband, 1979).

Various mechanisms of virus inactivation on solid surfaces are illustrated in *Figure 7* as summarized by Gerba (1984). Moore *et al.* (1982) found that intact reoviruses lose their specific infectivity during adsorption, suggesting that rapid inactivation may occur during contact with the surface of geological media. Murray and Laband (1979) observed the inactivation and degradation of polioviruses eluted from CuO and Al. Yeager and O'Brien (1979) proposed a mechanism of viral (poliovirus) inactivation irreversibly bound to moist and dried soils: first, viral nucleic acid and capsids are dissociated; and second, the viral genome is degraded in the soil environment by microorganisms. Furthermore, the viral nucleic acid may be damaged before dissociation from the capsid, so the degradation of nucleic acid by microorganisms occurs as soon as it is released (Gerba, 1984).

The rate of virus inactivation near-surface detected in studies may also be influenced by heterogeneity in site reactivities, virus aggregation, or the possibility that inactivation occurs mostly at impact (Murray and Laband, 1979). In summary, the main factors affecting virus inactivation in the subsurface besides virus-specific are types of geological media, temperature, microbial activity, and saturation condition (Schijven and Hassanizadeh, 2000).

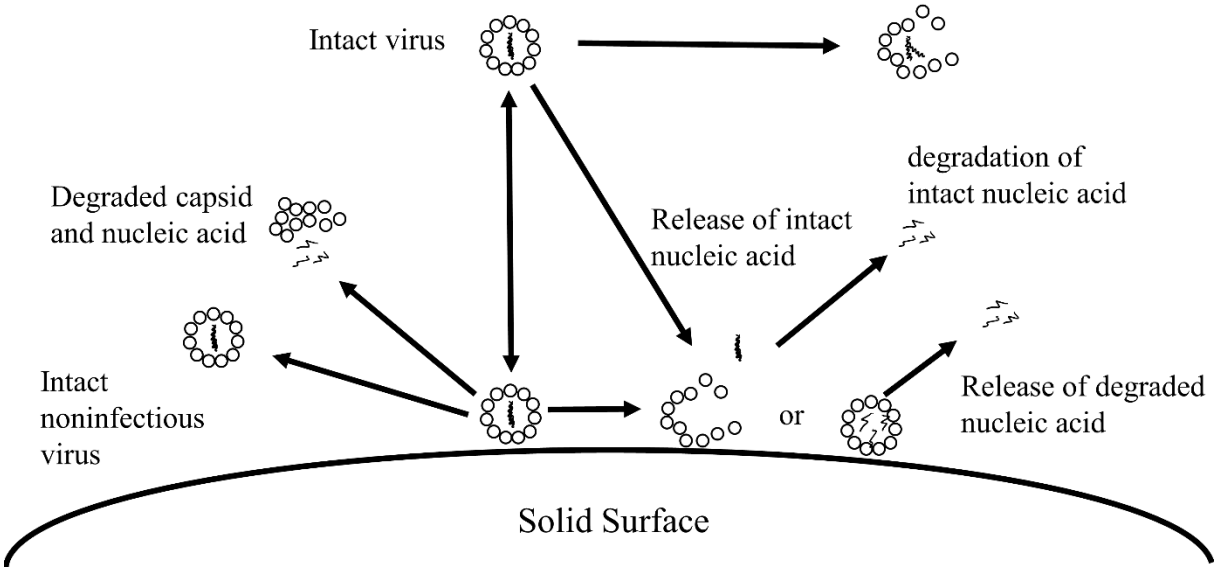

Figure 7 Mechanisms of virus inactivation during adsorption (adapted from Gerba, 1984).

1.4.2.3 Factors affecting virus inactivation during the underground passage

The most important factors associated with virus inactivation are believed to be temperature, adsorption to soil and particulate matter, unsaturated conditions, and microbial activity (Schijven and Hassanizadeh, 2000). Viruses remain infectious longer in the subsurface compared with surface water due to a combination of factors including lower temperature, lack of solar radiation, and lower microbial activity (Yates *et al.*, 1987).

Water temperature correlates significantly with virus inactivation in the aquatic environment: the higher the temperature, the higher the virus inactivation rate. Yates *et al.* (1985) studied virus inactivation using more than 100 groundwater samples. The temperature was the only significantly correlating variable among other tested variables like nitrate, ammonia, pH, temperature, total dissolved solids, turbidity, and hardness. Virus survival in batch experiments also indicated that temperature is a primary factor affecting virus inactivation (Allwood *et al.*, 2003; Bae and Schwab, 2008; Christon, 1988). High temperature can either directly degrade coat proteins or nucleic acids of viruses or indirectly enhance microbial antagonism in the aquatic environment (Feng *et al.*, 2003; Gordon and Toze, 2003; Yates *et al.*, 1987).

It is generally considered that adsorption to soil and particulate matter, as well as aggregation, prolongs virus survival compared with suspended virus particles (Schijven and Hassanizadeh, 2000; Yates *et al.*, 1987). Exceptions have also been reported by Gerba (1984) in the case where viruses adsorb to metal oxides or materials that bind viruses strongly.

Microbial activity can enhance virus inactivation (Yates *et al.*, 1987). Viruses have been found to remain infectious longer in groundwater than in surface water when incubated in batch at darkness and controlled temperature, suggesting microbial antagonism is a dominating factor to influence virus survival (Bae and Schwab, 2008). Microbially mediated antagonistic processes have been reported previously, including predation and release of chemicals toxic to viruses by bacteria, leading to reduction of allochthonous viruses and microbes (Feichtmayer *et al.*, 2017).

The unsaturated conditions, i.e., vadose zone, can retain and/or inactivate viruses during underground transport (Schijven and Hassanizadeh, 2000). The same scheme was also observed by Jin and Flury (2002): the higher the saturation, the higher the coliphage breakthrough curve. In addition to the aforesaid factors, viral inactivation rates remain low under anoxic conditions (Klitzke *et al.*, 2015).

1.5 The potential vulnerability of bank filtration to hydrological extremes

Studies have shown that the efficiency of bank filtration is vulnerable to climate change (Sprenger *et al.*, 2011). Climate change is predicted to lead to an increase in extreme hydrological events, including more frequent and extended droughts and heavy rainfalls (Eckert *et al.*, 2008; Herrador *et al.*, 2015). Hari *et al.* (2020) demonstrated that the occurrence of the 2018–2019 summer droughts in central Europe is unprecedented in the last 250 years. Patz *et al.* (2001) pointed out that waterborne diseases are one of the major problems associated with climate change.

Many previous studies have revealed that heavy rainfall and surface runoff lead to deterioration of raw surface water quality and related human disease outbreaks due to high concentrations of *Cryptosporidium* oocyst, *Giardia* cyst, fecal bacteria, and infectious viruses (Harvell *et al.*, 1999; Weniger *et al.*, 1983; Atherholt *et al.*, 1998; Mac Kenzie *et al.*, 1991). Because of the elevated concentration in surface water, increased survival at low temperatures, and reduced travel time during high water levels, flood scenarios pose a serious threat to the bank filtration system from waterborne pathogens. In addition, it is worth considering that groundwater can be contaminated by infiltrating precipitation and remobilization of viruses previously adsorbed to sediments (Drayna *et al.*, 2010). Variation in ionic strength of sediment pore water can exert an important influence on adsorption-desorption and mobilization of virus particles (Landry *et al.*, 1979). Therefore, it is crucial to understand the effects of low ionic strength water percolating through bank sediments on virus transport.

Similar to flooding scenarios, the efficiency of the bank filtration system in removing microorganisms and viruses is challenging by further issues. Recent studies indicate an increase in the relative amount of wastewater in rivers during hot summers (Karakurt *et al.*, 2019). Przybyłek *et al.* (2017) indicated that long-term hydrological droughts increase the rate and extent of riverbed clogging, which directly degrades attenuation efficiency for pathogens. Moreover, viruses have been assumed to be removed to a lesser extent under anoxic conditions compared with oxic conditions because they are less likely to be inactivated and adsorbed during underground passage (Frohnert *et al.*, 2014; Klitzke *et al.*, 2015; Wielen *et al.*, 2008).

1.6 Ecological monitoring of bank filtration and groundwater

Bank filtration, a widespread managed aquifer recharge method, is one of the most promising hydrological techniques for transferring surface water to aquifers and improving groundwater resources with respect to quality and quantity (Esfahani *et al.*, 2020). Thus, bank filtration can be considered a result of groundwater intrusion by surface water to varying degrees. Besides providing drinking water, the services delivered by the groundwater ecosystem that are beneficial for humans, such as purification of water, depend largely on the activities of organisms living in it, as they play a key role in degrading contaminants and inactivating or eliminating pathogens (Feichtmayer *et al.*, 2017; Griebler and Avramov, 2015). Nowadays, however, groundwater ecosystems are threatened by many factors, such as increasing industrial activities, intensive agriculture, urbanization, overexploitation, and extreme climate conditions (Retter *et al.*, 2021). Fortunately, in recent decades, legislative perceptions in parts of the world started to consider groundwater as a unique ecosystem that merits protection rather than a commodity, because of the collective effort of the scientific community (Griebler *et al.*, 2014). Therefore, the development of a suitable and universally applicable ecological assessment tool is needed to assist legislators in routine monitoring of groundwater ecosystems. This ecological assessment tool should meet the following criteria: i) the targeted parameters are easy and inexpensive to measure; ii) they are sensitive to a wide range of disturbing factors and globally applicable to different hydrogeological conditions; iii) the data are easy to analyze and interpret; iv) ideally, samples for measuring ecological parameters can be obtained together with samples for typical physico-chemical analyses without additional sampling efforts.

Previous research efforts already strived to develop practically applicable toolboxes and assessment schemes to detect the disturbance of groundwater ecosystems. Firstly, they tried to use the indicator organisms which have already been applied as ecological assessment criteria for monitoring surface water. However, these tools failed due to the characteristic environment conditions (e.g. light, nutrient levels) and repertoire of organisms (e.g., absence of macrophytes, algae) (Steube *et al.*, 2009). Later, the use of groundwater fauna was suggested. However, the sparse distribution of groundwater fauna and the requirement for oxygen concentrations complicate data collection and interpretation of these types of indicators, thus limiting their applicability mainly to oxic aquifers (Korbel and Hose, 2017; Stein *et al.*, 2010). Since prokaryotic microorganisms are ubiquitous in groundwater, even under harsh conditions, they are better suited than other organisms as bioindicators to monitor disturbances in groundwater ecosystems (Griebler and Lueders, 2009). Indeed, to date, numerous studies have considered

the inclusion of microbial parameters in ecological assessment schemes, although the method may either be applicable under certain conditions or require DNA-based lab work (Mermillod-Blondin *et al.*, 2013; Pearce *et al.*, 2011).

1.7 Knowledge gaps and Objectives

By recognizing the importance of bank filtration to public water supply and the great potential risk of pathogenic viruses to the bank filtration system, particularly in times of hydrological extremes, I attempted to close the knowledge gaps by stressing the following questions:

1. What is the effectiveness and contribution of individual attenuation processes during the underground passage of viruses?
2. What is the influence of hydrological extremes (e.g., heavy precipitation) on the attenuation efficiency of viruses during subsurface transport? What are the key mechanisms?
3. Are there suitable indicators of virus attenuation during bank filtration, especially during periods of varying hydrological events?
4. What is the influence of varying hydrological conditions on attenuation of pathogenic viruses, fecal and viral indicators during bank filtration?
5. Can we use prokaryotic cell density, microbial activity, and/or bioavailable carbon concentration to develop a universally applicable and user-friendly method to monitor groundwater?

After identifying the knowledge gaps, I designed and conducted stepwise experiments from mesoscale sediment column experiments to macroscale field experiments. In summary, this thesis includes the following three sections:

I) Adsorption, re-mobilization, and inactivation of bacteriophage MS2 during bank filtration following simulation of a high virus load and an extreme rain event

The purpose of this section is to fill knowledge gaps #1 and #2 by using a water-saturated sediment column fed with surface water to simulate a bank filtration system. First, we investigated the efficacy of the individual attenuation processes in virus transport through aquatic sediment after a high dose of MS2 contamination, including adsorption, inactivation, and decay. Subsequently, re-mobilization of previously adsorbed virus particles (reversible adsorption) was quantified particularly after a pulse of deionized water simulating an artificial heavy rainfall. The efficacies of individual attenuation processes during transport were

estimated by quantifying the abundance of sediment-adsorbed MS2, total MS2, and infectious MS2. During the whole experiment, the correlation between the physico-chemical parameters of the water, the abundance of autochthonous microbial biomass and its activity, and the abundance of suspended and sediment-adsorbed total and infectious allochthonous viruses were explored. Finally, we compared the main attenuation processes during these two events, i.e., the high virus load and the heavy rain event, and discuss the factors which may control the attenuation processes.

II) Dynamics of pathogens and fecal indicators during riverbank filtration in times of high and low river levels

This section addresses knowledge gaps #3 and #4, by conducting a 16 months' field study at the Rhine River, sampling surface water, corresponding bank filtrate, and neighbouring groundwater. To this end, the efficiency of riverbank filtration in attenuating adenovirus, fecal indicators, coliphages, and further viral and microbial variables following different river levels was studied. In this context, we evaluated which indicator performed best in predicting the fate of adenoviruses, and is of practical use to monitor the overall efficiency of riverbank filtration. In addition, possible correlations between biological indicators and water physico-chemical variables, especially at different river levels, were assessed and the potential relationship between these correlations and different river levels is discussed.

III) The D-A-(C) index: A practical approach towards the microbiological-ecological monitoring of groundwater systems

Part three of this thesis aims to close the knowledge gap #5 by introducing a universally applicable and user-friendly microbiological-ecological groundwater monitor tool based on three parameters: prokaryotic cell density (D), microbial activity (A), and/or the concentration of bioavailable carbon (C). First, we tested this tool for three groundwater threat cases: i) an organic contamination, ii) surface water intrusion into a shallow aquifer, and iii) groundwater quality influenced by different types of land use. Furthermore, the use of this monitoring tool revealed a microbiological-ecological fingerprint of regional groundwater when analyzing a large dataset of more than 200 samples collected from nine non-contaminated aquifers in different regions across Germany. Additionally, the sensitivity and robustness of using simultaneous multivariate analysis were explored by comparing with separate univariate analysis.

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2. Research

2.1 Attachment, re-mobilization, and inactivation of bacteriophage MS2 during bank filtration following simulation of a high virus load and an extreme rain event

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Synopsis

The study of virus transport through aquatic sediments is crucial because bank filtration of potentially contaminated surface water is a widely used method in drinking water production. Viral contamination of drinking water has always been a human health concern. In fact, previous studies almost exclusively addressed the fraction of infectious viruses and its fate during sediment passage, ignoring individual responsible attenuation process, i.e., inactivation, decay or adsorption. In this study, to investigate the efficacy and contribution of individual viral attenuation processes during sediment transport, we not only examined the spatial-temporal dynamics of total and infectious virus particles suspended in sediment pore water but also evaluated total and infectious virus particles adsorbed onto the sediment matrix. In this context, we first addressed the fate of a heavy virus pulse and later the effect of a heavy rainfall on the transport of virus particles. Bacteriophage MS2 was used as a surrogate for enteric viruses in this study.

The field experiment was distinguished into two phases to simulate the sudden occurrence of a severe viral contamination and the subsequent sudden heavy rainfall. Together with a pre-experiment focusing on MS2 inactivation and decay in still sterile surface water and water-saturated sediment, we found that virus decay (0.8 $\log_{10}$) and inactivation (1.7 $\log_{10}$) during sediment passage was higher, probably related to the activity of autochthonous microorganisms. In comparison with an earlier virus transport experiment where moderate virus load was applied,

we concluded that the removal of MS2 during sediment passage was highest (3.0 $\log_{10}$) at a low virus load, which can be explained by the colloid filtration theory and/or elevated virus aggregation at high virus concentration. Furthermore, although adsorption is considered one of the most effective attenuation processes during underground transport, this study showed that adsorbed infectious MS2 particles can re-enter sediment pore-water in the course of a sudden heavy rainfall event. This finding clearly demonstrates that sediment adsorption can maintain the infectivity and/or prolong the survival of viruses. In summary, in water quality assessment and monitoring schemes, attention should be paid not only to the initial viral contamination from surface water to the aquifer, but also to follow-up contaminations from re-mobilized viruses from the sediment matrix, stimulated by hydrological extremes.

Author contributions

Prof. Christian Griebler conceptualized the experiment. Dr. Judith Kaletta, Sigrid Kaschuba and Dr. Sondra Klitzke conducted the field sampling. He Wang, Dr. Judith Kaletta, Sigrid Kaschuba and Dr. Sondra Klitzke analyzed the samples. He Wang analyzed the data and visualized the data with the supervision of Prof. Christian Griebler. He Wang wrote the manuscript with the help of Prof. Christian Griebler, Dr. Sondra Klitzke, and Ingrid Chorus. All authors commented on the manuscript.

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Attachment, re-mobilization, and inactivation of bacteriophage MS2 during bank filtration following simulation of a high virus load and an extreme rain event

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Climate change

ABSTRACT

Viruses, including human pathogenic viruses, can persist in water. For producing drinking water from surface water via bank filtration, natural attenuation capacities and the fate of viruses during the passage of aquatic sediments are of particular interest. Moreover, the increasing frequency of extreme hydrological events necessitate re-evaluation of the sustainability and efficacy of processes removing viruses. For this purpose, we performed bank sediment filtration experiments using a mesocosm in a technical-scale experimental facility that simulates a field situation under more tightly controlled conditions. We used the bacteriophage MS2 as a surrogate for enteric viruses to study the transport of different viral loads through the bank sediment. Additionally, we simulated a heavy rain event to investigate the re-mobilization of initially attached virus particles. We quantified the abundance of infectious MS2 phages by plaque assay and the total number of MS2 particles by qPCR. Also, we differentiated pore water concentrations by depths of the sediment column and investigated attachment to the sediment matrix at the end of the individual experimental phases. Bank filtration over a vertical distance of 80 cm through sandy sediment revealed a virus removal efficiency of 0.8 $\log_{10}$ for total MS2 particles and 1.7 $\log_{10}$ for infectious MS2 particles, with an initial phage concentration of 1.84×10^8 gene copies mL^{-1}. A low load of infectious MS2 (1.9×10^6 plaque forming units mL^{-1}) resulted in a greater removal efficiency (3.0 $\log_{10}$). The proportion of infectious MS2 phages of the total MS2 particle mass steadily decreased over time, i.e., in the course of individual breakthrough curves and with sediment depth. The simulated pulse of rainwater caused a front of low ionic strength water which resulted in pronounced phage remobilization. The high proportion of infectious MS2 among the detached phages indicated that attachment to the sediment matrix may substantially conserve virus infectivity. Therefore, the re-mobilization of previously attached viruses owing to hydrological extremes should be considered in water quality assessment and monitoring schemes.

1. Introduction

Bank filtration of river and lake water has proven to be of great value for drinking water production by combining treatment and abstraction at low costs (Ahmed and Marhaba, 2017; Massmann et al., 2004; Krauss and Griebler, 2011; Kuehn and Mueller, 2000). The infiltrating surface water is physically, chemically, and microbiologically altered during sediment passage, and this generally improves water quality (Jaramillo, 2011). At some sites, bank filtration plants have been operating for more than 150 years (Kuehn and Mueller, 2000; Nagy-Kovács et al., 2019). For example, in Germany, the country in Europe with most sites of induced bank filtration, 9%–16% of drinking water is produced in this way (Gillefalk et al., 2018). However, if the efficiency of the natural subsurface attenuation processes is insufficient or highly variable, bank filtrate may be contaminated with pathogens, including viruses (Schijven et al., 2002; Tufenkji et al., 2002).

The contamination of raw and drinking water with pathogens is a widespread source of waterborne disease outbreaks (Yang et al., 2012).

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Previous studies found a range of human enteric viruses at densities of 10^2 to 10^3 gc (gene copies) L^{-1} in surface waters (Lodder et al., 2010; Prevost et al., 2015); these were introduced either by wastewater or via surface run-off from agricultural land where wastewater and manure were applied (Fong and Lipp, 2005). The reduction of pathogenic viruses during bank filtration is of particular interest because compared to most pathogenic bacteria, many viruses are more persistent in the aquatic environment. It is not rare for viruses to remain infective for more than 100 days in sediment, freshwater, and sewage (Fong and Lipp, 2005). Moreover, owing to their smaller size, viruses may travel longer distances through the porous subsurface, as underground physical attenuation processes may be less effective than for bacteria (Betancourt et al., 2014; Bition and Harvey, 1992). Viruses have been reported to be transported through subsurface media over distances as far as 1600 m and to a depth of 64 m below the land surface (Bales et al., 1989). For these reasons, a comprehensive understanding of the attenuation efficiency of bank filtration particularly for viruses is important for planning sustainable and safe drinking water production. This requires addressing the impact of dynamic changes in the hydrological cycle on virus transport and retention, including the impact of extended periods of droughts followed by heavy rain events and floods (Eckert et al., 2008).

Climate change is predicted to lead to an increase in extreme hydrological events, including more frequent and extended droughts and heavy rainfall. Understanding the impact of changes in hydrological conditions on the distribution of pathogens during bank filtration is therefore important for public health (Sprenger et al., 2011). Previous studies have already concluded that bank filtrate quality may be compromised by viral contamination induced by extreme precipitation owing both to shorter travel time in the subsurface and to elevated viral contamination in surface water (Rose et al., 2000; Sprenger et al., 2011). Additionally, Drayna et al. (2010) proposed that the re-mobilization (desorption) of formerly attached viruses in sediments can be a relevant process in the contamination of groundwater from infiltrating precipitation. Variation in the ionic strength of the sediment pore water may exert an important influence on virus attachment-detachment and (re-) mobilization (Landry et al., 1979). It is therefore important to understand how heavy rain events causing water with a low ionic strength to percolate through bank sediments affect virus transport.

Although bank filtration has been studied for many years, the focus was chiefly on the fate of a variety of chemical pollutants and pathogenic bacteria. The transport of pathogenic viruses through aquatic sediment has been less well studied, mainly owing to the challenges of virus detection and quantification (Dash et al., 2010; Doussan et al., 1997; Wyn-Jones and Sellwood, 2001). Moreover, studies have so far almost exclusively addressed the infectious viruses (i.e., detection by plaque assay or cell lines) suspended and transported in sediment pore water, while the transport of those which are no longer infectious has scarcely been addressed. However, including them provides information on virus inactivation, virus removal by irreversible attachment to sediment or decay, and virus retardation by attachment and re-mobilization (Loveland et al., 1996). Limiting research to the study of infectious virus particles may further underestimate the number of viruses involved in aggregation. In contrast, exclusively addressing total viruses, as is currently common with molecular tools, misses viral infectivity and inactivation (Hassard et al., 2016). Combining and comparing the fate of both the total virus load and the fraction which remains infectious is therefore the most comprehensive approach for elucidating the mechanisms of virus transport during sediment passage.

The primary objective of the current study was to elucidate the efficacy and contribution of individual natural attenuation processes as viruses travel through aquatic sediment, including irreversible attachment, retardation (attachment and re-mobilization), inactivation, and decay. This included, in particular, quantification of the re-mobilization of formerly attached viruses caused by rainwater infiltration. To achieve the aims of this study, bacteriophage MS2 was used as a surrogate because of its similar size (26 nm) and shape (icosahedral) to enteric viruses (especially enteroviruses), as well as its similar behavioral characteristics (persistence, resistance, no replication in aquatic environments, decay rate in surface water) that determine its fate in the subsurface (Boehm et al., 2019; Harvey and Ryan, 2004; Jofre et al., 2016; Pitol et al., 2018). Two scenarios in the main experiment focused on mimicking the infiltration of heavy viral contamination and a subsequent rainstorm event. Both scenarios are likely to occur frequently in fairly densely settled areas where bank filtrate is obtained from rivers and lakes that carry treated wastewater, e.g., by upstream discharge from sewage treatment plants or by stormwater overflow from mixed sewers. Unlike other studies, we not only looked at the fate of either total or infectious virus particles suspended in sediment pore water but also evaluated their combined dynamics over space and time and included total and infectious MS2 phages attached to the sediment matrix after each treatment phase, i.e., after the contamination event and after the heavy rain event. Moreover, key physical and chemical parameters, i.e., electrical conductivity (EC), dissolved oxygen concentration (DO), pH, redox potential, and temperature, and basic microbial parameters, i.e., adenosine triphosphate concentration (ATP) and total prokaryotic cell counts (TCC), were monitored in sediment pore water throughout the entire experiment.

2. Material and methods

2.1. Experimental facility

The study was conducted using a sediment column embedded in the facility for the simulation of riverbank and slow sand filtration (Fig. S1) at the German Environment Agency's field station in Berlin-Marienfelde. This is fed by water from a storage pond in which groundwater (from which Fe and Mn were removed through microbial precipitation) was exposed to the atmosphere (including natural rainfall and sunlight) so that natural flora and fauna could develop. After a residence time of 2–3 weeks this water entered the slow sand filtration basin, mimicking surface water with a pH of 8 ± 0.2 (29 replicate measurements) and a calculated ionic strength of 13.3 mM. Selected inorganic hydrochemical parameters of the infiltrating water are summarized in Table 1. Mean concentrations of total organic carbon recorded in the months of November/December of preceding years amounted to 2.0 mg L^{-1} (three replicates). A water-saturated sand column, similar to a lysimeter with a diameter of 113 cm (i.e., corresponding to a surface area of 1 m^2) and a height of 130 cm (Fig. 1) was embedded in the slow sand infiltration basin (dimensions 10 × 10 m) and fed with surrounding pond water. The supernatant, i.e., a water column of 10 cm (corresponding to 100 L of water) could be connected and disconnected to the surrounding pond water. The column was packed with 100 cm of natural coarse-grained medium sand (grain size distribution in Table S1) with a porosity of 41% (d_{10} = 0.14 mm, d_{60} = 0.33 mm, uniformity coefficient Cu = 2.36), on top of a supporting layer of 30 cm of gravel. As the ratio of the column diameter (d_{col}) to the effective particle diameter (d_{10}) of the media was

Table 1
Inorganic hydrochemical parameters of the infiltrating pond water in mg L^{-1} and mM (mean values of 14–27 analyses conducted over a period of 7 years).

Parameter	Concentration ± standard deviation (mg L^{-1})	Concentration ± standard deviation (mM)
Na^+	50.5 ± 4.6	2.2 ± 0.2
K^+	3.7 ± 0.9	0.09 ± 0.02
Ca^{2+}	123 ± 8	3.1 ± 0.2
Mg^{2+}	17.3 ± 1.4	0.71 ± 0.06
Fe_{tot}	0.018 ± 0.019	0.0003 ± 0.0003
Mn_{tot}	2.2 ± 1.6	0.04 ± 0.03
Cl^-	89 ± 6	2.5 ± 0.2
NO^{3-}	> 0.1	> 0.002
SO_4^{2-}	222 ± 12	2.3 ± 0.2

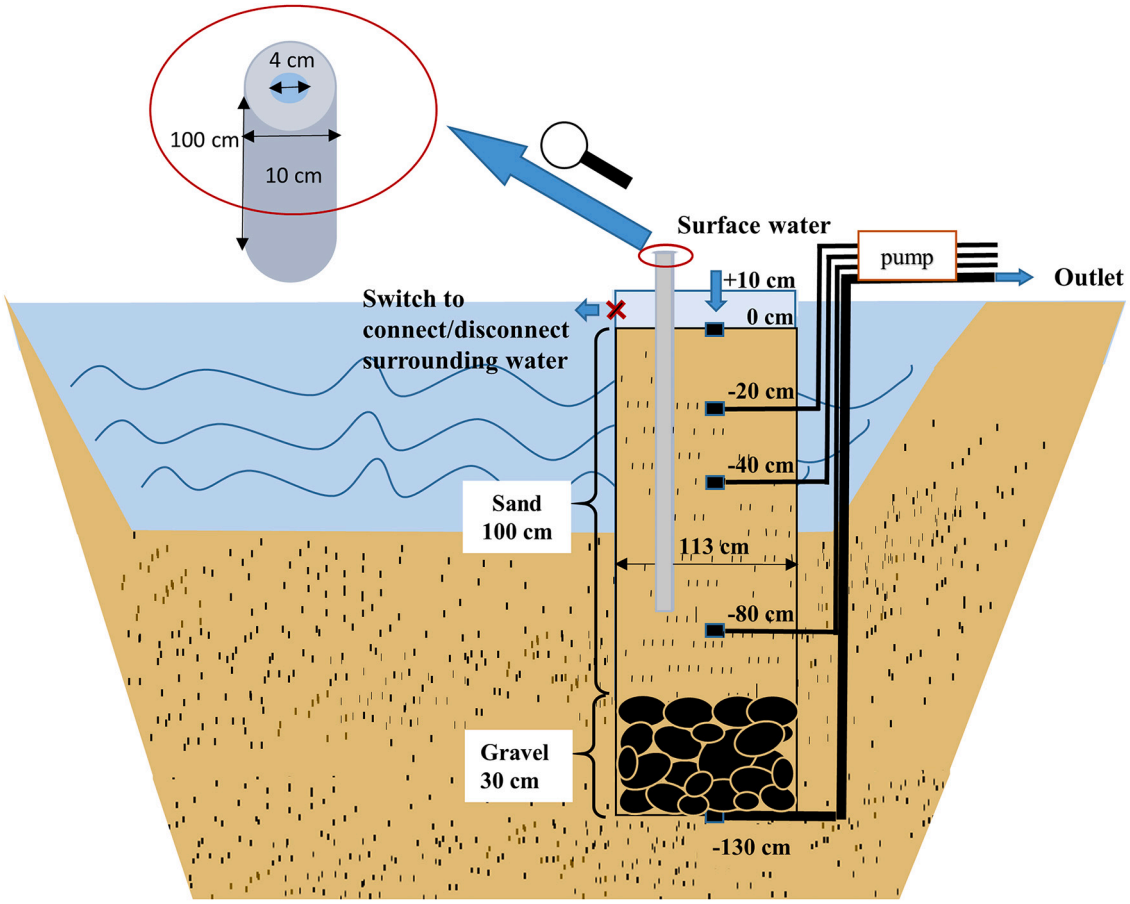

Fig. 1. Schematic drawing of the pond sediment column system.

far greater than 50, a potential wall effect can be neglected (Knappett et al., 2008). Sampling ports for sampling along the flowpath were installed at depths of 20 cm, 40 cm, and 80 cm. The system had been in operation for over 10 years, sufficient for the establishment of a natural bacterial community (Degenkolb et al., 2018).

2.2. Pre-experiments

Pre-experiments included (i) batch tests on the inactivation/decay of MS2 phages in sterile surface water and water-saturated sediment; and (ii) a virus transport experiment in the sediment column with a moderate virus load.

For the batch tests, MS2 was added to filter-sterilized (0.22 μm) Danube River water (pH: 8.19; EC: 401 μS cm^{-1}; DO: 12.76 mg L^{-1}, $n = 3$) or autoclaved water-saturated sand (natural bank sediment; grain size between 63 and 630 μm; porosity of 36%). Duplicate water samples contained an initial total MS2 concentration of 6.47 × 10^8 particles mL^{-1}. Duplicate sediment samples, each water-saturated, consisted of 17 g dry weight sediment with approximately 3.6 mL of pore water and received an initial total MS2 concentration of 1.52 × 10^8 particles mL^{-1}. These samples were incubated at 7 °C in the dark for 19–23 days.

The impact of a moderate virus load on MS2 transport was assessed 2 years before the main experiment, also in November and with the same column system. A pulse of pond water (200*L*) with a moderate virus load, i.e., a final concentration of 1.9 × 10^6 plaque forming units (PFU) mL^{-1} of infectious MS2 (total amount of infectious MS2 of 3.8 × 10^{11} PFU), was supplied with flow rate adjusted to a filter velocity of 1.1 m d^{-1} (corresponding to a pore water velocity of 2.2 m d^{-1}). After the pulse, the column was re-connected to the surrounding pond water just before the pulse solution was completely infiltrated. Water samples were collected before and after the respective treatments at a high temporal and spatial resolution (i.e., the supernatant, 40 cm, 80 cm, and the column outlet at 130 cm depth; Fig. 1). Since this pre-experiment was conducted two years before the main experiment, there was sufficient time for the sediment system to be flushed by MS2-free water.

2.3. Main experimental design

In the first of the two experimental phases, a 100 L pulse of pond water containing a high load of 1.84 × 10^8 gc mL^{-1} MS2 particles, as determined by quantitative real-time polymerase chain reaction (qPCR), and 1.5 × 10^8 PFU mL^{-1} of infectious phage, as determined by the plaque assay, was supplied to the column with a pore water velocity of 2.1 m d^{-1}. Nineteen days after the high virus load pulse, experimental phase 2 was initiated to simulate a heavy rainfall event (corresponding to 37 mm/h) via infiltration of a pulse of 100 L of deionized water. Deionized water was used because its low ionic strength is similar to that of natural rainwater. After each of the pulses, the column was reconnected to the surrounding pond water just before the pulse solution was completely infiltrated. Sampling depths for pore water included the surface (i.e., the supernatant), 20 cm (withdrawal rate: 1.3 L/h), 40 cm (withdrawal rate: 1.4 L/h), 80 cm (withdrawal rate: 1.4 L/h), and the column outlet (at 130 cm depth, withdrawal rate of 33.4 L/h; Fig. 1). From the intermediate ports and at the column outlet water was withdrawn continuously. Samples for infectious MS2, prokaryotic cell counts and microbial activity were collected every hour in the first 7 h and 12 h after the dose of MS2 pulse and the pulse of deionized water, respectively. Subsequently, the sampling intervals increased sequentially with time (see Tables S2 and S3 for details). At the mesocosm outlet in experimental phase 1, water samples for biological monitoring were first

collected 23 h after the supply of MS2 particles. Samples for the analyis of water chemistry were taken once a day during the first 10 days after MS2 dosing and for the entire experimental phase 2 after simulated rainfall. Additional water chemistry samples were taken at 15 and 16 days after MS2 donation.

Sediment samples were collected after the high virus load pulse (experimental phase 1) and the simulated rain event (experimental phase 2) using a double-wall sediment corer (high-density polyethylene, 10 cm outer diameter, 4 cm inner diameter, and 100 cm height), which was pushed into the sediment to a depth of 70 cm at the end of each experimental phase (day 15 and day 23 after the MS2 pulse). The inner core was withdrawn carrying the sediment, whereas the outer tube remained in place for the remainder of the experiment to maintain undisturbed hydrological conditions. The cored sediment samples were divided into portions of 5 cm depth and transferred into falcon tubes (50 mL) for the subsequent quantification of total and infectious MS2 particles. All experiments were conducted at the end of the calendar year (November/December).

2.4. Cultivation and quantification of MS2

Bacteriophage MS2 (DSMZ 13767) and MS2 host strain *Escherichia coli* (DSMZ 5695) were obtained from the DSMZ (German Collection of Microorganisms and Cell Cultures GmbH). Isolation and purification of MS2 phages after propagation with a co-incubation of *E. coli* 5695 were performed following the protocol described in Feichtmayer (2019) to produce a high-density phage suspension in buffer solution.

For the pre-experiment with the moderate virus load, infectious phages were quantified with the plaque assay in accordance with DIN EN ISO 10705–1:2001. For the pre-experiment batches and the main experiments, this was adapted to follow the double-agar overlay method of Kropinski et al. (2009). In brief, a solid agar base (10 mL of 1.5% agar, *w/v*) was overlaid with a mixture of soft agar (4 mL of 0.75% agar, w/v) and 1 mL of a well-mixed solution consisting of the (diluted) sample and host bacteria in the logarithmic growth phase (1:1, *v*/v). Serial dilutions of the mixture were incubated at 37 °C overnight and PFUs were counted in duplicate. The titer of infectious phages in the sediment samples was estimated after the elution of 0.5 mL of sediment with 1 mL of deionized water and vigorous shaking on a vortex mixer (3 times, each for 1 min, with 30 s between each mixing step). The limit of detection (LOD) for the plaque assays was 0.5 PFU mL^{-1}.

Triplicate quantification of the total number of phages was performed using qPCR analysis of phage RNA transcribed into cDNA. Before phage RNA extraction, all water samples were concentrated to a volume of 1 mL using Amicon® Ultra-15 Centrifugal Filter Units (Merck). Phage RNA was then extracted using the AllPrep PowerViral DNA/RNA Kit (Qiagen) and cDNA synthesis was performed using the Maxima First Strand cDNA Synthesis Kit (ThermoFisher). The isolation of phage RNA and the reverse transcription was conducted following the manufacturer's instruction. The qPCR targeted a 315 bp fragment using the MS2-specific primer sequences MS2-2717Fw (5′-CTG-GGC-AAT-AGT-CAA-A-3′) and MS2-3031Rv (5′-CGT-GGA-TCT-GAC-ATA-C-3′),and using the Brilliant III UltraFast SYBR Green QPCR Master Mix (Agilent). Then, 2 μL of cDNA were quantified in triplicate; each 25 μL reaction contained 12.5 μL of qPCR master mix, 0.375 μL of reference dye (1:500 v/v dilution in RNase-free water) and 0.5 μL of each primer (10 μM, Eurofins). The qPCR analyses were performed in a Stratagene MX3000P qPCR cycler (Agilent, $R^2 > 0.99$) and a Light Cycler 480 SW 1.5 (Roche, $R^2 > 0.98$) following a protocol modified from Dreier et al. (2005). Initial denaturation (95 °C, 10 min) was followed by 40 cycles of denaturation (95 °C, 15 s), annealing (50 °C, 30 s) and elongation (72 °C, 30 s). Afterwards, the melting curve was recorded between 55 °C and 95 °C to verify the specificity of the amplification products. Owing to the different sample volumes available for concentration, there was no single LOD; instead, the LOD range was determined as 5.8×10^2–5.6×10^3 gc mL^{-1}.

2.5. ATP and TCC analyses

To study the relationship between virus reduction and the amount and activity of autochthonous microorganisms, microbial activity and prokaryotic cell counts were monitored in both water and sediment samples throughout the experiment. The microbial activity in water and sediment samples was estimated using ATP concentration measurements using the BacTiter-Glo Microbial Cell Viability Assay kit (Promega) following the protocol described in Hammes et al. (2010), but with minor modifications. Water samples and the BacTiter-Glo reagent, prepared in accordance with the manufacturer's instructions, were prewarmed separately at 38 °C for at least 2 min before 1 mL of sample was mixed with 50 μL of reagent. The mixture was incubated at 38 °C for 1 min and luminescence measured on a GloMax 20/20 Luminometer (Promega). Concentrations were determined by comparison with external ATP standards dissolved in ATP-free water (Fisher Scientific) using ATP-free water as blank. To correct for the contribution of extracellular ATP in the samples the measurements were performed on an untreated sample fraction representing the total ATP concentration as well as a fraction of the same sample that was filtered (0.1 μm syringe filter, Merck Millipore) to remove microbial cells. The concentration of intracellular ATP was then calculated by subtracting the extracellular concentration from the total. All measurements were conducted in triplicate.

The total ATP in sediment samples was determined as follows: 50 μL of 0.1 μm filtered Milli-Q water was added to 200 μL of homogenized sediment. Next, 100 μL preheated (38 °C) BacTiter-Glo reagent was added and the sample was mixed using a Thermomixer (1400 rpm) at 38 °C for 2.5 min. Subsequently, 900 μL of 0.1 μm filtered Milli-Q water was added and the sample was briefly mixed using a vortex mixer and centrifuged. Finally, triplicate measurements of the supernatant were performed using a similar protocol to that for the water samples (see above). The extracellular ATP in sediment samples was quantified using a similar setup, but without the addition of 100 μL preheated (38 °C) BacTiter-Glo reagent to lyse the cells.

TCC was quantified using flow cytometry (FC 500 CYTOMICS, Beckman Coulter) as described in Fillinger et al. (2019) for pore water samples and as detailed in Bayer et al. (2016) for sediment samples.

2.6. Physicochemical conditions

Pore water temperature and pH were measured on-site using a temperature data logger (Voltcraft) and microprocessor pH meter, respectively. EC, redox potential, and DO were measured on-site using field sensors (WTW).

2.7. Data analysis

The recovery of total and infectious MS2 during experimental phase 1 and phase 2 was calculated to a depth of 80 cm. The deeper zone of the mesocosm including the gravel layer was not included, thus avoiding inconsistency due to its large sediment heterogeneity and the risk of having missed the MS2 peak concentration at the mesocosm's outlet. For samples with a total MS2 concentrations below the detection limit, the infectious MS2 concentration was used instead for calculation. The number of total and infectious sediment-attached MS2 was calculated as the product of the total or infectious MS2 concentration detected at various depths of the sediment cores and the volume of the corresponding column layer. The phage particle concentrations collected at depths between 65 cm and 70 cm were assumed to be representative of the depth interval of 65–80 cm.

To test the relationships between different biological and physicochemical variables, a Spearman rank correlation analysis was performed with the Hmisc package in R (Harrll et al., 2018) and applied to all available variables collected for pore water and sediments encompassing data from both experimental phases (Tables S4 and S5).

Student's *t*-test ("t.test" in R) and the Wilcoxon test ("wilcox.test" in R) were used to analyze the significance of difference between samples using t with a significance level of 0.05.

3. Results and discussion

3.1. Pre-experiments

3.1.1. Inactivation and decay of MS2 in batch incubations

Following the MS2 inoculum of 6.5 × 10^8 gc mL^{-1} in sterilized surface water for 23 days in batch experiments (Fig. 2) showed the concentration of total MS2 particles, as measured by qPCR, to remain constant, i.e., without any significant reduction (*Wilcoxon test*, $n = 12$, $P = 0.5$; Fig. 2). This indicates that no MS2 decay occurred during incubation. However, the fraction of infectious MS2 showed some decline at 19 days after inoculation, i.e. from 5.6 × 10^8 to 9.8 × 10^7 PFU mL^{-1}, accounting for 15% of the total MS2 and corresponding to a 0.8-log reduction of the initial infectious MS2 concentration. This decrease in infectious MS2 during incubation is assumed to be due to inactivation and implies an inactivation rate of MS2 in the sterilized surface water at 7 °C was 0.045 $\log_{10}$ day^{-1} (Fig. 2), similar to the inactivation rate of MS2 (0.043 $\log_{10}$ d^{-1}) determined at 4 °C in raw groundwater by Ogorzaly et al. (2010).

Similar to the experiments only in sterilized water, no reduction in total MS2 was observed after inoculation into water-saturated sediments (*Wilcoxon test*, $n = 12$, $P = 1$). The initial concentration of attached total MS2 in the sterile water-saturated sediment mixture was 1.5 × 10^8 gc mL^{-1}. Infectious MS2 attached to the sediment with an initial concentration of 8.9 × 10^7 PFU mL^{-1} declined at an inactivation rate of 0.035 $\log_{10}$ d^{-1} (Fig. 2). The assumed inactivation rate of attached MS2 phages was lower than that of the suspended MS2, indicating that although attachment is an important process for attenuating MS2 during the subsurface passage, phage activity is preserved. Prolonged survival of the virus by attachment to sediment was also observed by Melnick et al. (1980) and Schijven and Hassanizadeh (2000).

3.1.2. Transport of a moderate MS2 pulse through the sediment column

In the pre-experiment that lasted for 50 h, we observed a significant decrease in the peak concentrations of the infectious virus breakthrough curves with depth, from 1.9 × 10^6 PFU mL^{-1} at the surface to 2.1 × 10^3 PFU mL^{-1} at a depth of 80 cm (Fig. 3). After 80 cm of sediment passage, the attenuation or removal of infectious MS2 amounted to approximately 3.0 $\log_{10}$. No significant difference in the concentration of infectious MS2 was detected at the depth of 80 cm and the outlet at the depth of 130 cm (*t*-test, $t = 0.97$, $df = 9.7$, $P = 0.35$).

3.2. Main experiments

3.2.1. Dynamics of physico-chemical water quality

During the entire course of the main experiment, the pond water infiltrating into the bank sediment differed significantly in temperature (*Wilcoxon test*, $n = 34$, $P < 0.05$), DO concentration (*t-test*, $t = -3.6$, $df = 31$, $P < 0.01$) and pH (*t-test*, $t = -4.0$, $df = 24.6$, $P < 0.001$) from the water collected at the outlet after 130 cm of sediment passage. Compared to the surface water temperature ($mean_{surface}$ = 5.9 °C ± 2.4 °C) water temperature at the column outflow was higher and more constant ($mean_{outlet}$ = 7.8 °C ± 2.0 °C; Fig. 4). This difference was partially due to the transition from autumn to winter and the delayed response of the sediment temperature to the decrease in air temperature during the course of the experiment. The mean DO concentration decreased from 13.9 ± 1.8 mg L^{-1} ($mean_{surface}$) to 11.5 ± 2.2 mg L^{-1} ($mean_{outlet}$) during the passage of pore water through the column, which was likely due to higher oxygen solubility at lower temperature and microbial processes, e.g., aerobic respiration (Diem et al., 2013). A peak value of 17.2 mg L^{-1} DO in the infiltrating surface water on day 9 corresponded to a sudden drop in temperature (Fig. 4). The pH decreased from a mean value at the surface of 8.1 ± 0.1 to a mean value at the outlet of 7.8 ± 0.3 (Fig. 4). Heterotrophic biological processes result in DO consumption and oxidation of organic matter with the production of CO_2 and/or organic acids which generally reduces pH (Kuehn and Mueller, 2000). During sampling, the mean redox potential at the surface (350 ± 71 mV) was not significantly different from that at the column outlet (347 ± 67 mV; *Wilcoxon test*, $n = 32$, $P > 0.5$). As redox

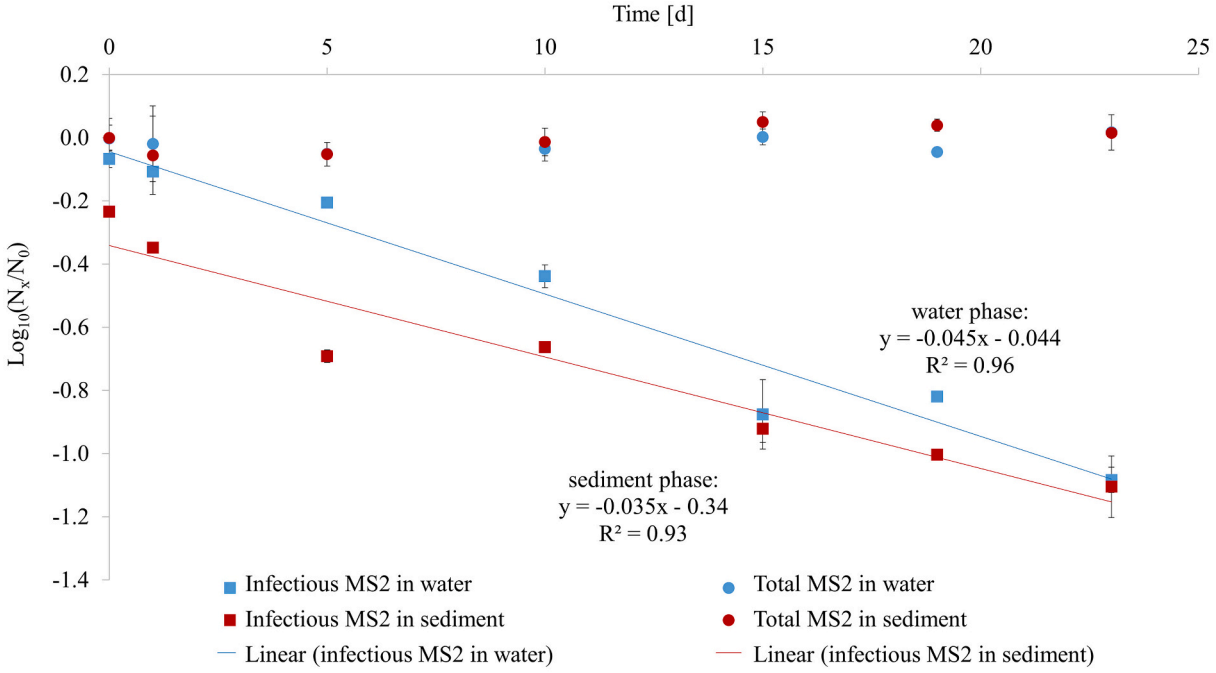

Fig. 2. The variation of total and infectious bacteriophage MS2 in sterilized surface water or sterilized water-saturated sediment (the abundance of total MS2 at time 0 in water or in sediment was recorded as N_0 and the abundance of total or infectious MS2 detected at the corresponding time point in water or in sediment was defined as N_x).

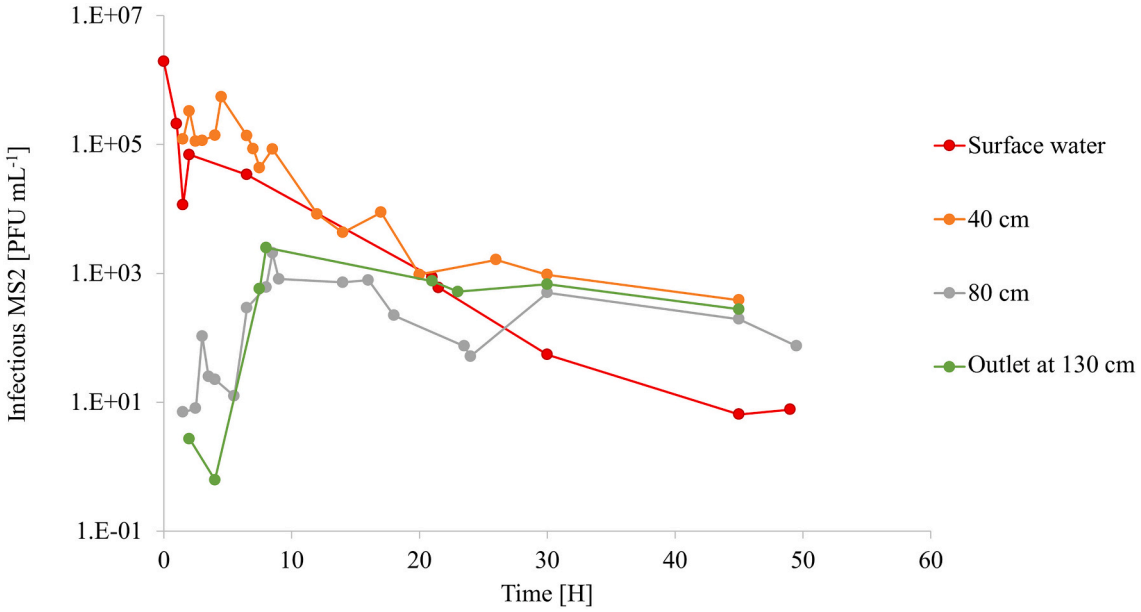

Fig. 3. Breakthrough of infectious MS2 at different depths of the column, as determined from the transport pre-experiment.

conditions remained oxic throughout the entire experiment effects on the survival of the virus by a redox shift are likely to have been negligible (Feichtmayer et al., 2017). EC remained constant during the experiment ($mean_{surface} = 878 \pm 223$ µS cm^{-1}, $mean_{outlet} = 879 \pm 192$ µS cm^{-1}) and was independent of sediment depth, with one exception, namely the supply of deionized water at the beginning of phase 2 which caused a temporary shift throughout the sediment column. The lowest EC caused by deionized water was 15.1 µS cm^{-1} at the surface; a higher value of 135.8 µS cm^{-1} was detected at the outlet. The simulated heavy rainfall, as intended, led to a reduction in the ionic strength of the sediment pore water. Overall, the differences in DO and pH indicated the activity of biological processes during bank filtration (Kuehn and Mueller, 2000; Diem et al., 2013).

3.2.2. *Experimental phase 1 – virus contamination*

3.2.2.1. Fate of MS2 during transport through the bank sediment. The peak concentrations of total MS2 in the breakthrough curves decreased with increasing depth and distance to the surface (Fig. 5), i.e., from 1.84 × 10^8 gc mL^{-1} in the surface water to 3.2 × 10^7 gc mL^{-1} at 80 cm. Thus, the peak of total virus concentration was reduced by 0.8 log units through 80 cm of sediment passage. A total amount of 1.75 × 10^{13} MS2 particles was estimated to be transported through the 80 cm sediment column (Table 2), i.e., at the end of experimental phase 1, only 5% of the total MS2 particles remained in the column. At 219 h after the inoculation with MS2, 10^3 total MS2 particles mL^{-1} were detected in the surface water, corresponding to a log decrease rate of 0.0185 h^{-1}. We attribute these remaining MS2 particles to kinetic phage attachment to and detachment from the top sediment, taking into account the mixing and non-uniform infiltration of the pond water (Frohnert et al., 2014; Schijven and Hassanizadeh, 2000).

As with the concentration of total MS2 phage particles, the peak concentration of infectious MS2 in pore water decreased with increasing distance from the surface, i.e., from 1.5 × 10^8 PFU mL^{-1} in the surface water to 2.9 × 10^6 PFU mL^{-1} measured at depth of 80 cm (Fig. 5) corresponding to a removal of approximately 1.7 log units. With a reduction in peak concentration of 0.8 log units for total MS2 particles and 1.7 log units for infectious MS2 particles, the overall reduction of virus in our main experiment was in the range of that reported in other studies using MS2 as a model agent, i.e., a 0.1–1.9 log unit reduction over a filtration distance of 0.4–1.5 m as reviewed by Bauer et al. (2011). With the moderate virus load applied in the pre-experiment, which was run under the same conditions as the main experiment, a 3.0 log unit removal of infectious MS2 in pore water was found, exceeding the removal in the main experiment.

The lower removal efficiency in the main experiment can be explained by a conceptual model for colloid transport derived from column studies and micromodel observations, as suggested by Bradford et al. (2009). At high initial input concentrations, a lower relative mass transfer to the solid phase occurs because weakly associated colloids are knocked off the solids' surface owing to more frequent collisions. Moreover, Bradford et al. (2009) found that only a small fraction of the porous media play an active role in colloid retention, the reason being that colloid retention is highest near grain–grain contact where low-velocity areas form. Furthermore, another possible explanation is the enhanced aggregation caused by the higher virus concentration, leading to less interaction of the individual viruses with the grain surfaces. Indeed, all virus particles within one aggregate must be inactivated before this aggregate group will not appear as active in the plaque assay (Schijven and Hassanizadeh, 2000). The high virus load we applied reflects a worst-case scenario with a high share of sewage in river flow, as raw sewage is reported to contain between 10^9 and 10^{12} infectious coliphages per L (Lodder and de Roda Husman, 2005). Applying a high virus load also enabled the reliable detection of total and infectious MS2 particles over time and space.

19 days after the major MS2 pulse 0.1% of MS2 particles that were transported through the 80 cm sediment column were still infective. Compared to the infectious MS2 fraction of the sterilized water in batch experiments, a much higher proportion of infectious MS2 particles lost their infectivity during the sediment passage in the main experiment. We assumed that the higher fraction of inactivated MS2 phages in the column pore water relative to the sterile batch tests was mainly attributable to the interplay of pore water flow and attachment, as well as to autochthonous microorganisms and their activity. For example, Hurst et al. (1980) and Schijven et al. (2002) found that the presence of aerobic microorganisms, especially sediment-attached microorganisms, could affect virus activity and may promote virus decay. Yates et al. (1990) provided an early review of the mechanisms through which microorganisms may cause the inactivation of viruses. For example, certain bacteria release proteolytic enzymes that destroy the protein coating of virus particles (Cliver and Herrmann, 1972). Deng et al. (2014) indicated that the number of infectious MS2 phages may be

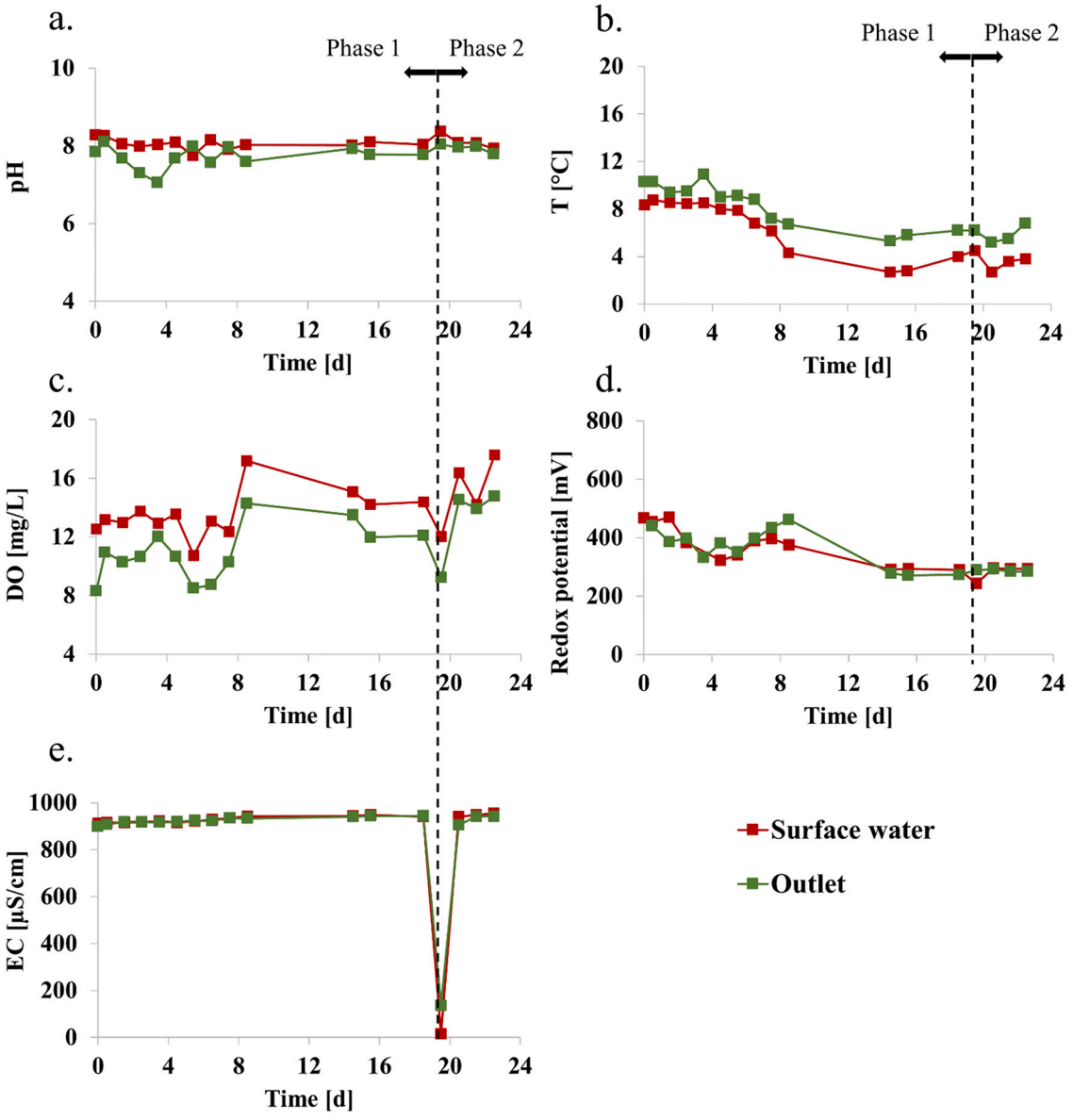

Fig. 4. Physico-chemical conditions in the surface water and column outlet over the course of the main virus transport experiment. T: temperature; DO: dissolved oxygen; EC: electrical conductivity.

reduced by grazing protozoa. More recently, Feichtmayer et al. (2017) provided a comprehensive review of the antagonistic microbial processes inactivating and/or eliminating viruses in aquatic ecosystems.

Importantly, the average percentage of infectious MS2 in pore water decreased with increasing distance from the point of infiltration (Fig. 5). Moreover, the proportion of infectious MS2, 0.4%–79%, also changed over time in the breakthrough water. The highest proportion of infectious MS2 particles was found near or at the peak of the individual breakthrough curves, independently of sediment depth (i.e., 79% at 0 cm, 59% at 20 cm, 47% at 40 cm, and 30% at 80 cm).

3.2.2.2. Fate of MS2 attached to the sediment in phase 1 core samples. The concentration of sediment-attached total MS2 particles was distributed rather evenly with depth in the sediment column at the end of phase 1 (i.e., 15 days after the inoculation with MS2) (Fig. 6). Starting with a total concentration of attached MS2 phages of 1.5×10^5 gc mL^{-1} in the uppermost sediment layer, the concentration increased to 4.8×10^5 gc mL^{-1} to a depth of 15 cm and then remained constant, independently of further sediment depth. This pattern indicated that the top sediment layer experienced significant detachment owing to the ongoing infiltration of MS2-free surface water whereas sediments below 15 cm experienced a MS2 saturation with a balance between the attachment and detachment of MS2 particles.

The distribution of infectious MS2 particles followed a slightly different trend, with a stable concentration in the top 45 cm and a moderate decrease in concentration and proportion between 50 cm and 70 cm. The percentage of infectious MS2 phages was thus high at the top (15% at 5 cm), decreased with depth, and became lowest at the bottom of the column (0.8% at 70 cm). Both the attached and suspended infectious MS2 decreased with depth, suggesting MS2 experienced more inactivation than decay during the sediment passage. This result also suggests that the retention of infectious phage particles is higher than that for the total MS2 particles because inactivation reduces particle size and alters the physico-chemical characteristics of the phage surface, facilitating transport (Ghanem et al., 2018).

3.2.2.3. Microbial biomass and activity in phase 1. The abundance of prokaryotic cells and microbial activity in the pore water at individual

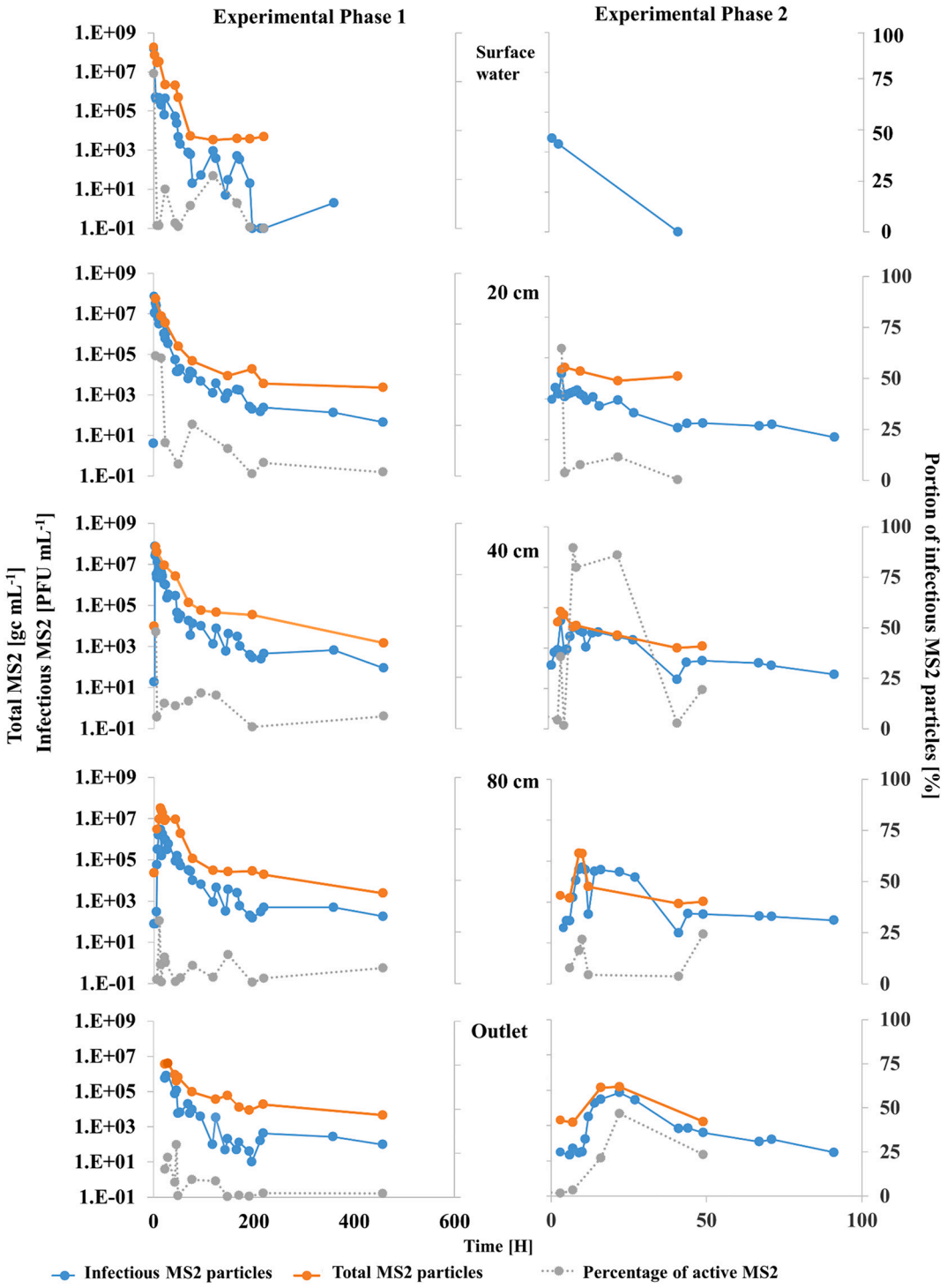

Fig. 5. Breakthrough of total and infectious MS2 particles at various sediment depths in pore water during experimental phases 1 and 2.

sediment depths remained stable during phase 1 (Fig. 7). This indicates that the inoculation of surface water with MS2 did not significantly alter other microbial patterns in sediment pore water. As expected, the concentration of prokaryotic cells in the pore water decreased with sediment depth, from 8.12×10^5 cells mL^{-1} in the surface water to 5.39×10^4 cells mL^{-1} at 100 cm. A similar pattern was observed for pore water microbial activity (Fig. 7). Prokaryotic cell counts and microbial activity of the attached microbial communities from core sampling were also highest in the top-most sediments (Fig. 8). Decreasing prokaryotic cell numbers with increasing sediment depth were consistent with previous studies (as reviewed by Hassard et al., 2016), while the stable microbial activity throughout the column below 10 cm the presence of a well-established depth-independent active bacterial community (Degenkolb et al., 2018). The activity of indigenous microbes is assumed to have a negative impact on the survival of allochthonous viruses and bacteria, particularly those that do not have a host in the environment, such as human pathogenic viruses (Sobsey et al., 1980; Feichtmayer et al., 2017).

Table 2
Estimate of total and infectious MS2 during the main experiment down to a column depth of 80 cm (Phase 1: dose of MS2; Phase 2: pulse of deionized water).

Experimental phase	Samples	Total MS2 (gc)	Distribution of inoculum	Proportion of infectious to total MS2
	Inoculum	1.84×10^{13}	100%	79%
Phase 1	Sediment-attached	3.3×10^{11}	1.8%	4%
	Flow through 80 cm	1.75×10^{13}	95%	6.7%
	Retained–MS2	3.3×10^{11}	1.8%	4%
Phase 2	Sediment-attached	4.2×10^{11}	2.4%	2.9%
	Flow through 80 cm	3.3×10^{10}	0.2%	69%

3.2.3. *Experimental phase 2 – Phage re-mobilization through a simulated heavy rainfall event*

As described in Section 2.2, the simulated heavy rainfall event was initiated 19 days after the supply of a high load of MS2. The infiltration of 100 L of deionized water was equivalent to a recharge of precipitation of 37 mm/h, which represents an extreme rainfall for the Berlin area. The pulse of deionized water caused a significant decrease in EC, i.e., a reactive front of low ionic strength water migrating down through the sediment column (Fig. 4). After 1 day, the EC values were fully recovered, not only in the surface water but also at the outlet of the column.

3.2.3.1. Re-mobilization of attached MS2 phages. Although the number of total MS2 particles had dropped below or close to the detection limit in sediment pore water immediately before the simulated rainfall event, the pulse of deionized water caused a new moving front of MS2 phages re-mobilized from the sediment migrating through the sediment column. In contrast to experimental phase 1, the peak concentrations of total MS2 particles in pore water increased with depth, reaching concentrations as high as 10^5 gc mL^{-1} (Fig. 5).

The fraction of infectious MS2 phages followed a distribution pattern similar to that of the total MS2 phages (Fig. 5). The average percentage of infectious MS2 in pore water samples at different depths was in the range of 13%–40% and hence significantly higher than in the first experimental phase (where the range was 7.6%–21%). Notably, in experimental phase 2, 3.3×10^{10} MS2 particles in total were transported through the 80 cm sediment column after the application of deionized water, and a significant portion (on average 69%) were still infectious (Table 2). This suggested that attachment somehow conserves infectivity of phage particles better than transport in suspension.

Similar findings were obtained in the batch experiments (Fig. 2) and in earlier studies (Yates et al., 1987; Melnick et al., 1980; Schijven and Hassanizadeh, 2000). Gerba (1984) proposed that the mechanisms protecting viruses attached to sediment might include shielding from proteolytic enzymes or other substances that inactivate and degrade viruses. The attachment of virus particles to the soil and sediment surface is mediated by a combination of electrostatic and hydrophobic interactions (Gerba, 1984). A decrease in pore water salt concentration is known to increase the surface potential of the attached viruses owing to the expansion of the electrostatic double layer surrounding the virus particles and soil particle surfaces, causing virus detachment (Quanrud et al., 2003). A rapid shift to low ionic strength conditions has a strong desorption effect on phages and other particles from sediment surfaces (Yates et al., 1987; Gerba, 1999). Deionized water, as shown in this experiment, can cause a quantitative re-mobilization of previously immobilized virus and thus a second pulse of contamination (Guber et al., 2006; Landry et al., 1979). Although the reactive front of the low ionic strength water pulse initiated the desorption of part of the attached phages, the phage peak in the column outflow was broader. We assumed that some virus particles underwent re-attachment further down the sediment column, as observed by Wellings et al. (1975).

3.2.3.2. Phages remaining attached to sediment after simulated heavy rain. A significant number of total MS2 particles (around 10^{11} gc) remained attached to the sediment after both phases of the experiment (Table 2). After the artificial rain event, the abundance of sediment-attached total MS2 particles was moderately lower at the top of the sediment column than in experimental phase 1, down to a depth of 20 cm, but below that it remained constant and similar to the numbers found at the end of experimental phase 1 (Figs. 6 and 8). Indeed, the

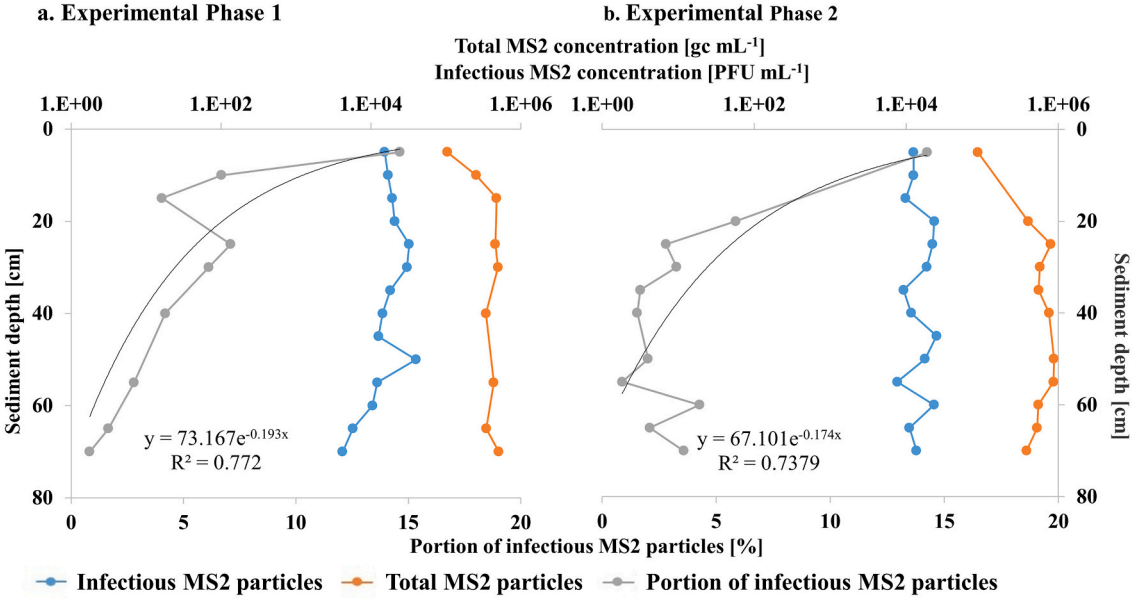

Fig. 6. Distribution of total and infectious sediment-attached MS2 particles with depth (a: sediment samples taken from core samples after 15 days, at the end of experimental phase 1; b: sediment samples taken from core samples 23 days after the application of deionized water, at the end of experimental phase 2; the grey solid line models the proportion of infectious MS2 collected from the sediment core against the sediment depth).

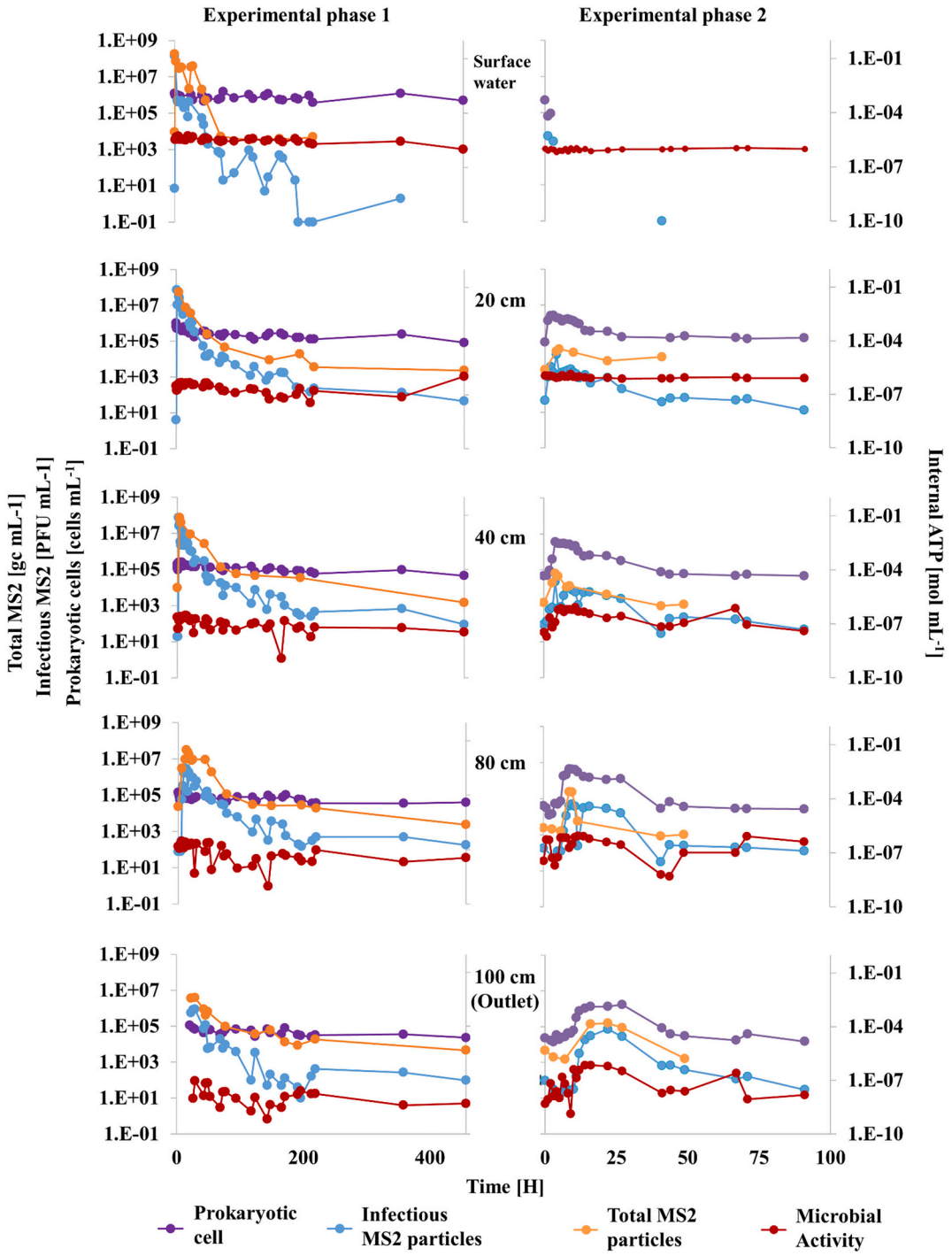

Fig. 7. Spatio-temporal distribution of bacteriophage MS2 (total and infectious), microbial activity, and prokaryotic cell concentration in sediment pore water of the column for both experimental phases (virus pulse and simulated rain event) and in various depths of pore water.

number of sediment-bound infectious MS2 phages after the simulated rainfall event was not significantly different from that in experimental phase 1 (*t-test, t = 0.80, df = 26, P = 0.43*). Infectious MS2 particles attached to the sediment matrix were distributed more evenly with depth than total phage particles, ranging from 7.6×10^3 to 2.5×10^4 PFU mL^{-1}. Modelling the proportion of infectious MS2 collected from the sediment core against the sediment depth showed that the decrease in the proportion of infectious MS2 phages with depth was less steep after the artificial rainfall than in experimental phase 1 (Fig. 6), indicating that infectious MS2 particles were preferentially re-mobilized.

3.2.3.3. Effects of rain on the distribution of bacteria and microbial activity. The artificial heavy rainfall not only mobilized sediment-attached MS2 particles but also caused prokaryotic cells to detach (Fig. 7). This result supported the findings of earlier studies, which reported that the contamination of groundwater wells was promoted by the detachment of bacteria from surrounding sediments in periods of heavy rainfall (Bition and Harvey, 1992; Zyman and Sorber, 1988). Interestingly, in our study, no apparent changes were observed in microbial activity down to a depth of 40 cm, indicating that cells with no or low activity were predominantly dislodged from the sediment following rainfall. However, at deeper zones of the sediment column, the microbial

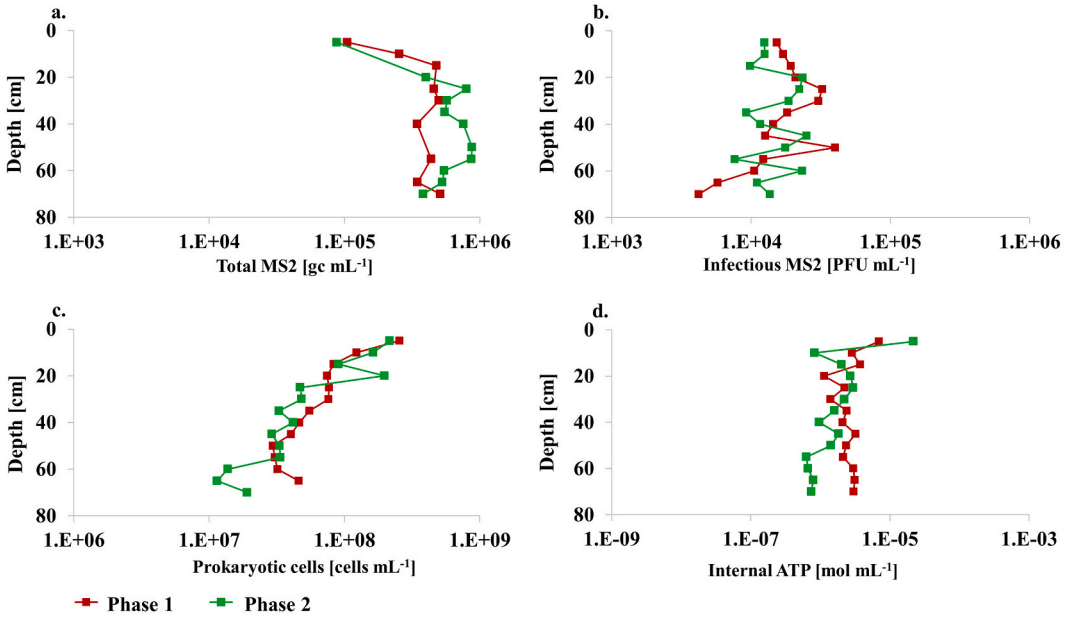

Fig. 8. Vertical distribution of sediment-attached bacteriophage MS2 (total: a and infectious: b), prokaryotic cell concentration (c), and microbial activity (d) in the enclosure at the end of experimental phase 1 (virus pulse) and phase 2 (simulated rain event).

activity appeared to follow the dynamics of the bacterial numbers (Fig. 7). The peak concentration of prokaryotic cells in the sediment pore water following the simulated rainfall was more than 10 times higher than the pore water concentrations detected in experimental phase 1. However, at 91 h after the simulated rainfall, lower concentrations of prokaryotic cells were detected in pore water compared with phase 1, but the overall pattern was similar: fewer prokaryotic cells were present further down the column.

As in experimental phase 1, the sediment-attached prokaryotic cell concentration was highest at the top of the column (5 cm sediment depth) and 10 times lower at a depth of 70 cm. After the rainfall, although the numbers of sediment-attached microorganisms were lower mainly in the top sediments, the overall abundance of bacteria that remained attached to the sediment was lower in experimental phase 2 than in experimental phase 1. Although a breakthrough of prokaryotic cells migrating through the sediment column was observed following the simulated rain event, the total number of sediment-attached cells did not change significantly (*Wilcoxon test*, $n = 28$, $P = 0.38$), as shown by the cell count, which was two orders of magnitude lower in pore water than attached to the sediment matrix. Similar patterns were found for microbial activity (Fig. 8). The simulated rainfall significantly increased the activity of the sediment-attached microbial consortia in the top sediment. Apart from this exception, most of the sediment-bound microbial activity was lower in phase 2, with a significant difference between phase 1 and phase 2 revealed (*Wilcoxon test*, $n = 28$, $P = 0.003$).

3.3. A comprehensive perspective on the main experiment

Although it may be assumed that the virus concentrations applied in the experiment rarely occur in natural environments, in face of the rather small sample volumes available and the LOD of the methods applied for the quantification of total and infectious MS2, the seeding of high phage numbers allowed the spatio-temporal resolution of several processes involved in the retardation and attenuation of virus in bank sediment infiltration, including attachment, detachment, and inactivation. Our study revealed that high peak concentration of viruses in recharge water can lead to exceedance of the retention capacity of bank sediments, presumably caused by the displacing interactions between attached MS2 and suspended MS2 in the water phase. In our main experiment, only a minor fraction of the supplied virus particles was retarded by attachment, and for the peak concentrations of total MS2 and infectious MS2 phage particles the reductions achieved amounted only to of 0.8 and 1.7 log units, respectively, for a sediment passage of 80 cm. In contrast, for the lower initial MS2 concentration in the supply water, the sediment removal efficiency was much higher, amounting to 3.0 $\log_{10}$. If we only consider infectious virus particles to be a health hazard, our results are more promising. We showed that with travel distance through the sediment and water residence time, the proportion of non-infectious virus particles steadily increased. After a sediment passage of 80 cm, 1.2 $\log_{10}$ of the total MS2 particles were inactivated and could no longer be detected by the plaque assay. Indeed, compared with MS2, other viruses, particularly many human pathogenic viruses, tend to be attached more efficiently, especially within the first centimeters of sediment passage (Schijven and Hassanizadeh, 2000). Thus, the breakthrough of viruses of health concern is likely to be lower than for the bacteriophage MS2.

The attachment of a virus particle does not imply that it will be inactivated and decay. Our data showed that re-mobilized virus particles were characterized by a higher proportion of infectious particles (0.3%–89.4%) than that found in the suspended virus fraction during the initial contamination, i.e., experimental phase 1 (Fig. 5). The inactivation of viruses during attachment is reported to be virus-specific (Frohnert et al., 2014). Yates et al. (1987) reviewed further factors that could affect the transport and inactivation of viruses during soil and sediment passage, including temperature, pore water pH, sediment organic matter content, autochthonous microbial activity, and ionic strength. While earlier studies commonly agree that viruses remain infective and intact for longer at low temperatures (Yates et al., 1987; Feng et al., 2003) and inactivation rates increase with an increase in temperature (John and Rose, 2005; Krauss and Griebler, 2011), during our experiment, temperature showed a positive correlation to both infectious MS2 and total MS2 phage concentrations in pore water (Table S4). However, this correlation is caused by a decrease of virus particles over time and a simultaneous seasonal decrease of ambient temperature in the sediment mesocosm. Moreover, water temperatures detected during our experiment remained $<10\,^{\circ}C$, a temperature range characterized by a very low inactivation rate of MS2 (Schijven and Hassanizadeh, 2000). EC was negatively correlated with infectious MS2 phage concentrations. This supports the view that compared with total MS2, the desorption of infectious MS2 is more sensitive to ionic strength. For the sediment

samples tested, prokaryotic cell abundance and microbial activity were significantly negatively correlated with the concentration of total MS2 particles rather than the concentration of infectious MS2 particles (Table S5). This indicates an antagonistic relationship between the autochthonous microbial communities and the allochthonous phages. Microbial mediated antagonistic processes that lead to a reduction of allochthonous viruses and microbes have been reported (Feichtmayer et al., 2017). A further possibility is competition between total viruses and autochthonous bacteria for attachment sites in the aquifer medium.

The simulated recharge of rainwater clearly showed that a significant proportion of the initially attached viruses was re-mobilized, resulting in a second pulse of virus-contaminated pore water moving through the bank sediment and thus demonstrating that extreme hydrological events indeed have the potential to cause a temporary impact on water quality. As the low EC of the simulated rainwater exceeded 130 cm of sediment passage (Fig. 4), the re-mobilization of attached viruses can be expected to have an impact over a distance of several meters, depending on the time and intensity of the rain event, water flow velocity, and pore-water ionic strength (Quanrud et al., 2003).

4. Conclusions

Attachment, inactivation, and decay are important processes in the attenuation of viruses, as exemplified with the bacteriophage MS2, during bank sediment passage. Even when challenged with an extreme virus concentration of 10^8 particles mL^{-1}, at the upper end of loads to be expected in rivers, bank filtration can be efficient in reducing a viral contamination by 1–2 log units after only a short transport distance of less than one meter. At lower concentrations of viruses infiltrating bank sediments and a lower water flow velocity (i.e., high water residence time), and/or higher temperatures, the natural attenuation efficiency is likely to be much higher, in the range of 3–4 log units. Equally important, only a small fraction of virus particles remained infectious after the sediment passage, indicating that virus inactivation was a quantitatively important process in bank filtration. Extreme hydrological events, such as heavy rainfall, will cause the re-mobilization of previously attached virus particles and autochthonous microorganisms from the sediment matrix. As such, rain and flood events may pose a hazard for drinking water production, and they require particular attention in risk assessment and management.

Author statement

He Wang: Sample and data analysis, visualization, writing of the MS. Judith Kaletta: Field work, sample analysis. Sigrid Kaschuba: Field work, sample analysis. Sondra Klitzke: Field work, sample analysis, writing of the MS. Ingrid Chorus: Writing of the MS. Christian Griebler: Conceptualization, supervision, writing of the MS.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jconhyd.2022.103960.

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Supporting Materials

Attachment, re-mobilization and inactivation of a surrogate pathogenic virus during bank filtration simulating an accidental spill and an extreme rain event

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Figure caption

Figure S1 The linear regression between total MS2 concentration and infectious MS2 concentration.

Table caption

Table S1 Grain size distribution of the coarse-grained medium sand in the enclosure

Table S2 The temporal resolution water samples for biological parameters in experimental phase 1 (blue: infectious MS2; orange: total MS2; red: microbial activity; purple: prokaryotic cell counts).

Table S3 The temporal resolution water samples for biological parameters in experimental phase 2 (blue: infectious MS2; orange: total MS2; red: microbial activity; purple: prokaryotic cell counts).

Table S4 The coefficient of spearman correlation analysed from infiltration water samples during the whole experiment

Table S5 The coefficient of spearman correlation analysed from sediment samples collected at the end of both experimental phases

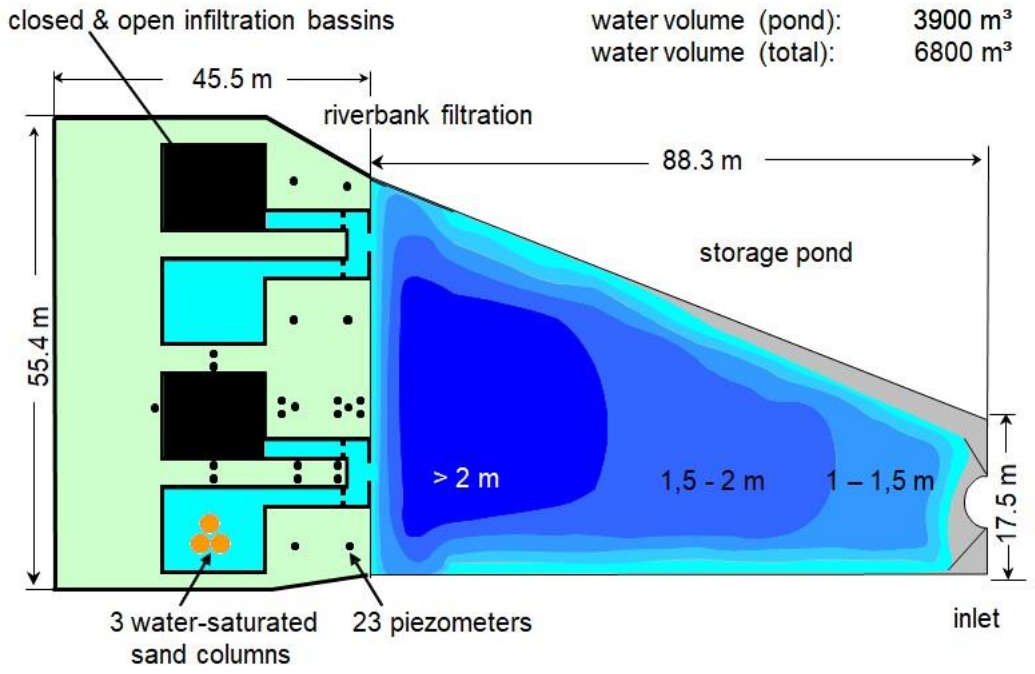

Figure S1 Sketch of facility for simulation of riverbank and slow sand filtration

Table S1 Grain size distribution of the coarse-grained medium sand in the enclosure.

Grain size (mm)	< 0.002	0.002 - 0.0063	0.0063 - 0.02	0.02 - 0.063	0.063 - 0.2	0.2 - 0.63	0.63 - 2.0
Fraction (%)	0.0	0.0	0.1	0.4	14.8	73.8	10.9

Table S2 The temporal resolution water samples for biological parameters in experimental phase 1 (blue: infectious MS2; orange: total MS2; red: microbial activity; purple: prokaryotic cell counts).

sampling date	sampling time	time	time interval	surface	20 cm	40 cm	80 cm	outlet
[m/d/y]	[h:m]	[h]	[h]					
11/2/2011	13:30	0		××××	××××	××××	××××	
11/2/2011	14:30	1	1	××	××××	××××	××	
11/2/2011	15:30	2	1	××	××××	××××	××××	
11/2/2011	16:30	3	1		××××	××××	××××	
11/2/2011	17:30	4	1	××××	××××	××××	××××	
11/2/2011	18:30	5	1	××××	××××	××××	××××	
11/2/2011	19:30	6	1	×	××××	××××	××××	
11/2/2011	20:30	7	1	××××	××××	××××	××××	

11/2/2011	22:30	9	2	××	×××	×××	×××	
11/3/2011	0:30	11	2	×××	×××	×××	××××	
11/3/2011	2:30	13	2	×	×××	×××	××××	
11/3/2011	4:30	15	2	××	××××	×××	××××	
11/3/2011	6:30	17	2	×	×××	×××	××××	
11/3/2011	10:30	21	4	××	×××	××××	××××	×
11/3/2011	12:30	23	2	×××	××××	×××	××××	××
11/3/2011	15:30	26	3	××	×××	×××	×××	×××
11/3/2011	18:30	29	3	××	×××	×××	×××	××××
11/4/2011	8:30	43	14	××××	×××	××××	××××	××××
11/4/2011	11:30	46	3	×××	×××	×××	×××	××××
11/4/2011	14:30	49	3	××××	××××	×××	×××	××××
11/4/2011	18:30	53	4	×××	×××	×××	×××	×××
11/5/2011	10:30	69	16	×××	×××	××××	×××	×××
11/5/2011	14:30	73	4	××××	×××	×××	×××	×××
11/5/2011	18:30	77	4	×××	××××	×××	××××	××××
11/6/2011	11:30	94	17	×××	×××	××××	×××	×××
11/7/2011	11:30	118	24	××××	×××	×××	××××	×××
11/7/2011	17:30	124	6	×××	×××	××××	×××	××××
11/8/2011	12:30	143	19	×××	×××	×××	×××	×××
11/8/2011	17:30	148	5	×××	××××	×××	××××	××××
11/9/2011	11:30	166	18	××××	×××	×××	×××	×××
11/9/2011	16:30	171	5	×××	×××	×××	×××	××××
11/10/2011	12:30	191	20	××××	×××	×××	×××	××××
11/10/2011	17:30	196	5	×××	××××	××××	××××	×××
11/11/2011	10:30	213	17	×××	×××	×××	×××	×××
11/11/2011	16:30	219	6	××××	××××	×××	×××	××××
11/17/2011	11:30	358	139	×××	×××	×××	×××	×××
11/21/2011	14:30	457	99	××	××××	××××	××××	××××

Table S3 The temporal resolution water samples for biological parameters in experimental phase 2 (blue: infectious MS2; orange: total MS2; red: microbial activity; purple: prokaryotic cell counts).

sampling date	sampling time	time	time interval	surface	20 cm	40 cm	80 cm	outlet
[m/d/y]	[h:m]	[h]	[h]					
11/21/2011	18:00	1	1	×××	××××	×××	××	
11/21/2011	19:00	2	1	×	××××	×××	××	××
11/21/2011	20:00	3	1	×××	×× ××	××××	×××	××
11/21/2011	21:00	4	1		××××	××××	×××	××
11/21/2011	22:00	5	1		××××	××××	×××	××
11/21/2011	23:00	6	1		×××	×××	××××	×××

11/22/2011	0:00	7	1		×××	×××	×××	××××
11/22/2011	1:00	8	1		×××	××××	×××	××
11/22/2011	2:00	9	1		×××	××××	××××	×××
11/22/2011	3:00	10	1		××××	×××	××××	×××
11/22/2011	4:00	11	1		×××	×××	×××	×××
11/22/2011	5:00	12	1	××	×××	×××	××××	×××
11/22/2011	7:00	14	2		×××	×××	×××	×××
11/22/2011	9:00	16	2		×××	×××	×××	××××
11/22/2011	15:00	22	6	××	××××	××××	×××	××××
11/22/2011	20:00	27	5		×××	×××	×××	×××
11/23/2011	10:00	41	14	×××	×××	××××	××××	×××
11/23/2011	13:00	44	3		×××	×××	×××	×××
11/23/2011	18:00	49	5	××	××××	×××	××××	××××
11/24/2011	12:00	67	18		×××	×××	×××	×××
11/24/2011	16:00	71	4		×××	×××	×××	×××
11/25/2011	12:00	91	20	××	××××	××××	××××	××××

Table S4 The coefficient of spearman correlation analysed from infiltration water samples during the whole experiment. (DO: oxygen concentration; EC: electrical conductivity; T: water temperature; E_h: redox potential; IMS2: infectious MS2 concentration by plaque assay; TMS2: total MS2 concentration by qPCR; TCC: prokaryotic cell number by flow cytometry, different probablity shown with a different color, yellow: P < 0.001; orange: 0.001< P < 0.01; white: 0.05<P).

	TMS2	IMS2	TCC	ATP	E_h	DO	EC	T
pH	0.25	0.29	0.56	0.64	0.01	0.31	-0.03	-0.32
T	0.67	0.52	0.02	-0.21	0.60	-0.71	-0.72	
EC	-0.45	-0.77	-0.36	-0.10	-0.53	0.50		
DO	-0.34	-0.22	0.10	0.32	-0.26			
E_h	0.64	0.38	0.26	0.22				
ATP	0.10	0.13	0.77					
TCC	0.27	0.13						
IMS2	0.75							

Table S5 The coefficient of spearman correlation analysed from sediment samples collected at the end of both experimental phases. (IMS2: infectious MS2 concentration by plaque assay; TMS2: total MS2 concentration by qPCR; TCC: prokaryotic cell number by flow cytometry different probablity shown with a different color: blue: 0.01< P < 0.05; white: 0.05<P).

	TCC	iATP	TMS2
IMS2	0.12	-0.01	-0.01
TMS2	-0.49	-0.53	
iATP	0.48		

2.2 Dynamics of pathogens and fecal indicators during riverbank filtration in times of high and low river levels

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Synopsis

Riverbank filtration is a popular method of managed aquifer recharge that not only enhances the quantity of groundwater but also provides a cleaning of recharged surface water which allows for a cost-effective and reliable drinking water production. However, riverbank filtration systems are considered vulnerable to contamination with pathogens, especially viruses, and in particular under hydrological extremes. According to previous studies, this threat from pathogenic viruses is mainly due to their small size, long transport distance, high persistence, and low infectious dose. Furthermore, the threat to riverbank filtrate quality from pathogenic viruses is exacerbated by shortened travel times and elevated virus concentrations in surface waters during extreme hydrological events. To better understand the effect of different hydrologic events on the efficiency of riverbank filtration in attenuating different biological indicators, we investigated the spatial-temporal dynamics of a range of biological parameters including pathogenic virus, fecal bacteria, coliphages, total prokaryotic cell counts, total viral-particle like counts, and microbial activity. In this study, we sampled the water not only from the Rhine River but also from the observation wells built by waterwork Flehe, Düsseldorf. The observation wells are along the riverbank filtration and with different travel distances from the river. The whole study was over 16 months, thus we acquired the samples distributed in different hydrological conditions, i.e., high, medium and low river levels. In addition, important physico-chemical parameters (i.e., water temperature, electrical conductivity, pH, dissolved oxygen, dissolved organic carbon, water stable isotope ratios, major cations, and anions) were monitored in the river, bank filtrate and groundwater to explore correlations between biological indicators and physico-chemical variables, particularly at different river levels. Furthermore, the predictability of hygienic contamination (i.e., adenoviruses) via other biological parameters and their practical use in monitoring riverbank filtration efficiency were also targeted.

First, we found that after >72 m of passage of river water through bank sediments, all biological indicators significantly decreased, with the largest reduction in coliforms (3.6 $\log_{10}$) and *E. coli* (>2.9 $\log_{10}$). Most of the reduction occurred between the early sediment passage, i.e., first >32 m (from the river to wells A). Comparing the reduction of biological parameters during the early sediment passage, we observed a similar removal rate for *E. coli* (2.6 $\log_{10}$), coliforms (2.4 $\log_{10}$), somatic coliphages (2.3 $\log_{10}$), and adenovirus (2.3 $\log_{10}$). However, the attenuation mechanisms may have differed with the individual agents because of their different size, surface charge, inactivation, and decay. A clear pattern was observed when we dynamically separated the biological indicators during riverbank filtration based on the defined different river levels. Most indicators were reduced most efficiently at high river levels likely due to the elevated surface water contamination and shortened travel time. Although all biological parameters correlated with each other during riverbank filtration, the degree of correlation varied with different river levels. Based on our results, the combination of *E. coli*, coliforms, and somatic coliphages is recommended to predict the performance of riverbank filtration in adenovirus removal. No single biological parameter correlated with adenovirus at the different river levels. Moreover, ignoring river levels, pH and travel time were additional factors that correlated with the abundance of all biological parameters in the riverbank filtration system.

In conclusion, this study stresses the importance of assessing the effect of hydrologic events on riverbank filtration efficacy on an event basis.

Author contributions

He Wang conceptualized and designed the experiment with the supervision of Prof. Christian Griebler, Prof. Irina Engelhardt, Prof. Christine Stumpp; field sampling with the help of Dustin Knabe, Dr. Björn Droste, Prof. Hans-Peter Rohns; analyzed and visualized data with the supervision of Prof. Christian Griebler and with the help of Prof. Johannes Ho, Dr. Björn Droste, Prof. Hans-Peter Rohns. He Wang wrote the manuscript with input from Prof. Christian Griebler, Dustin Knabe, Prof. Christine Stumpp, Prof. Johannes Ho. All authors commented on the manuscript.

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Dynamics of pathogens and fecal indicators during riverbank filtration in times of high and low river levels

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ABSTRACT

Riverbank filtration is an established and quantitatively important approach to mine high-quality raw water for drinking water production. Bacterial fecal indicators are routinely used to monitor hygienic raw water quality, however, their applicability in viral contamination has been questioned repeatedly. Additionally, there are concerns that the increasing frequency and intensity of meteorological and hydrological events, i.e., heavy precipitation and droughts leading to high and low river levels, may impair riverbank filtration performance. In this study, we explored the removal of adenovirus compared with several commonly used bacterial and viral water quality indicators during different river levels. In a seasonal study, water from the Rhine River, a series of groundwater monitoring wells, and a production well were regularly collected and analyzed for adenovirus, coliphages, *E. coli, C. perfringens*, coliform bacteria, the total number of prokaryotic cells (TCC), and the number of virus-like particles (TVPC) using molecular and cultivation-based assays. Additionally, basic physico-chemical parameters, including temperature, pH, dissolved organic carbon, and nutrients, were measured. The highest $\log_{10}$ reduction during the >72 m of riverbank filtration from the river channel to the production well was observed for coliforms (>3.7 $\log_{10}$), followed by *E. coli* (>3.4 $\log_{10}$), somatic coliphages (>3.1 $\log_{10}$), *C. perfringens* (>2.5 $\log_{10}$), and *F+* coliphages (>2.1 $\log_{10}$) at high river levels. Adenovirus decreased by 1.6–3.1 log units in the first monitoring well (>32 m) and was not detected in further distant wells. The highest removal efficiency of adenovirus and most other viral and bacterial fecal indicators was achieved during high river levels, which were characterized by increased numbers of pathogens and indicators. During low river levels, coliforms and *C. perfringens* were occasionally present in raw water at the production well. Adenovirus, quantified via droplet digital PCR, correlated with *E. coli*, somatic coliphages, TCC, TVPC, pH, and DOC at high river levels. At low river levels, adenoviruses correlated with coliforms, TVPC, pH, and water travel time. We conclude that although standard fecal indicators are insufficient for assessing hygienic raw water quality, a combination of *E. coli*, coliforms and somatic coliphages can assess riverbank filtration performance in adenovirus removal. Furthermore, effects of extreme hydrological events should be studied on an event-to-event basis at high spatial and temporal resolutions. Finally, there is an urgent need for a lower limit of detection for pathogenic viruses in natural waters. Preconcentration of viral particles from larger water volumes (>100 L) constitutes a promising strategy.

1. Introduction

Groundwater is the primary water source for drinking water supply in Europe and many regions worldwide (Völker and Borchardt, 2019). When groundwater is insufficient or of low quality, surface water is used. However, since the surface water quality is generally low, purification is required. Wherever possible, natural purification processes are used instead of technical treatment. Along large rivers and lakes,

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induced riverbank filtration is a common approach for drinking water production (Kuehn and Mueller, 2000). In this study, surface water is guided through riverbed sediments and the adjacent shallow aquifer to a production well. Although riverbank filtration appears efficient, providing adequate water conditioning most of the year, extreme hydrological events, which tend to become more frequent due to climate change, can pose a serious risk to the quality of bank-filtrated water and the safe operation of drinking water supply (Sprenger et al., 2011).

Drinking water supply from surface water via riverbank filtration is widely used not only in Europe but also in North and South America and Asia (Gillefalk et al., 2018). In Germany, approximately 16% of drinking water is produced from induced riverbank filtration (Gillefalk et al., 2018). At numerous sites, riverbank filtration has a long history of more than 150 years of operation (Schubert, 2002). With ongoing industrialization and population growth, there has been widespread pollution of surface water resources. Although the establishment of high standards in wastewater treatment has improved water quality over the past decades, pollutants such as pathogenic viruses may exhibit high resistance to current water treatment techniques, which poses a continuing concern in drinking water production (Fong and Lipp, 2005).

In riverbank filtration, the reduction in the concentration of pollutants is achieved by hydrodynamic (e.g., dilution, dispersion, and diffusion), mechanical (e.g., filtering, straining, and sedimentation), physicochemical (e.g., sorption, precipitation, and redox reaction), and biological (e.g., biodegradation) processes (Jaramillo, 2011). Numerous studies have focused on the efficiency of riverbank filtration in removing organic compounds (Hamann et al., 2016; Glorian et al., 2018) and organic matter (Derx et al., 2013; Romero-Esquivel et al., 2017), as well as the immobilization of metals and other inorganic compounds (Ibrahim et al., 2015; Sandhu et al., 2019). The hygienic aspects of water quality have always been of particular concern. Recent studies have investigated the reduction of pathogens, including protozoans (e.g., *Giardia lamblia* and *Cryptosporidium parvum*; Freitas et al., 2017), pathogenic bacteria (e.g., *Clostridium sp*. and *Pseudomonas aeruginosa*; Weiss et al., 2003; Nagy-Kovács et al., 2019), and viruses (e.g., adenovirus and norovirus; Betancourt et al., 2014; Sprenger et al., 2014).

Pathogenic viruses pose a particular risk to water quality because of their persistence in the aqueous environment, resistance to disinfection methods, small colloidal size, and low infectious doses (Fong and Lipp, 2005). To date, the efficiency of riverbank filtration in removing pathogenic viruses has mainly been studied using viral surrogates or fecal indicators such as bacteriophages, rather than following the fate of human pathogenic viruses, with only a few exceptions (Betancourt et al., 2014). Reasons for using surrogates are manifold, including difficulties associated with the direct detection of pathogenic viruses (McMinn et al., 2017). Commonly used fecal indicators, such as *E. coli* and coliforms, are reliable in assessing bacterial pathogen contamination. However, their application to assess and monitor viral contamination is limited (Ashbolt et al., 2001). Even the use of bacteriophages (e.g., coliphages) as indicators for assessing water contaminated with human pathogenic viruses lack predictive power (Jofre et al., 2016). Alternatively, adenoviruses, which cause several gastroenteric diseases, have been proposed as monitoring targets (WHO, 2006). They are shed into wastewater in significant amounts, along with the feces of infected patients (Bauer et al., 2011).

Riverbank filtration systems must be considered highly vulnerable to pathogen contamination under extreme hydrological conditions. Floods and low water situations are expected to occur more frequently in Europe in the future because of climate change (Sprenger et al., 2011; Eckert et al., 2008; IPCC, 2014). Hari et al. (2020) demonstrated that the occurrence of the 2018–2019 summer drought in central Europe is unprecedented in the last 250 years. It has been repeatedly recommended to evaluate the impact of extreme hydrological events on water quality and riverbank filtration efficiency, particularly the impact on the natural attenuation of bacterial and viral pathogens (Masse-Dufresne et al., 2021).

The primary objective of this study was to investigate the dynamics in attenuation efficiency of riverbank filtration for adenoviruses, viral indicators, and other fecal and microbial indicators with respect to the impact of extreme hydrological events. We targeted the predictability of hygienic contamination via individual fecal and/or viral indicators, and its practical use for monitoring riverbank filtration efficiency. Correlations between microbiological and physico-chemical variables with a focus on water quality parameters were assessed. For 16 months, water samples were collected from the banks of the Rhine River and from various groundwater monitoring wells along an observation transect from the river to the extraction well and beyond at the Flehe waterworks in Düsseldorf, Germany. Samples were analyzed for the presence of adenoviruses, coliphages, bacterial indicators (*Escherichia coli*, coliforms, and *Clostridium perfringens*), the total number of prokaryotic cells (TCC) and virus particles (TVPC), microbial activity (ATP), dissolved organic carbon (DOC), and key physico-chemical parameters, including temperature, electrical conductivity (EC), pH, dissolved oxygen (DO), major ions, and water stable isotope ratios ($^{2}H/^{1}H$ and $^{18}O/^{16}O$).

2. Material and methods

2.1. Study site

The Flehe waterworks in the city of Duesseldorf, Germany, is located on an outer bend of the Rhine River between kilometers 731.5 and 732.1 (Fig. 1A). Here, the Rhine River is 400 m wide with a flow velocity of 1–1.4 m s^{-1}, a hydraulic gradient of 0.2 m km^{-1}, and a median discharge of 2100 $m^3 s^{-1}$ (Sharma et al., 2012). Samples were collected between January 2018 and May 2019. During this period, the mean river level was 29.5 ± 1.6 m.a.s.l. (meters above sea level; Fig. 1C). Thus, we assumed river levels above 31.1 m.a.s.l. as "high" (flood), below 27.9 m.a.s.l. as "low" (drought), and river levels between 27.9 and 31.1 m.a.s.l. as medium river levels in our study. River water quality was monitored at a nearby station (kilometer 732.1) by the local water authorities.

The hydrogeology at the site is marked by a shallow quaternary aquifer consisting of heterogeneous sand-gravel mixtures (d_{50} = 0.5–9.4 mm) with an effective porosity of 20% and a range in k_f values from 2×10^{-2} to 4×10^{-3} m s^{-1} (Schubert, 2002). The aquifer is about 20 m thick and confined below by a tertiary silty fine sand aquitard. Numerous groundwater monitoring wells surround the riverbank filtration plant. Eleven wells have been selected for our study because of their locations with increasing distance to the Rhine River and the extension to various depths, which allowed following the fate of the bank filtrate from the river to one of the production wells (PW5). PW5 is part of a well gallery located parallel to the river, which forces river water through the riverbank and the shallow aquifer (Fig. 1B). The bank filtrate obtained from PW5 was a mixture of water from the well gallery. Between the river and PW5, two sets of monitoring wells (A and B) are situated at a horizontal distance of 40.2 and 20.4 m to the production well (Fig. 1C). According to Schubert (2002), the minimum travel distance from the Rhine River to well row A is 32.2 m with river levels above 26 m.a.s.l.

Another set of monitoring wells (C) and a single well (OO8) are located opposite PW5 and the river, where land side groundwater flows toward the production well (Fig. 1). Each well row, i.e., A, B, and C, comprises three individual wells (1–3) with 1 m filter screens at different depths to allow a depth-dependent sampling (Fig. 1C). The actual pumping rate during the period of our study varied between 177 and 540 $m^3 h^{-1}$ (excluding one extreme outlier value of 39 $m^3 h^{-1}$) with a mean of 322 $m^3 h^{-1}$, excluding a shut-down period from April 10 to May 23, 2018 (Figure S1).

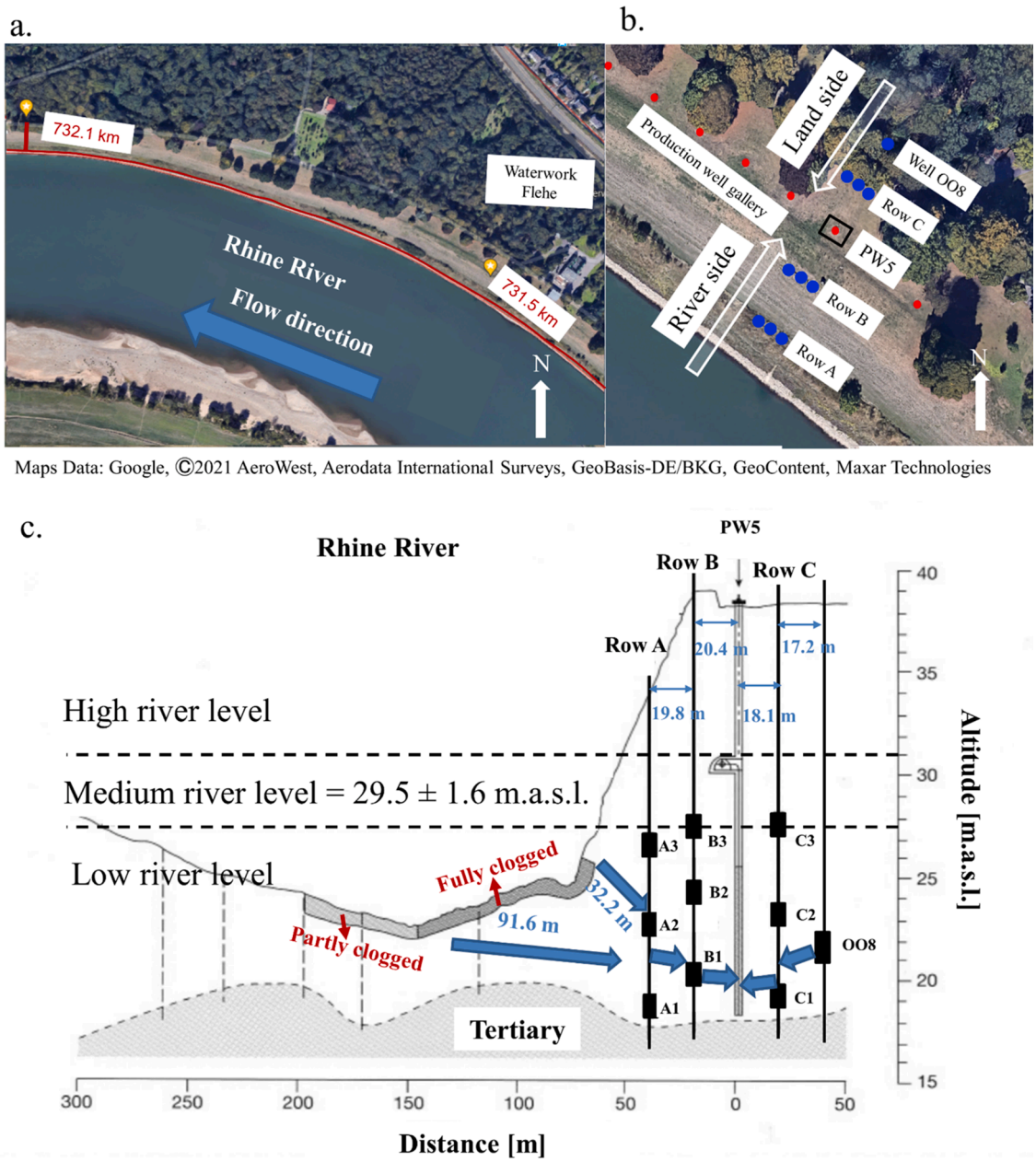

Fig. 1. (a) The Duesseldorf-Flehe waterworks; geographic location. (b) Position of sampling wells. (c) Position of the filter screens in the groundwater monitoring wells. River are categorized as high, medium, and low. Travel distances during infiltration have been calculated according to Schubert (2002).

2.2. Sampling and analysis

Water samples from the Rhine River and observation wells were collected at least once a month from January 2018 to May 2019; sampling started only in July 2018 for well PW5.

For molecular analysis and coliphage tests, 10 L water was collected from all sites in sterile high-density polyethylene carbons. Further water samples from all sites were collected in prebaked 500 mL glass bottles to analyze DOC, fecal indicators (*E. coli*, coliforms, and C. *perfringens*), and ATP. To quantify TCC and TVPC, water samples (15 mL) were fixed with glutaraldehyde (0.5%, v/v) and stored at 4 °C or put on dry ice and kept at −80 °C until further processing.

Key physico-chemical parameters such as pH, water temperature, DO, and EC were measured in situ with WTW field sensors. Groundwater levels were recorded with a level sensor, and river level information was obtained from the Federal Institute for Hydrology (https://www.bafg.de/DE/Home/homepage_node.html).

For most variables a single sample was collected and analyses in the lab were done in duplicates or triplicates (technical replicates). The coefficient of variation for the data obtained were within 10% if not mentioned otherwise.

2.2.1. Water isotopes, major ions, and DOC analysis

Water collected for stable water isotope analysis was filtered through 0.22 µm syringe filters (Merck Millipore) and then analyzed with a laser-based water stable isotope analyzer (Picarro L2130-i). A two–point calibration with laboratory reference material calibrated against VSMOW-SLAP (Vienna Standard Mean Ocean Water-Standard Light Antarctic Precipitation) was used. Each sample was measured up to nine times. Precision of the instrument (1σ) was better than 0.15 ‰ and 0.6 ‰ for $\delta^{18}O$ and $\delta^{2}H$, respectively. Results are in delta notation in ‰ relative to the Vienna Standard Mean Ocean Water.

Chemical analyses for major cations (K^{+}, Na^{+}, Ca^{2+}, and Mg^{2+}) were performed with a 7900 ICP-MS (Agilent) following DIN EN ISO

17,294–2 (2005), and major anions (Cl^-, Br^-, NO_3^-, and SO_4^{2-}) were analyzed in an ion chromatograph (ICS 1600, Dionex) following DIN EN ISO 10,304–1 (2009).

For DOC measurements, 8 mL water was filtered through a pre-rinsed 0.45 μm syringe filter (Merck Millipore), followed by acidification with HCl to pH ≤ 2. The non-purgeable organic carbon was analyzed in a TOC analyzer (Shimadzu TOC-5000A) with MQ water as blank.

2.2.2. TVPC, TCC, and ATP measurements

Water samples for TVPC were first filtered through a 0.22 μm syringe filter (Merck Millipore) and then fixed with 0.5% (v/v) glutaraldehyde and stored at −80 °C until further analysis following the protocol of Brussaard (2014). Later, samples were unfrozen and diluted appropriately with filtered (0.1 μm syringe filter) MQ water to achieve a particle concentration of approximately 10^6 virus-like particles per milliliter. Samples were then stained with 1 × SYBR Gold nucleic acid dye and incubated for 10 min at 80 °C in the dark prior to measurement.

For TCC analysis, river water and groundwater samples were fixed with 0.5% (v/v) glutaraldehyde and then stored at 4 °C for a maximum of 10 days before further processing. The samples were then diluted appropriately with filtered MQ water (0.1 μm syringe filter) to achieve a cell concentration of approximately 10^5 cells per milliliter. The samples were then stained with 10 × SYBR Green nucleic acid dye and incubated for 13 min at 37 °C in darkness prior to measurement (Bayer et al., 2016).

TCC and TVPC were quantified via flow cytometry (FC 500 CYTOMICS, Beckman Coutler) equipped with an air-cooled 488-nm Argon ion laser in biological and technical replicates. As an internal reference, fluorescent beads (Trucount) were added to each sample. Using the StemCXP Cytometer software (v2.2), the analysis and evaluation of the samples were performed. Filtered MQ (0.1 μm filter) was used as blank for these two analyses.

Intracellular ATP concentrations were derived from the measured concentrations of total and extracellular ATP in water samples. ATP analysis followed the protocol of Hammes et al. (2010) using the BacTiter-Glo Microbial Cell Viability Assay kit (Promega). Freshwater samples were analyzed within 3 days after storage at 4 °C in the dark. Additional samples collected between regular intervals were frozen for subsequent analysis. With these samples, intracellular ATP was calculated from measurements of total ATP only using the linear relationship between total ATP and extracellular ATP found in regular groundwater ($R^2 = 0.40$, $p << 0.05$) and river water ($R^2 = 0.25$, $p << 0.05$) samples. Autoclaved MQ water were used as blank for extracellular and total ATP analysis.

2.2.3. Biological indicators

The detection and enumeration of *E. coli* and coliforms were performed using the Colilert-18 method (IDEXX) according to ISO 9308–2 (1990). The detection and enumeration of *Clostridium perfringens* were performed using the membrane method according to ISO 14189 (2013). The detection limit for all indicator bacteria was 1 MPN or 1 CFU per 100 mL sample.

Analysis of somatic and *F+* coliphages from 10 L water samples followed the protocol of Binder (2013). The titer of infectious coliphages in water samples was determined using the double-agar overlay method (plaque assay; Kropinski et al., 2009). Briefly, a solid agar base (10 mL of 1.5% agar, w/v) was overlaid with a mixture of soft agar (4 mL of 0.75% agar, w/v) and 1 mL of a well-mixed solution of (diluted) sample and host bacteria (*E. coli Famp* for *F+* coliphages and *E. coli CN-13* for somatic coliphages) harvested in the logarithmic growth phase (1:1, v/v). Serial dilutions of samples were incubated at 37 °C overnight and quantified in duplicates. The detection limit for coliphages was 0.07 PFU per 100 mL.

2.2.4. Quantification of adenovirus

We quantified the free and particle-adsorbed fraction (containing viruses on and in organic and inorganic particles > 0.22 μm) of adenovirus particles in water samples. Adsorbed viruses in 10 L freshwater were first filtered onto 0.22 μm PES filters (Thermofisher). The filtrate containing the fraction of free virus particles was dedicated to a $FeCl_3$ addition (2 mg L^{-1}) to concentrate virus particles along with Fe precipitation (John et al., 2011). After ≥1 h of sedimentation, the precipitate was collected on 0.45 μm filters (Whatman). The adsorbed viruses (together with microbial biomass collected on 0.22 μm filters) and free viruses (Fe precipitate collected on 0.45 μm filters) were both frozen at −80 °C for later processing. For data analysis, virus abundance values from the free and adsorbed fraction were combined.

The AllPrep PowerViral DNA/RNA Kit (Qiagen) was used to extract adenovirus DNA. For the adsorbed adenovirus fraction, small pieces of the 0.22 μm PES filters were directly processed according to the instruction of the manufacturer. For the free adenovirus fraction, first, the iron precipitate was dissolved using an ascorbate buffer (John et al., 2011), and then, the resuspension was concentrated via Amicon® Ultra-15 Centrifugal Filter Units (Merck). The concentrate was then used for DNA extraction.

For quantitative estimation of adenovirus particles, a droplet digital PCR (ddPCR) was applied with the following details in accordance with the digital MIQE guidelines (Huggett, 2020). The detection was based on a 131-bp fragment using the primer sequences 5′-GCC-ACG-GTG-GGT-TTC-TAA-ACT-T-3′, 5′-GCC—CCA-GTG-GTC-TTA-CAT-GCA-CAT-C-3′, and a probe 5′-HEX-TGC-ACC-AGA-CCC-GGG-CTC-AGG-TAC-TCC-GA-BHQ1–3′ (Heim et al., 2003). Primers and the probe were obtained from ThermoFisher Scientific. The ddPCR was performed on the QX200 Droplet Digital PCR System (BioRad) using ddPCR Supermix for Probes (Bio-Rad Laboratories). Reactions were set up in a final volume of 20 μL. The reaction mixture consisted of ddPCR Supermix for Probes, 900 nM of each primer, 250 nM probes, 4 U HaeIII, (ThermoFisher Scientific), 1 μL template and nuclease-free water. DNA digestion was performed to improve ddPCR efficiency according to the manufactures recommendations for ddPCR Supermix (Bio–Rad Laboratories). The mixture was combined with 70 μL droplet generation oil and droplets were prepared using the Droplet Generator QX200 (Bio-Rad Laboratories). The resulting droplets were transferred to a 96-well plate and the PCR was then performed in a C1000 Touch Cycler at 95 °C for 10 min, followed by 40 cycles at 94 °C for 30 s and 60 °C for 1 min, and then, a final incubation at 98 °C for 10 min and a hold step at 4 °C until reading on the QX200 Droplet Reader (Bio-Rad Laboratories). No-template control and a positive control (human Adenovirus Type 2 strain DNA) were included in each ddPCR assay. QuantaSoft Analysis Pro (BioRad) were used to manually threshold and export the data. Analyses with a droplet number below 8000 were repeated with an additional tenfold dilution. For selected samples, up to four replicates were performed with an average coefficient of variation of 23%. All detections were performed in the same lab and with the same methods. Based on the initial sample volume, and the volume of DNA extract used in our study, a detection limit between 10.9 and 397 particles L^{-1}, was calculated; without taking into account losses during concentration and extraction.

2.3. Water travel time analysis

Mean travel times for conservative solute transport from the river to the observation wells, as influenced by the river level changes and the pumping rate, was estimated from the measured piezometric pressure heads of the observation wells. For this, we calculated the mean particle velocity in the groundwater on the basis of Darcy's law, taking the hydraulic gradient between observation wells A1 and B1:

$$v(t) = \frac{1}{n}\cdot\left(k\cdot -\frac{dh(t)}{dl}\right) = \frac{1}{n}\cdot\left(k\cdot\frac{h_{A1}(t) - h_{B1}\ (t)}{\Delta l_{A1/B1}}\right)$$

where $v(t)$ represents the mean particle velocity in the groundwater at

time t [m d^{-1}], n represents the effective porosity of the aquifer [–], k represents the hydraulic conductivity of the aquifer [m d^{-1}], $dh(t)/dl$ represents the hydraulic gradient at time t [–], $h_{A1}(t)$ and $h_{B1}(t)$ represent piezometric pressure heads at time t at the observation wells A1 and B1 [m], and $\Delta l_{A1/B1}$ represents the distance between wells A1 and B1 [m]. Following the modeling results of Sharma et al. (2012) for the same field site, we assumed $n = 0.2$ and $k = 1.59 \times 10^{-3}$ m s^{-1}.

Assuming flow is only in the direction perpendicular to the river, the mean travel time $T(s,t)$ from the river to an observation well at a distance s from the river and time t can be calculated using the following equations:

$$s = \int_{t-T(s,t)}^{t} v(t')dt' = V(t) - V(t - T(s,t)),\ V(t) = \int v(t)dt,$$

$v(t)$ is assumed to be stepwise constant based on the hydraulic gradients available at the sampling dates. Then, the integral $V(t)$ can be given with

$$V(t) = \left(\sum_{i=0}^{j-1}(v_i \cdot dt_i)\right) + v_j \cdot \left(\left(\sum_{i=0}^{j} dt_i\right) - t\right),\ t > \sum_{i=0}^{j-1} dt_i \text{ and } t \le \sum_{i=0}^{j} dt_i.$$

Then,

$$s = V(t) - V(t - T(s,t))$$

$$s = v_k \cdot \left(\left(\sum_{i=0}^{k+1} dt_i\right) - (t - T(s,t))\right) + \left(\sum_{i=k+1}^{j-1}(v_i \cdot dt_i)\right) + v_j \cdot \left(\left(\sum_{i=0}^{j} dt_i\right) - t\right)$$

$T(s,t)$ can be calculated by finding k∈[0,1,…, N], where N represents the highest timestep number so that these two equations are satisfied:

$$s > \left(\sum_{i=k+1}^{j-1}(v_i \cdot dt_i)\right) + v_j \cdot \left(\left(\sum_{i=k}^{j} dt_i\right) - t\right),$$

$$s < \left(\sum_{i=k}^{j-1}(v_i \cdot dt_i)\right) + v_j \cdot \left(\left(\sum_{i=k}^{j} dt_i\right) - t\right).$$

Then,

$$T(s,t) = \frac{s - \left(\sum_{i=k+1}^{j-1}(v_i \cdot dt_i)\right) + v_j \cdot \left(\left(\sum_{i=0}^{j} dt_i\right) - t\right)}{v_k} + t - \sum_{i=0}^{k+1} dt_i.$$

Note that $T(s,t)$ is only an estimate for the travel time since (i) the distance between the river and the observation wells slightly varies over time due to river level changes, (ii) flow will have a vertical component close to the river groundwater, (iii) our limited sampling frequency prevent us from fully capturing the dynamically changing the hydraulic gradient, (iv) and spatial heterogeneities in n and k are neglected.

The mean travel time T was calculated for observation wells at A (s = 40 m), B (s = 60 m), and PW5 (s = 80 m) with the river as a reference for the riverside groundwater flow. Using the hydraulic gradient between observation wells C and OO8, T is also calculated for observation wells C (s = 17 m from OO8) and PW5 (s = 35 m from OO8).

During the shut-off of PW5 in Spring 2018, the river level and the groundwater level at well OO8 indicate that natural effluent conditions have returned; thus, no travel time could be calculated. Excluding the switch-off of PW5, the attenuation efficiency of riverbank filtration for viral and bacterial indicators was evaluated for three hydrological situations, i.e., the high, medium, and low river levels. These hydrological events were categorized by river levels, as described in Section 2.1, whereas the water samples from the observation wells were categorized according to the hydrological event at the commencement of its underground passage considering the estimated mean travel time (Figure S2).

2.4. Statistical analysis

All statistics were implemented with software packages in the R environment (R Foundation; http://www.r-project.org/), with the significance level set as α = 0.05. Differences and similarities in abiotic and biotic variables within and between the individual well rows were tested through Kruskal–Wallis tests ("kruska.test") and ANOVA ("oneway.test"). Spearman rank correlation matrices (using "Hmisc" package) were used to explore relationships between biological (abundance of viral and bacterial indicators, ATP, TCC, and TVPC), and chemical (ion concentrations) and physico-chemical factors (pH, DO, EC, and temperature). The log reduction of biological indicators during riverbank filtration was calculated by comparing the composition of groundwater and river water at different hydrological events (Figure S2). Principal component analysis (PCA) was performed, considering the selected biological variables and physico-chemical parameters, using the "prcomp" function.

3. Results

3.1. Water level dynamics

The river level during the 16-month sampling period showed a typical seasonality with high levels in winter and low levels in summer (Fig. 2). The highest levels were monitored at the beginning of the field study with 33 meter above sea level (m.a.s.l). Lowest river levels (<27.3 m.a.s.l.) were recorded in late October 2018. The groundwater levels in the different observation wells were very similar but dampened dynamic, as those in the river (Fig. 2). The river level was always above the groundwater levels indicating infiltrating conditions, except for the shutdown period of the waterworks facility.

3.2. Bank filtration water travel times

Travel times of the infiltrated river water are strongly related to the river level (Fig. 3). Extended travel times occur when the river level is receding over a longer period, for example, during the regression of the high river level. Short travel times are caused by sudden river level increases, as observed in winter 2018/2019. When PW5 was shut-off for maintenance, the direction of water flow between the river and the production well could have reversed, resulting in groundwater discharge to the river and negative water flow velocities. When river levels are medium to low, which was the case for most of the year, groundwater from the land side (area opposite to the river with respect to the extraction well) flows toward PW5, passing well OO8 and row C (Fig. 3). During high river levels, river water infiltrates far beyond the production well, reversing the water flow direction at the land side.

3.3. Water stable isotope signatures

The $\delta^{18}O$ values, as well as $\delta^{2}H$ values (Figure S3) in river water, remained constant throughout the year, with only small variations (−9.5 ± 0.3) (Fig. 4a). Compared with surface water, groundwater-specific isotopic signatures at OO8 and row C showed higher values from March to November 2018 (Fig. 2). Samples taken at OO8 and row C during high water levels showed similar isotope ratios to river water. At low river levels, only water in rows A, B, and PW5 contained river water, as depicted from the isotope ratios, whereas wells opposite to the production well (C, OO8) contained land side groundwater.

3.4. Dynamics in physico-chemical conditions

The river water temperature showed a seasonality with low values down to 3.9 °C in winter and high temperatures up to 25.7 °C in summer

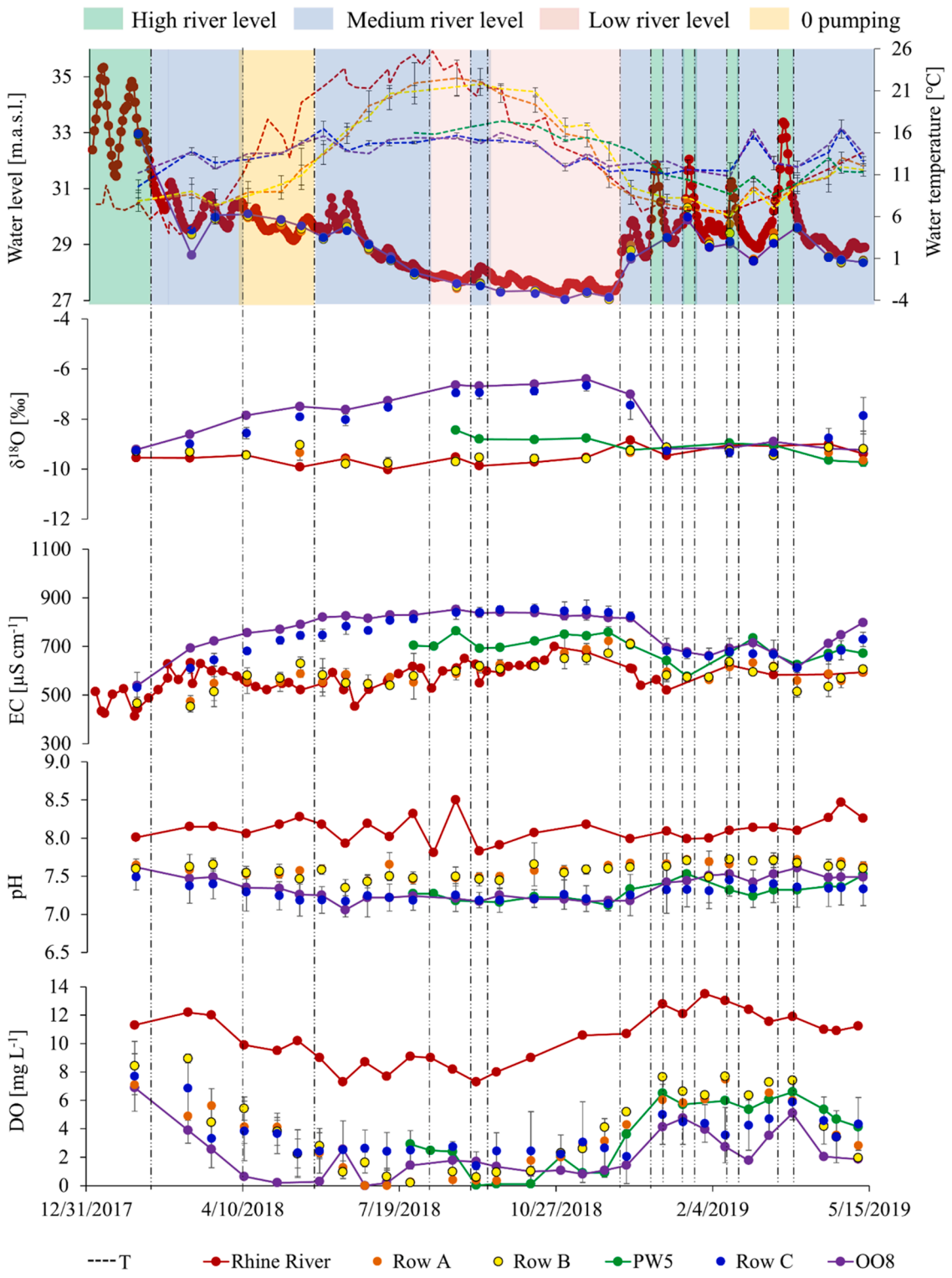

Fig. 2. Seasonal dynamics of water levels, $^{18}O/^{16}O$ water signature, and selected physico-chemical variables, as monitored in the Rhine River and at different observation wells. Error bars depict the variance of individual wells within one well row (e.g., A1, A2, and A3). Datapoints of the selected sites (river, PW5, and OO8) are connected with a line. *T* = river or groundwater temperature.

(Fig. 2). The mean river water temperature during the period of sampling was 14.2 °C ± 7.1 °C. The groundwater temperature at OO8 varied between 10.8 °C and 16.6 °C (13.7 °C ± 1.6 °C). At PW5, as recorded between July 2018 and May 2019, the water temperature ranged between 8.6 °C and 17.4 °C with a mean of 13.4 °C ± 3.0 °C.

EC in the river water showed no significant variation (Fig. 2) with a mean value of 566 ± 61 µS cm^{-1}. Groundwater in OO8 had its minimum of 539 µS cm^{-1} during high river levels and its highest values (up to 852 µS cm^{-1}) when disconnected from the river during medium and low water levels. The overall pattern of EC in water from the observation wells was consistent with stable water isotope analysis results. The EC pattern was also consistent with selected ions, exemplarily displayed for Ca^{2+} and SO_4^{2-} (Figure S4).

Generally, pH values did not show strong fluctuations during the

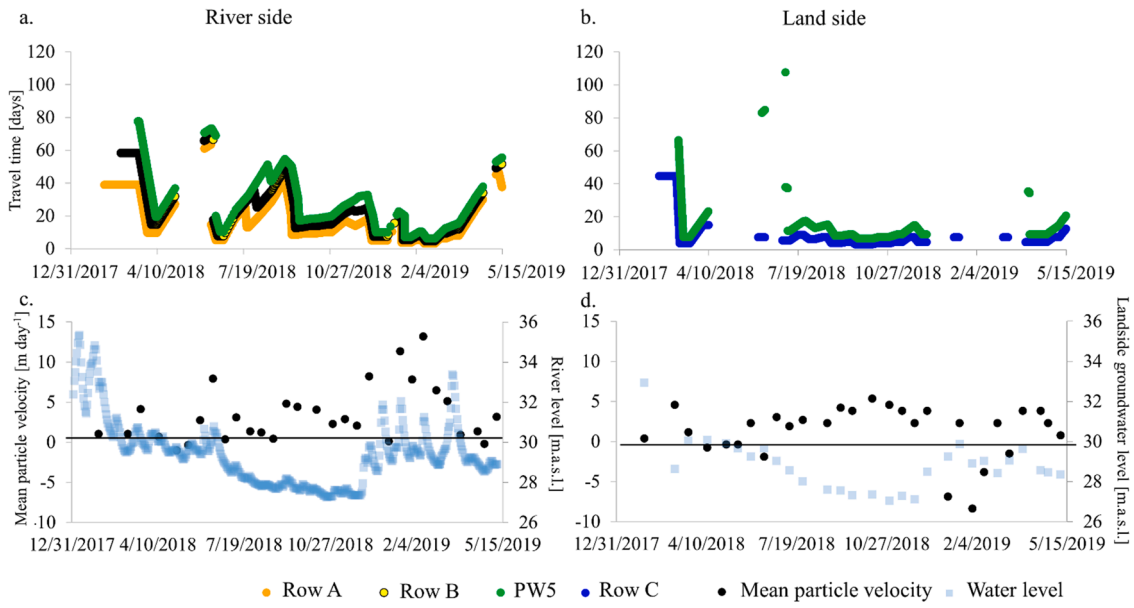

Fig. 3. Mean travel time from river and from land side (a and b), respectively, as well as mean particle velocity of bank filtrate at individual observation wells (c and d).

study, but a clear gradient between the river (8.1 ± 0.2) and water in OO8 (7.3 ± 0.2). The mean pH value at PW5 was similar to that at OO8 with 7.3 ± 0.1. The DO concentration in the water samples exhibited a clear seasonality decreasing with increasing water temperature (Fig. 2). Compared with the land side groundwater, high DO concentrations were observed in river water ranging from 7.3 to 13.5 mg L^{-1} with a mean of 10.4 ± 1.8 mg L^{-1}. The concentration of DO in groundwater at OO8 and PW5 ranged from below detection to 6.9 mg L^{-1}, with a mean of 2.2 ± 1.7 mg L^{-1} and 3.5 ± 2.3 mg L^{-1}, respectively. DO decreased as river water passed through the riverbank (Fig. 4f). The decline in DO was also mirrored to a lesser degree in a decrease in DOC (Figure S3). Further distant from the river, the DOC concentration remained constant. Noticeably, the DO concentration in the riverbank filtrate dropped to zero several times during summer. However, in connection to the local disappearance of DO, no obvious change in nitrate concentrations was observed (Figure S3).

3.5. Dynamics of bacterial indicators

The total number of TCC in the river ranged from 3.0 × 10^8 to 2.8 × 10^9 cells L^{-1} positively correlated with the water temperature (Fig. 5). As water flows through the riverbank, TCC readily declined 16-fold on average between the river and wells in row A. Although the annual mean values of TCC in water from the different observation wells were in the same order of magnitude, there was a significant difference between different locations along the transect to the production well, PW5 (*Table S1*). The lowest TCC values were observed in groundwater from row C. A similar pattern was found with microbial activity (ATP) (Fig. 6a). The highest ATP values were found in the river water, on average 6.4 × 10^{-10} ± 5.2 × 10^{-10} M. ATP decreased not only with distance to the river but also with distance to well OO8; hence, the lowest ATP was found in water in PW5 (2.4 × 10^{-12} ± 8.1 × 10^{-12} M).

The fecal indicators, *E. coli* and coliforms, were frequently detected in river water, but only occasionally in water from the observation wells (Fig. 5). There was no obvious trend in the dynamics of *E. coli* or coliforms in the river, depending on the season. The mean *E. coli* concentration in river water was 803 MPN/100 mL with a large variance (±1148 MPN/100 mL). Only 12 of 283 water samples from the observation wells were positive for *E. coli*, and *E. coli* was never found in any of the land side groundwater samples (Fig. 5). The concentration of coliforms in the river was approximately 5 times higher than *E. coli*, with an average of 4884 ± 6027 MPN/100 mL. Coliforms were observed more frequently in water from the observation wells (in 83 of 283 samples). Considering hydrological situations and travel time, coliforms were detected in water in PW5 at low river level (Figure S5).

The occurrence of *C. perfringens* in river water did not show a seasonal trend, similar to other bacterial indicators. Compared with *E. coli* and coliforms, the average concentration of *C. perfringens* was lower, with 197 ± 163 CFU/100 mL. *C. perfringens* was also detected in the monitoring wells of row A, even when the river was at medium levels. In total, 38 of 268 water samples from the observation wells were *C. perfringens* positive.

3.6. Viral indicators

A mean of 3.2 × 10^{10} ± 1.2 × 10^{10} virus particles L^{-1} was detected in the river, which was on average is 23 times higher than the TCC of the same samples and 38 times higher than TVPC in row A. The lowest TVPC was found in water samples from row C (5.7 × 10^8 ± 3.7 × 10^8 virus particles L^{-1}).

Human enteric adenoviruses were found in the Rhine River during the entire sampling period. The mean concentration of adenovirus in surface water was 1.2 × 10^4 ± 3.0 × 10^4 particles L^{-1}, whereas the virus concentration in well A2 was already two to three orders of magnitude lower with 5.8 × 10^1 ± 3.6 × 10^1 particles L^{-1}. No adenovirus was above the limit of detection (LOD) in the selected samples of well B1.

The occurrence of *F*+ coliphages and somatic coliphages in the river differed. *F*+ coliphage concentration was the highest in January 2018 with 12 PFU/100 mL overall with a mean concentration of 2.6 ± 3.8 PFU/100 mL. Effective attenuation of *F*+ coliphages during riverbank filtration was observed between the river and rows A and B. Only a few samples were detected as positive in wells A2 and B1 in spring 2018 (Fig. 7). No *F*+ coliphages were found in groundwater from other wells. Only 5 of 74 water samples from the observation wells contained active *F*+ coliphages above the LOD. The highest concentration of somatic coliphages detected in the river was 116 PFU/100 mL in February 2019, and other samples exceeding a concentration of 40 PFU/100 mL were detected only in January and December 2018. The mean concentration of active somatic coliphages in river water was 24.1 ± 28.0 PFU/100

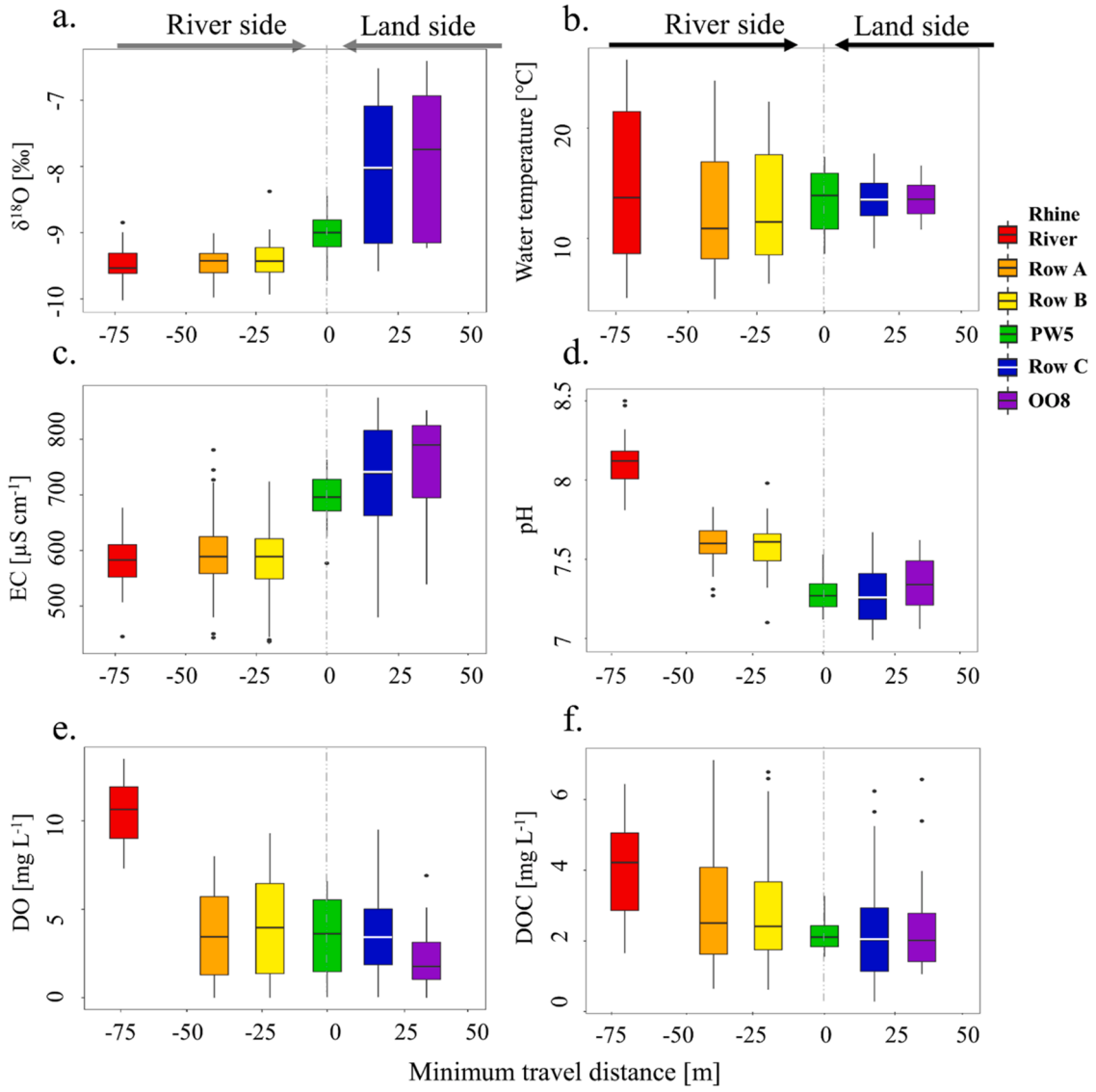

Fig. 4. Gradients of selected physico-chemical variables monitored in water from the Rhine River and from selected groundwater observation wells.

mL, 9-fold higher than *F*+ coliphages. Only 7 of 74 water samples from the observation wells were positive for somatic coliphages. However, somatic coliphages were occasionally detected in groundwater at C1 in spring 2018.

3.7. River water, bank filtrate, and groundwater in comparison

Spearman rank correlation analysis revealed that all biological variables (including adenovirus) recorded for the riverbank filtration transect are significantly correlated (Figure S6). Noticeably, all biological indicators positively correlated with pH and negatively correlated with the travel time. Furthermore, all biological indicators negatively correlated with $\delta^{18}O$ and EC, except adenovirus. The DO concentration positively correlates with viral indicators, *E. coli* and *C. perfringens*, rather than coliforms and TCC. The river and groundwater level co-varied with three bacterial indicators, i.e., *E. coli*, coliforms, and *C. perfringens*. A covariation was also discovered between TCC, TVPC, coliforms, and temperature.

The correlation analysis exhibited different patterns when data from surface water and observation wells were analyzed separately. For the river samples, fewer correlations were obtained although all biological indicators were detected all year round. For bacterial indicators, the concentration of coliforms correlated with both *C. perfringens* and *E. coli*. A positive correlation occurred between the viral indicators *F*+ coliphages and somatic coliphages, as well as for TVPC and *F*+ coliphages. However, none of the biological variables correlated with the abundance of adenovirus. The relationships between the biological indicators in the riverbank filtrate and groundwater were different. *C. perfringens* correlated with most of the biological indicators, including *F*+ coliphages, coliforms, *E. coli*, TCC, TVPC, and ATP. Additionally, the number of coliforms correlated with *E. coli* and TCC. The two groups of coliphages did not correlate with each other; nonetheless, somatic coliphages correlated positively with ATP, whereas *F*+ coliphages correlated with *E. coli* and *C. perfringens*. Once more, no correlation between adenovirus and other biological indicators was found.

Taking a specific look at the correlations between adenovirus and individual physico-chemical parameters at different hydrological situations, pH was the only variable correlating independent of the hydrological condition. The abundance of adenovirus during bank filtration correlated positively with pH. Elevated DOC concentrations found at high river levels correlated positively with an increased abundance of adenovirus. At medium river levels several factors (i.e., travel time, temperature, pH, DO) were found to correlate with adenovirus. At low river levels it was only pH and travel time that revealed a statistical

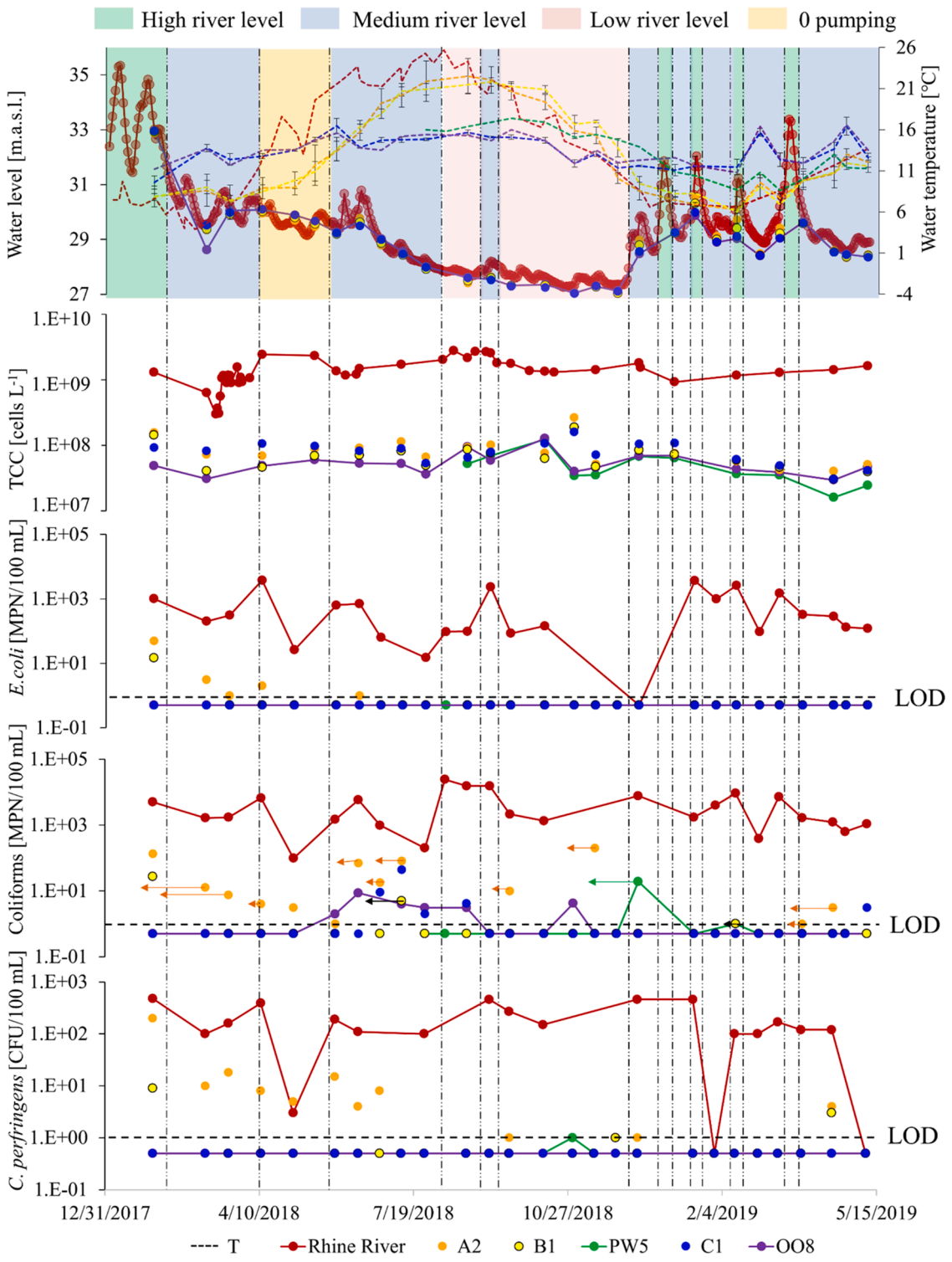

Fig. 5. Seasonal dynamics of selected bacterial indicators, as monitored in the Rhine River and water from the different groundwater observation wells. *T* = water temperature; LOD = limit of detection. The arrows with the coliform data exemplarily show the affiliation to specific hydrological situations when accounting for the time delay caused by the travel time to wells A2, B1, and PW5. Data from selected sampling sites (river, PW5, and OO8) are connected with a line.

significant correlation to adenovirus. With respect to other biological indicators, adenoviruses correlated with *E. coli*, somatic coliphages, TCC, and TVPC during high river levels, with coliforms, somatic coliphages, ATP at medium river levels, and with coliforms, TVPC, and ATP at low river levels, respectively.

Principal component analyses (PCA) (Fig. 8) depict the spatial and temporal differences within water samples collected from the river and observation wells with respect to the selected physico-chemical and biological variables. The difference between river water and water from the observation wells was explained by principal components with 47.1% of the total variance and PC2 explained another 17.4% (Fig. 8a). Although river water had significantly higher concentrations of coliforms, *E. coli, C. perfringens*, ATP, TCC, TVPC, and DOC, the riverbank filtrate and groundwater samples were closely clustered in the PCA plot

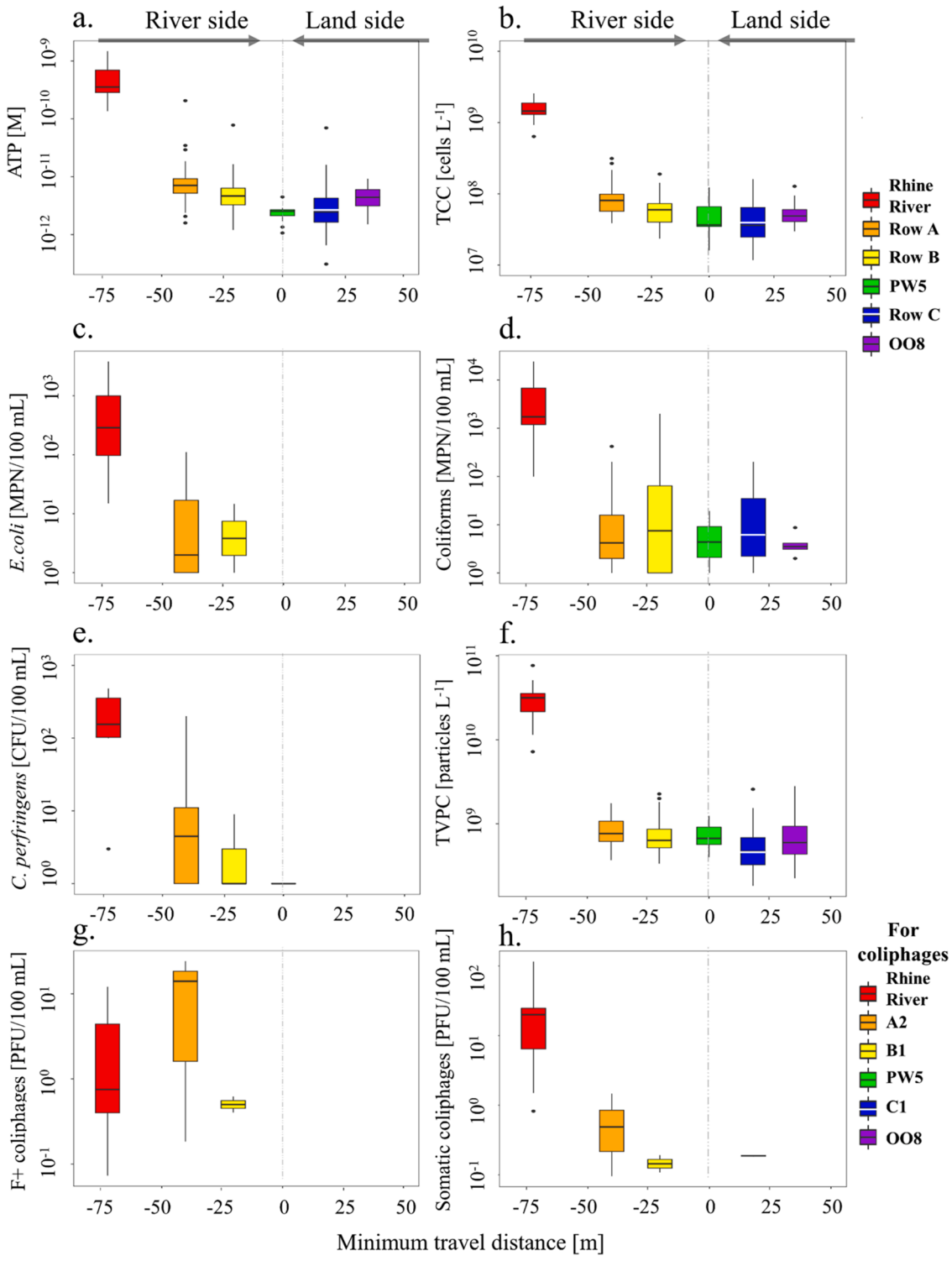

Fig. 6. Gradients of selected bacterial and viral indicators monitored in water from the Rhine River and from selected groundwater observation wells.

(Fig. 8a). Excluding river samples from the analysis, the trend in water samples could be explained by PC1 with 29.5% and PC2 with 19.4% (Fig. 8b). PC1 is mainly represented by the distance from the river, whereas seasonality (represented by temperature and DO) is the dominant factor for the variance in PC2.

3.8. Efficiency of riverbank filtration in removing pathogens and fecal indicators

We observed that in surface water concentrations of several bacterial and viral indicators, i.e., *E. coli, C. perfringens*, adenovirus, *F+* coliphages, and somatic coliphages, were higher during high river levels (flood situation) than during low and medium river levels (Fig. 9).

The absolute removal efficiency during riverbank filtration, in terms of $\log_{10}$ reduction, was higher during high river levels than in other

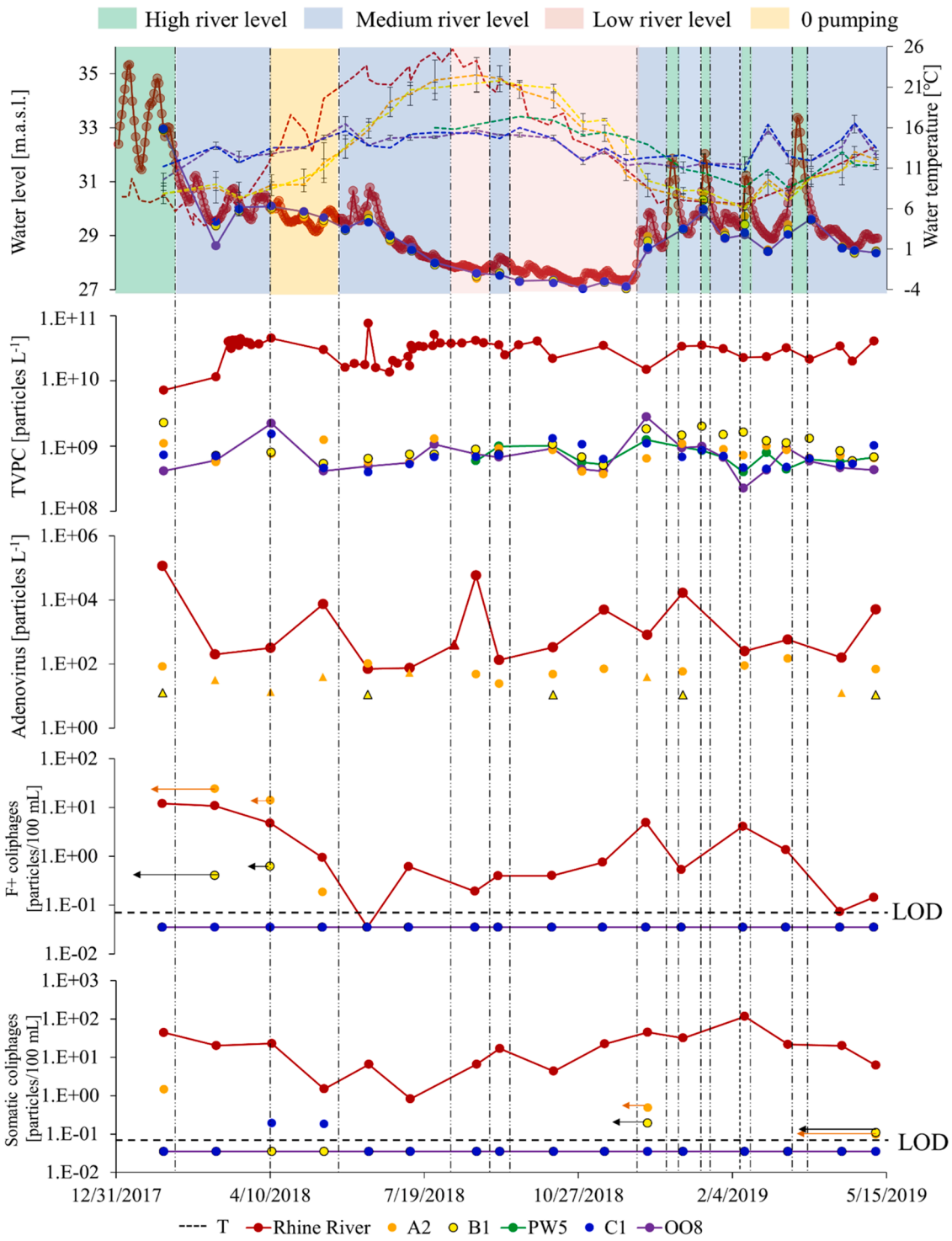

Fig. 7. Seasonal dynamics of selected viral indicators, as monitored in the Rhine River and water from different groundwater observation wells. T = water temperature; 'LOD' and triangles with adenovirus data exemplary show the limit of detection. The arrows with F^+ and somatic coliphages data exemplarily show the affiliation to specific hydrological situations when accounting for time delay caused by the travel time to wells A2, B1, and PW5. Data from selected sampling sites (river, PW5, and OO8) are connected with a line.

hydrological phases for most biological indicators (Fig. 9). However, most of the biological indicators could not be detected in the production well PW5, regardless of the hydrological conditions. The highest $\log_{10}$ reduction during passage through the riverbank was observed for coliforms (>3.7 $\log_{10}$), followed by *E. coli* (>3.4 $\log_{10}$), somatic coliphages (>3.1 $\log_{10}$), *C. perfringens* (>2.5 $\log_{10}$), and *F*+ coliphages (>2.1 $\log_{10}$) at high river levels. The concentration of adenovirus decreased by 3.1 log units between the river and observation well row A.

It is obvious from our data that the most pronounced removal of viruses and bacteria takes place in the early sediment passage from the river channel to well row A (Table 1). For this first stretch of sediment passage, low river levels had a beneficial effect on the removal of *C. perfringens*, TVPC, and *F*+ coliphages, with a reduction of >2.3 $\log_{10}$, 1.8 $\log_{10}$, and >0.8 $\log_{10}$, respectively. TCC, *E. coli*, and somatic

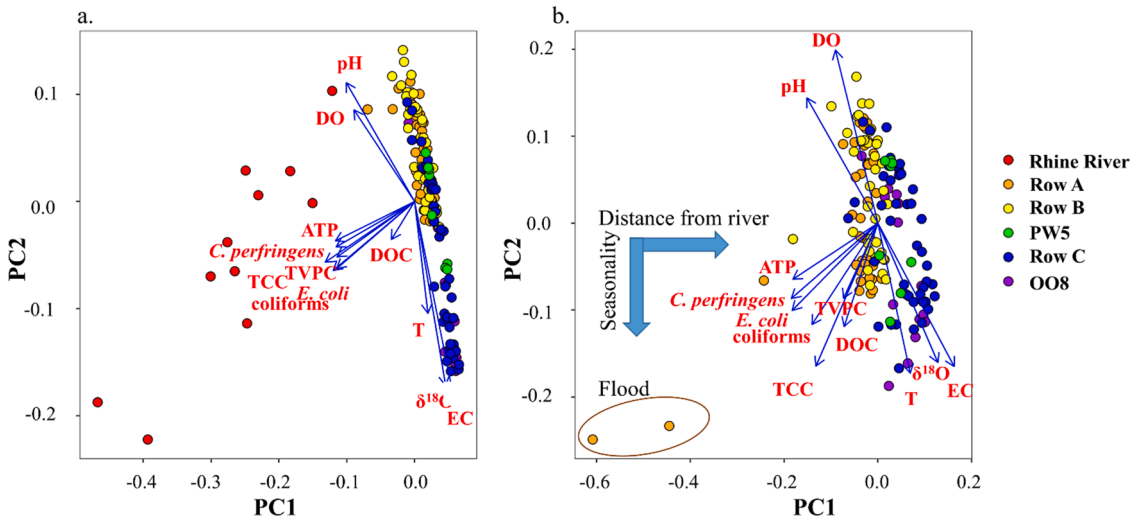

Fig. 8. PCA of all water samples and sampling sites (a) and of samples from the observation wells only (b).

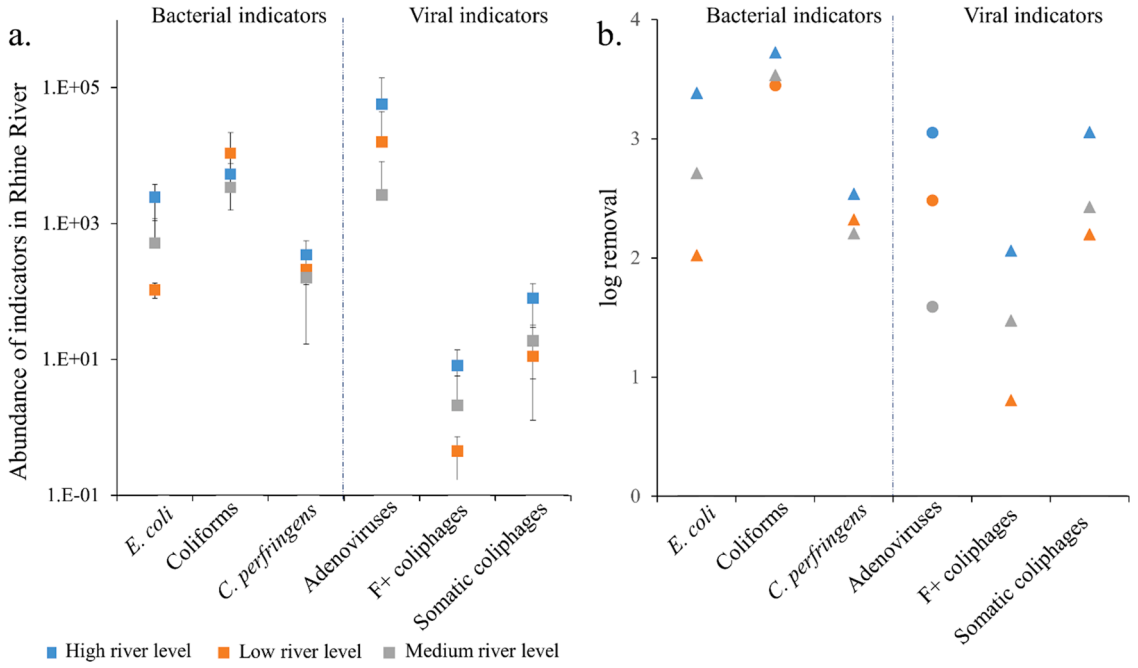

Fig. 9. Abundance of bacterial and viral indicators in the Rhine River (a), and its removal efficiencies by riverbank filtration (b). The abundances of *E. coli*, coliforms, *C. perfringens*, adenovirus, F^{+}, and somatic coliphages are in MPN/100 mL, MPN/100 mL, CFU/100 mL, particles L^{-1}, PFU/100 mL, and PFU/100 mL, respectively. Triangles in chart (b) refer to the minimum log removal. Calculation of the log removal in adenovirus was limited to the reduction between the river and well A2.

coliphages were reduced most efficiently at medium water levels by 1.3 $\log_{10}$, >2.7 $\log_{10}$, >2.4 $\log_{10}$. Adenovirus was the only agent that exhibited the highest removal efficiency (3.1 $\log_{10}$) between the river to well row A at high river levels (Table 1).

4. Discussions

4.1. Attenuation of adenovirus and other viral and bacterial indicators during riverbank filtration

In our study, we investigated the fate of adenovirus compared with several viral and bacterial fecal indicators during riverbank filtration across a transect of >72 m from the Rhine River to the production well of a waterwork facility (Fig. 1). Using ddPCR, we detected adenovirus all year round in Rhine River water at concentrations of 7.1×10^{1} to 1.2×10^{5} particles L^{-1}. Although adenovirus could occasionally still be detected in the riverbank filtrate water after >32 m of sediment passage at A2 (Fig. 7), all samples analyzed from the observation well B1 (>52 m travel distance from the river) were adenovirus negative, given the LOD of approximately 10 particles L^{-1}. Consequently, we assume the risk of adenovirus contamination at concentrations >1 virus particle L^{-1} in water at the production well (PW5) to be very low; however, an occasional occurrence at lower concentrations cannot be excluded. In agreement with this observation, none of the other tested viral indicators, such as coliphages, were detected in water from the production well and individual bacterial fecal indicators, such as *C. perfringens* and coliforms, were detected only rarely.

Besides the risk assessment of adenovirus contamination in raw water at the waterworks in Duesseldorf, Germany, a major focus of our study was on the suitability of individual viral and bacterial indicators.

Table 1
Mean log removal of selected bacterial and viral indicators with riverbank filtration during different hydrological conditions (n/a: the value is not available).

	Location	High river level	Low river level	Medium river level
TCC	RR-A2	1.2	1.1	1.3
	A2-B1	0.08	0.1	0.09
	B1-PW5	0.3	0.2	0.1
E. coli	RR-A2	2.6	>2.0	>2.7
	A2-B1	0.5	n/a	n/a
	B1-PW5	>0.3	n/a	n/a
Coliforms	RR-A2	2.4	2.4	2.4
	A2-B1	0.7	>1.6	>1.2
	B1-PW5	>0.6	n/a	n/a
C. perfringens	RR-A2	1.0	>2.3	1.9
	A2-B1	1.2	n/a	>0.3
	B1-PW5	>0.3	n/a	n/a
TVPC	RR-A2	1.4	1.8	1.6
	A2-B1	n/a	n/a	n/a
	B1-PW5	0.2	0.08	0.2
Adenovirus	RR-A2	3.1	2.5	1.6
	A2-B1	>0.7	>0.7	>0.8
F+ coliphages	RR-A2	0.2	>0.8	0.2
	A2-B1	1.8	n/a	1.4
	B1-PW5	>0.06	n/a	>0.1
Somatic coliphages	RR-A2	2.4	1.8	>2.4
	A2-B1	>0.7	>0.4	n/a
	B1-PW5	n/a	n/a	n/a

Expectedly, viral and bacterial indicators when transported through aquatic sediments differ in terms of attenuation including processes such as straining, adsorption, persistence, and decay (Schijven and Hassanizadeh, 2000). Although straining is considered one of the most important processes for attenuating bacteria, it has been shown that it mainly occurs when the ratio of the particles of concern (viruses 0.02–0.08 μm; bacteria 0.2–2 μm) and the medium grain size (this study 0.5 to 10 mm) is >0.5% (Bradford et al., 2004). As such, straining is considered to be negligible for our setting. Previous studies revealed that total adenovirus particles had the highest persistence (decay rate of 0.008–0.027 $\log_{10}$ day^{-1}), followed by other selected indicators, i.e., *C. perfringens* (inactivation rate of 0.027 $\log_{10}$ day^{-1}), infectious coliphages (inactivation rate of 0.03–1.0 $\log_{10}$ day^{-1}), *E. coli* and coliforms (inactivation rate of 0.02–1.5 $\log_{10}$ day^{-1}), when incubated at 15 °C in groundwater or autoclaved surface water (Medema et al., 1997; John and Rose, 2005; Ogorzaly et al., 2010). Moreover, the persistence of selected indicators decreases to varying degrees at elevated temperatures, the presence of active autochthonous microbes, and unsaturated conditions (Feichtmayer et al., 2017; Schijven and Hassanizadeh, 2000). The adsorption of viruses and bacteria is particle-specific (i.e., surface charge and hydrophobicity), mineral surfaces-specific (i.e., surface charge heterogeneity) and depends on water chemistry (i.e., pH, ionic strength, cations, and organic matter) as summarized in the DLVO (Derjaguin–Landau–Verwey–Overbeek) theory (Schijven and Hassanizadeh, 2000). Therefore, even among viruses of a similar size, different transport and survival behavior have to be expected and documented (A. Bosch et al., 2006). Nevertheless, numerous studies claim that individual fecal indicators, such as *E. coli*, can adequately account for bacterial and viral contamination (Donn et al., 2020; Love et al., 2014). Meanwhile, at first glance, the different indicators tested in our study provided similar information. However, at a closer look, numerous differences are found. From the perspective of removal efficiency during the sediment passage from the river to the production well, the routinely used indicators coliforms and *E. coli* experienced a stronger reduction, i.e., 3.6 $\log_{10}$, and >2.9 $\log_{10}$, respectively, than viral indicators; i.e., somatic coliphages (>2.5 $\log_{10}$), *F+* coliphages (>1.6 $\log_{10}$), and total viral particles (TVPC, 1.6 $\log_{10}$). *Clostridium perfringens* performed worse than somatic coliphages (>2.3 $\log_{10}$), and the total number of TCC revealed the least reduction (1.4 $\log_{10}$) of all indicators tested. The main reduction in viral and bacterial indicators generally takes place in the early sediment passage (Schijven and Hassanizadeh, 2000). This is consistent with our findings. Focusing only on the transect between the Rhine River and row A and ignoring various hydrological situations, the mean reduction of adenovirus was 2.3 log units, which is comparable with the removal of *E. coli* (2.6 $\log_{10}$), coliforms (2.4 $\log_{10}$), and somatic coliphages (2.3 $\log_{10}$), and higher than that of other selected microorganisms. A higher spatial resolution would have been advantageous to explore the attenuation pattern, i.e., exponential vs. linear reduction, in more detail.

A large range in the reduction of adenovirus from approximately 1 to 5 log units is reported for riverbank filtration of comparable distances (31–92 m) from previous studies (Betancourt et al., 2014; Verbyla et al., 2016; Sprenger et al., 2014). Removal efficiencies are very site-specific (Partinoudi and Collins 2007; Rohns et al., 2006; Weiss et al., 2003, 2005). Variations in reduction are mainly caused by the different adenovirus source concentrations, sediment properties, water residence times, and the LOD of quantification methods used. The same is true for other indicators. A reduction of coliphages by >1.9 to >4.4 log units after a sediment infiltration distance of 27–177 m has been documented (Verbyla et al., 2016; Weiss et al., 2003, 2005; Rohns et al., 2006). Note that an approximate 5-$\log_{10}$ reduction of somatic coliphages after only 3.8 m of riverbank passage was reported by Sprenger et al. (2014) for site water infiltration is not forced by pumping, implying the water residence time to be a key factor (Sprenger et al., 2011). The removal efficiency of bacterial fecal indicators (i.e., coliforms, *E. coli*, and *C. perfringens*) during riverbank filtration has been investigated in several studies. A reduction of 2–4 log units has frequently been observed for sediment travel distances of 12–55 m and source concentrations $\leq 10^4$ PFU/100 mL (Partinoudi and Collins, 2007; Ren et al., 2019; Weiss et al., 2003). Although studies that directly compared the removal efficiency for viral and bacterial pathogens and/or indicators are rare, bacterial indicators showed a higher reduction level than viral indicators same as found in our study (Weiss et al., 2003, 2005).

Besides specific bacterial and viral indicators, we monitored the behavior of TVPC and TCC during riverbank filtration, which are variables that have rarely been considered in previous studies, especially for the underground passage at field scale. Compared with adenovirus and other indicators, TVPC and TCC exhibited the lowest log removal during the passage of river water to the production well. Similar to the other indicators, most of the removal occurred in the sediment passage between river and well row A. A low removal of TVPC and TCC, comparable with that in the present study, ranging between 1 and 1.7 $\log_{10}$ units after 1.1 m of vertical infiltration and 70 m of horizontal infiltration through aquatic sediments were observed by Mindl et al. (2015) and Fillinger et al. (2020). Contrary to other more specific indicators, TVPC and TCC may contain offspring from microbes and viruses, which can replicate in the bank sediment. Thus, they may not be suitable indicators for monitoring attenuation efficiency for pathogens during riverbank filtration.

WHO (2017) regarded 10^{-5} pathogenic viruses L^{-1} as a safe threshold concentration for drinking water, which is six orders of magnitude below our LOD for adenovirus (<10 particles L^{-1}). There is no routine technique to concentrate viruses from water by greater than or equal to six to seven orders of magnitude (Pei et al., 2012; Seidel et al., 2016). Hence, this target quality of riverbank filtration can be evaluated considering the viral load in river water and predicted reduction during riverbank filtration. The removal of adenovirus and the viral indicators tested meet the minimum removal for viruses of >2.1 $\log_{10}$ expected in riverbank filtration, as stated by WHO (2017). Additionally, the reduction of most other targeted bacterial fecal indicators exceeded the minimum removal of 2 $\log_{10}$ for bacteria with the sediment passage from the river to the production well.

4.2. Correlations between biological indicators and physico-chemical variables

As human pathogenic viruses cannot propagate in an aquatic environment, the abundance of adenovirus in river likely correlates with the fraction of wastewater from the runoff of sewage treatment plants, which may be increased during and after heavy rain events (Passerat et al., 2011) and low river level periods (Karakurt et al., 2019). Concretely, our study revealed increased numbers of coliphages in river water at high levels when compared with other hydrological conditions. Note that there was a strong negative correlation between the number of cultivable coliphages and river water temperature, suggesting that low temperatures experienced during high river levels may have favored virus survival, as viruses generally experience thermal destabilization and gradation of the viral capsid when exposed to higher temperatures (John and Rose, 2005). Additionally, low temperatures reduce the activity of antagonistic microbes and biological mechanisms contributing to virus survival (Gordon and Toze, 2003; Yates et al., 1990). TCC and TVPC, mainly reflecting the autochthonous microbes and viruses, exhibited a higher density at high temperatures, resulting in a high concentration in river water during low river levels. TCC was also positively correlated with temperature, as reported in a previous study by Liu et al. (2013). Furthermore, we observed a negative correlation between *C. perfringens* and EC in river water. Heavy rainfall accompanied by high river levels with low EC in river water due to dilution effects has the potential to mobilize adsorbed microorganisms from river bed sediments and soils of the catchment (Landry et al., 1979). Moreover, albeit a high concentration of coliforms in river water was observed at low river levels, no correlation with any drought-specific physico-chemical parameter was found. Noticeably, the occurrence and abundance of adenovirus in the river water and bank filtrate did not correlate with any of the bacterial and viral indicators monitored nor with any of the physico-chemical parameters tested. Thus, based on the fate of fecal bacterial indicators (*E. coli*, coliforms), viral surrogates (coliphages), TCC, and TVPC, it would not have been possible to predict the occurrence and abundance of pathogenic viruses in either river water or bank filtrate.

4.3. Impact of hydrological extremes on riverbank filtration efficiency

Another focus of our study was the effect of different river levels on the transport and reduction of adenovirus and viral and bacterial fecal indicators during riverbank filtration. The water travel time through bank sediments is a complex function of parameters, including hydraulic conductivity of subsurface sediments, pumping rate at the production well, land side groundwater levels, and river stages. Expectedly, with longer travel times, all selected biological indicators were reduced. This correlation supports the typical hypothesis that a decrease in travel time caused by short–termed increases of the river stage increases the risk of viruses reaching the production well. *Vice versa*, at slow changing and generally low river levels, reduced water flow velocity and increased water residence time can be assumed, improving removal efficiency for pathogenic agents.

High river levels are believed to impair the natural water purification efficiency in riverbank filtration mainly due to potentially elevated concentrations of viruses and bacteria in surface water, a reduced travel time and less dilution with groundwater. In our study, concretely, most of the targeted viral and microbial indicators either showed the highest concentrations in surface water during flood events or concentrations were in the same range in all hydrological situations. We found travel times from the Rhine River to the production well of less than 10 days at high river levels, compared to maximum travel times of up to 78 days. Mean travel time was 31 days. Previous studies and well-established raw water protection schemes recommend a mean water travel time of 10–60 days to be sufficient for efficiently removing pathogenic viruses and bacteria. Worth mentioning, based on our $\delta^{18}O$ data, we can show that water from the production well entirely originated from the river at high water levels, while the river water share was 84% and 77% during medium and low water levels, respectively. Furthermore, at times of high river levels, a full sediment passage from the river to the production well was required to efficiently remove the tested biological indicators, whereas, under other hydrological situations, particularly during low river levels, a sediment passage from the river to the nearby observation wells at row A was already sufficient to reduce most targeted indicators to below the LOD. Another aspect related to high river level situations was raised by Irmscher and Teermann (2002), who claimed that low activity filter media do not remove pathogens efficiently, and, as the river level rises, the infiltrating surface water reaches unsaturated subsoil zones unconditioned for effective natural attenuation. Additionally, factors, including low ionic strength, elevated DOC, and high flow velocities, caused by floods may not only lower the adsorption of microorganisms and viruses to the sediment matrix, significantly decreasing the attenuation capacity of riverbank filtration, but also trigger a remobilization of previously adsorbed bacteria and viruses, promoting their long-distance underground transport (Sprenger et al., 2011; Maliva, 2019).

The highest removal efficiency for adenovirus and most viral and bacterial indicators tested in our study was observed during high river levels. Following the above-mentioned findings and assumptions, this is surprising. However, the data obtained are worth a second look. The higher removal efficiency for pathogens and indicators by riverbank filtration during high river levels are related to higher absolute numbers of respective agents in river water during flood situations (Fig. 9). In fact, high coliform and coliphage numbers in combination with high water levels, as reported by Rohns et al. (2006), could indeed pose a serious risk to water quality at the production well, exceeding the capacity of riverbank filtration.

Taking a specific look to the different hydrological situations, the abundance of adenovirus during bank filtration correlated positively with pH, supporting the idea that virus particles attach to a lesser extent at a higher pH, due to increased electrostatic repulsion according to the DVLO theory (Schijven and Hassanizadeh, 2000). Elevated DOC concentrations found at high river levels correlated positively with an increased abundance of adenovirus. In fact, dissolved organic matter was shown to compete with viruses for sorption sites on sediment surfaces (Gerba, 1984). At low river levels it was only pH and travel time that revealed a statistical significant correlation to adenovirus. No common biological indicators were found to correlate with the abundance of adenovirus during sediment passage at different river levels. While at high river level, adenoviruses correlated with *E. coli*, somatic coliphages, TCC, and TVPC, a correlation with coliforms, TVPC, and ATP was revealed at low river levels. In consequence, for the site investigated, a combination of *E. coli*, coliforms and coliphages seems safe to monitor raw water quality.

The only contamination of raw water at the production well was the occasional occurrence of coliforms and *C. perfringens* during the long period of extremely low river levels in the summer of 2018. Low river levels entail the risk of river bed sediment clogging. Here, clogging includes excessive biomass accumulation, sedimentation of accumulated suspended particles, and precipitation of Mn^{4+} or Fe^{3+} after consumption of DO and denitrification (Sprenger et al., 2011; Engesgaard et al., 2006; Diem et al., 2013). Clogging may not only reduce the bank filtrate quantity but may also lead to preferential flow paths and impaired removal efficiency of pathogens and microbiological indicators (Jaramillo, 2011). Conversely, an increased number of fine particles and iron oxide minerals may constitute an effective barrier leading to an increased adsorption of viruses (Schijven and Hassanizadeh, 2000). Additionally, viruses and bacteria have been demonstrated to be less inactivated and removed under anoxic conditions, which we have observed during the long-term drought, when compared with oxic conditions (Klitzke et al., 2015; Frohnert et al., 2014).

Thus, we summarize that both high river levels with reduced

sediment travel times for water carrying a huge particle load and low water levels with increased water temperature and the risk of bank sediment clogging may pose a risk to raw water quality at the production well. This conclusion is supported by earlier findings of Rohns et al. (2006), who investigated the same riverbank filtration site.

5. Conclusion

In this study, we compared the performance of a riverbank filtration site for the removal of adenovirus and several commonly used bacterial and viral water quality indicators during different hydrological situations. Surprisingly, high river levels were characterized not only by an increased number of pathogens and indicators but also by the highest removal efficiency with riverbank filtration. During drought and low river levels, coliforms and *C. perfringens* were occasionally present in raw water at the production well of the waterworks. Adenovirus, quantified via droplet digital PCR, correlated with *E. coli*, somatic coliphages, TCC, TVPC, pH, and DOC at high river levels. At low river levels, adenoviruses correlated with coliforms, TVPC, pH, and water travel time. For the site investigated, a combination of *E. coli,* coliforms and coliphages for assessing raw water quality was proved safe. For adenovirus, and probably other human pathogenic viruses, viral fecal indicators such as coliphages may occasionally fail predictability, as shown for low river levels, which is an issue that awaits a more detailed exploration. Extreme hydrological events and their influence on the performance of riverbank filtration should be studied on an event-to-event basis at a significantly higher spatial and temporal resolution. This is a difficult task because flood and drought periods cannot be precisely forecasted when planning a field study. Finally, there is an urgent need for lower LODs of pathogenic viruses in natural waters. Since molecular tools such as the ddPCR cannot be significantly improved, the pre-concentration of viral particles from larger water volumes (>100 L) is the way to achieve higher sensitivity.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2021.117961.

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Supporting information

Dynamics of pathogens and fecal indicators during riverbank filtration in times of high and low river levels

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Table caption

Table S1 probability values of significant difference test in selected parameters between different row A, row B and row C.

Table S2 probability values of significant difference test in selected parameters between different wells in one row.

Figure caption

Figure S1 The variation of pumping rate in PW5 during the course of the field study.

Figure S2 Hydrological conditions of the river and for the different observation wells.

Figure S3 The variation of δ^2H, DOC and nitrate in the Rhine river and selected observation wells.

Figure S4 Gradients of Ca^{2+} and SO_4^{2-} monitored in water from the Rhine river and selected groundwater observation wells.

Figure S5 Dynamics of selected biological indicators considering the retention time between river and selected groundwater observation wells.

Figure S6 Heat map of spearman rank correlations between biological variables and water physico-chemical parameters during riverbank filtration system (a), in river water (b), and in observation well water (c). A statistical correlation is indicated in yellow: $P < 0.001$; orange: $0.001 < P < 0.01$; and blue: $0.01 < P < 0.05$). TT: travel time; Adv: adenoviruses; S. coliphage: somatic coliphage; T: water temperature; WL: water level.

Figure S7 Heat map of spearman rank correlations between biological variables and water physico-chemical parameters during riverbank filtration obtained from water in river, row A, row B and PW5 for different hydrological events: at high river level (a); at medium river level (b); at low river level (c). A statistical correlation is indicated in yellow: $P < 0.001$; orange: $0.001 < P < 0.01$; and blue: $0.01 < P < 0.05$). TT: travel time; Adv: adenoviruses; S. coliphage: somatic coliphage; T: water temperature; WL: water level.

Table S1 Probability values of significant difference test in selected parameters between different row A, row B and row C.

Statistical test	Contextual factors	P values
Kruskal-Wallis	Water level	0.9
	Temperature	0.05
	δ18 O	4.2E-15
	DOC	0.06
	Oxygen concentration	0.6
	pH	<2.2E-16
	Cellular ATP	1.0E-08
	TVPC	1.0E-09
	TCC	1.0E-08
	Na+	0.002
	K+	3.5E-12
	NO3-	1.0E-05
	Cl-	0.008
	Br-	0.5
	Mg2+	0.8
	Ca2+	<2.2e-16
	SO42-	<2.2e-16

Table S2 Probability values of significant difference test in selected parameters between different wells in one row.

Statistical test	Contextual factors	P values		
		Row A	Row B	Row C
Kruskal-Wallis	Water level	1.0	1.0	0.9
Kruskal-Wallis	Temperature	0.7	0.9	1.0
ANOVA	δ^{18} O	0.9	0.8	0.5
Kruskal-Wallis	DOC	0.3	0.9	0.8
Kruskal-Wallis	Oxygen concentration	0.1	1.0	3.0E-09
Kruskal-Wallis	pH	0.9	0.002	1.2E-14
Kruskal-Wallis	Electrical conductivity	0.8	0.8	0.7
Kruskal-Wallis	Cellular ATP	0.007	0.6	3.9E-07
Kruskal-Wallis	TVPC	0.03	1.7E-05	0.0001
Kruskal-Wallis	TCC	0.8	0.1	2.9E-08
Kruskal-Wallis	Na^+	0.7	0.9	6.3E-07

Kruskal-Wallis	K^+	0.1	0.6	2.8E-08
Kruskal-Wallis	Cl^-	0.7	0.9	0.1
Kruskal-Wallis	Br^-	0.8	0.9	0.7
ANOVA	NO_3^-	0.2	0.8	2.0E-05
Kruskal-Wallis	Mg^{2+}	0.1	0.5	0.01
Kruskal-Wallis	Ca^{2+}	0.7	0.7	0.1
ANOVA	SO_4^{2-}	0.5	0.7	0.7

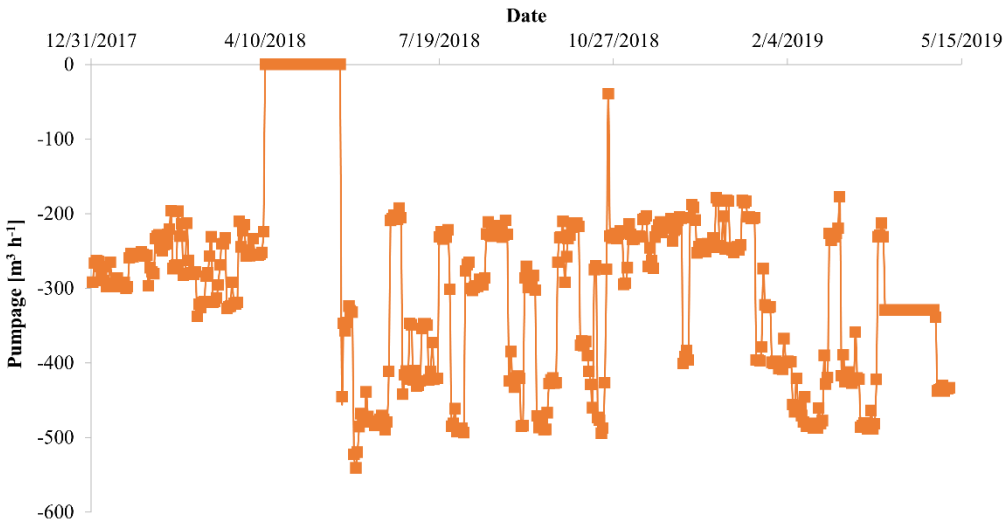

Figure S1 The variation of pumping rate in PW5 during the course of the field study.

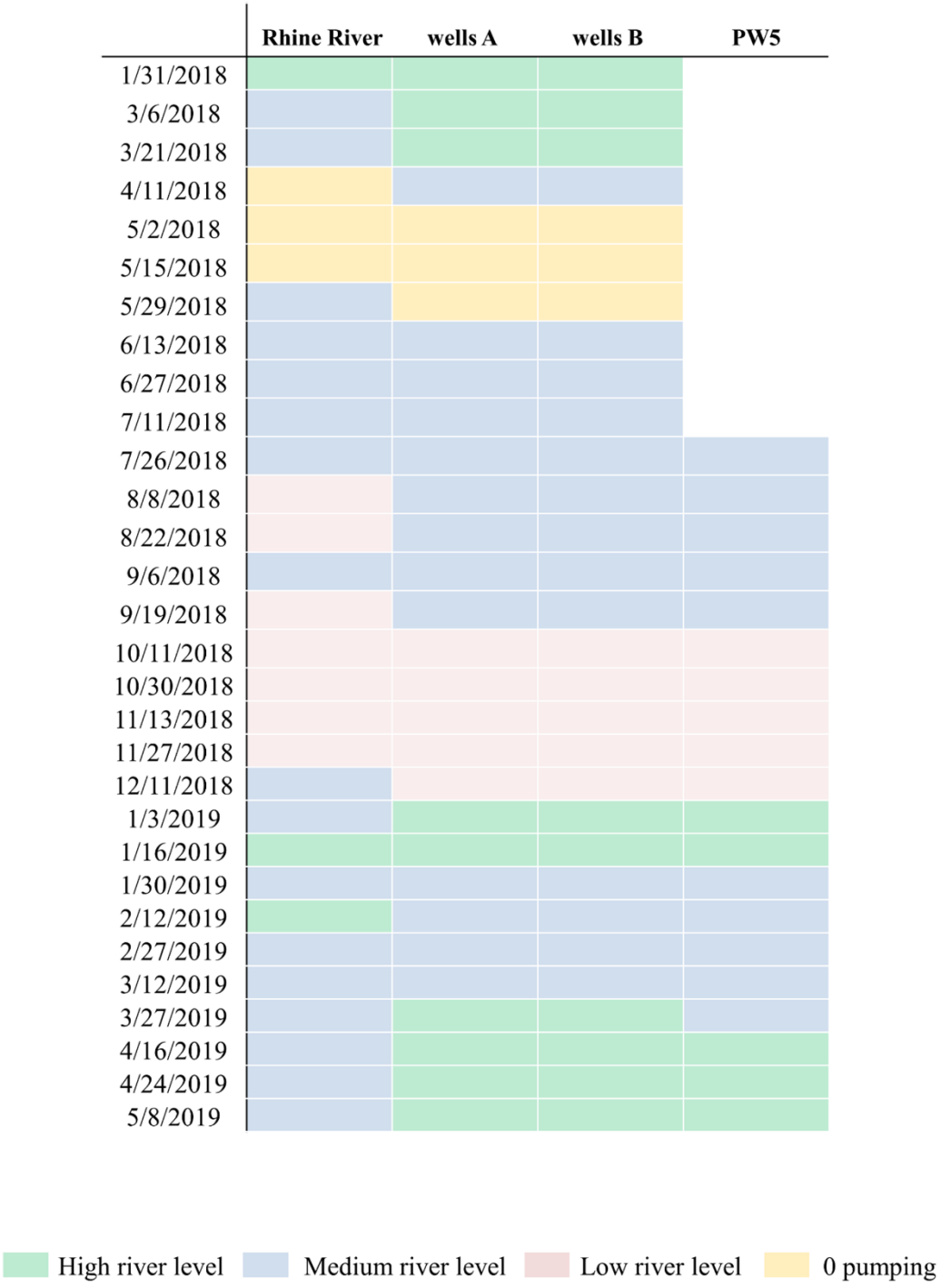

Figure S2 Hydrological conditions of the river and for the different observation wells.

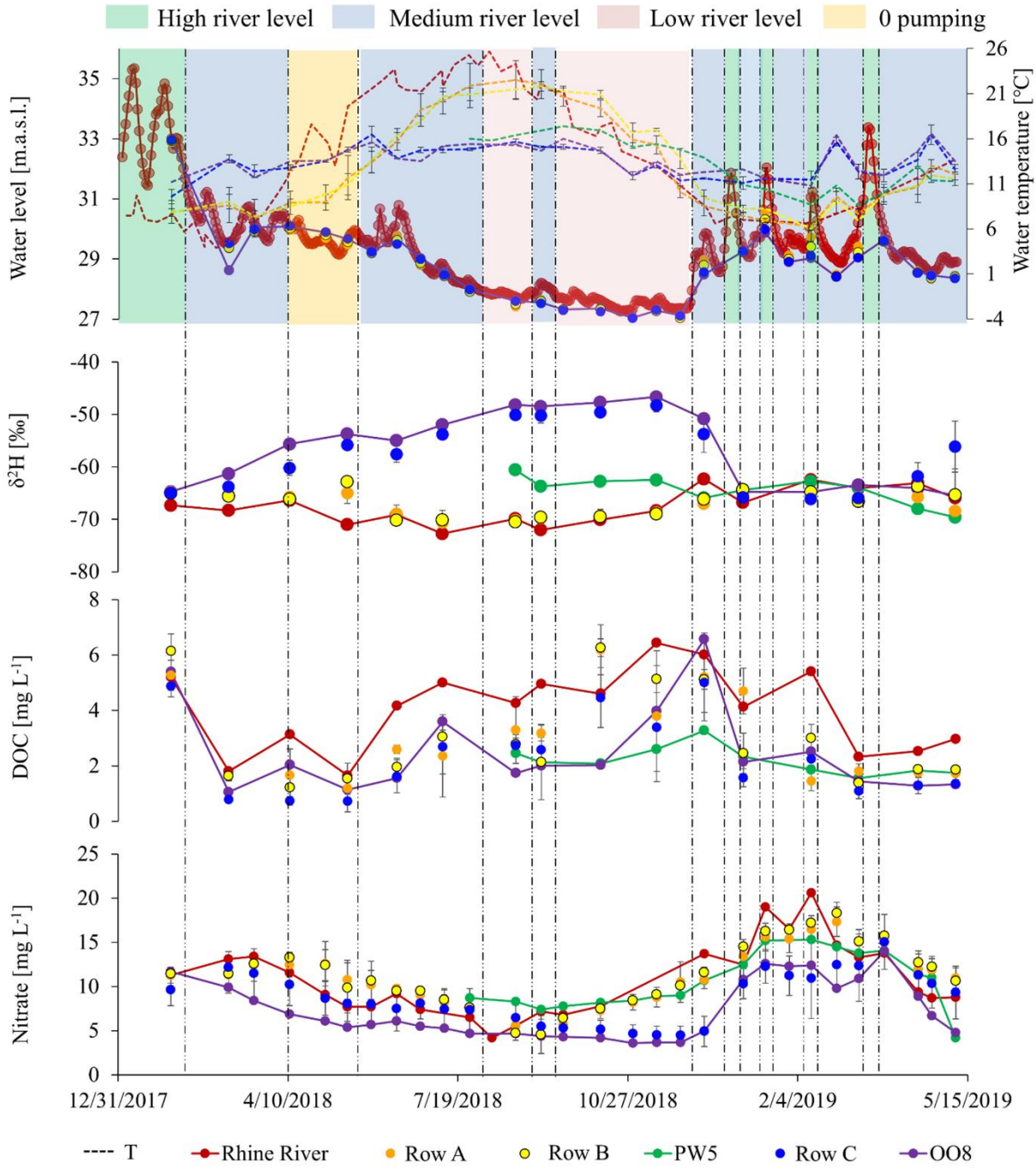

Figure S3 The variation of δ^2H, DOC and nitrate in the Rhine river and selected observation wells.

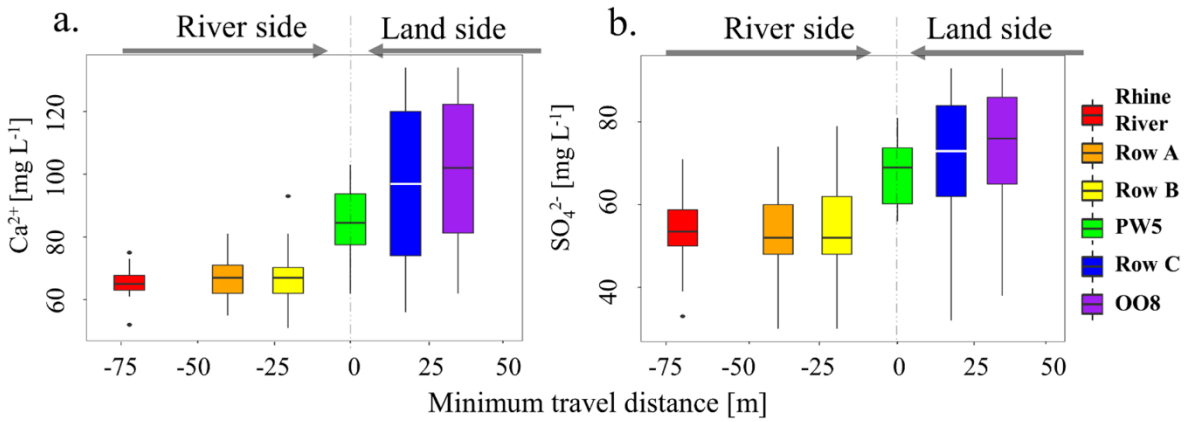

Figure S4 Gradients of Ca^{2+} and SO_4^{2-} monitored in water from the Rhine river and selected groundwater observation wells.

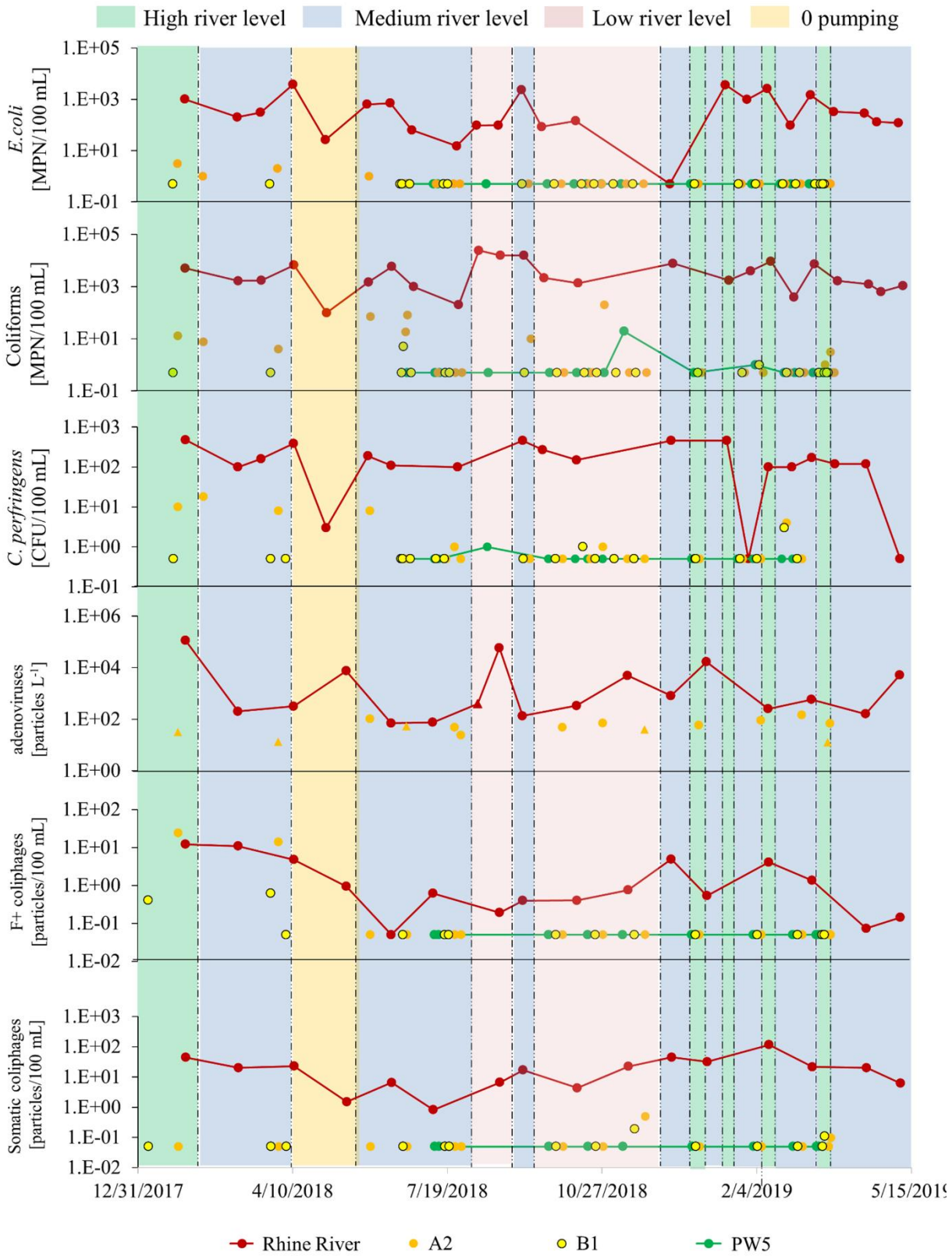

Figure S5 Dynamics of selected biological indicators considering the retention time between river and selected groundwater observation wells.

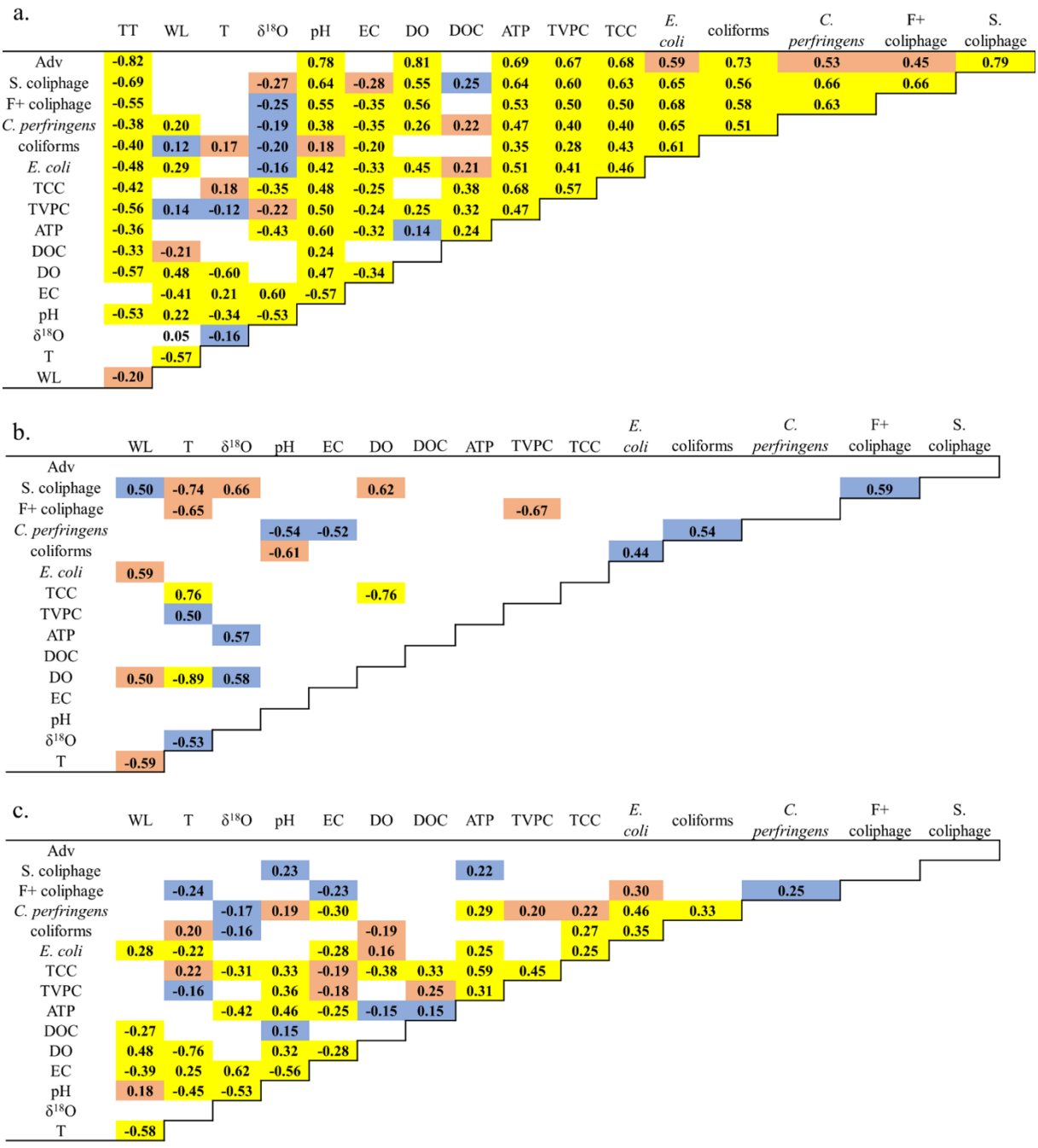

Figure S6 Heat map of spearman rank correlations between biological variables and water physico-chemical parameters during riverbank filtration system (a), in river water (b), and in observation well water (c). A statistical correlation is indicated in yellow: P < 0.001; orange: 0.001< P < 0.01; and blue: 0.01 < P < 0.05). TT: travel time; Adv: adenoviruses; S. coliphage: somatic coliphage; T: water temperature; WL: water level.

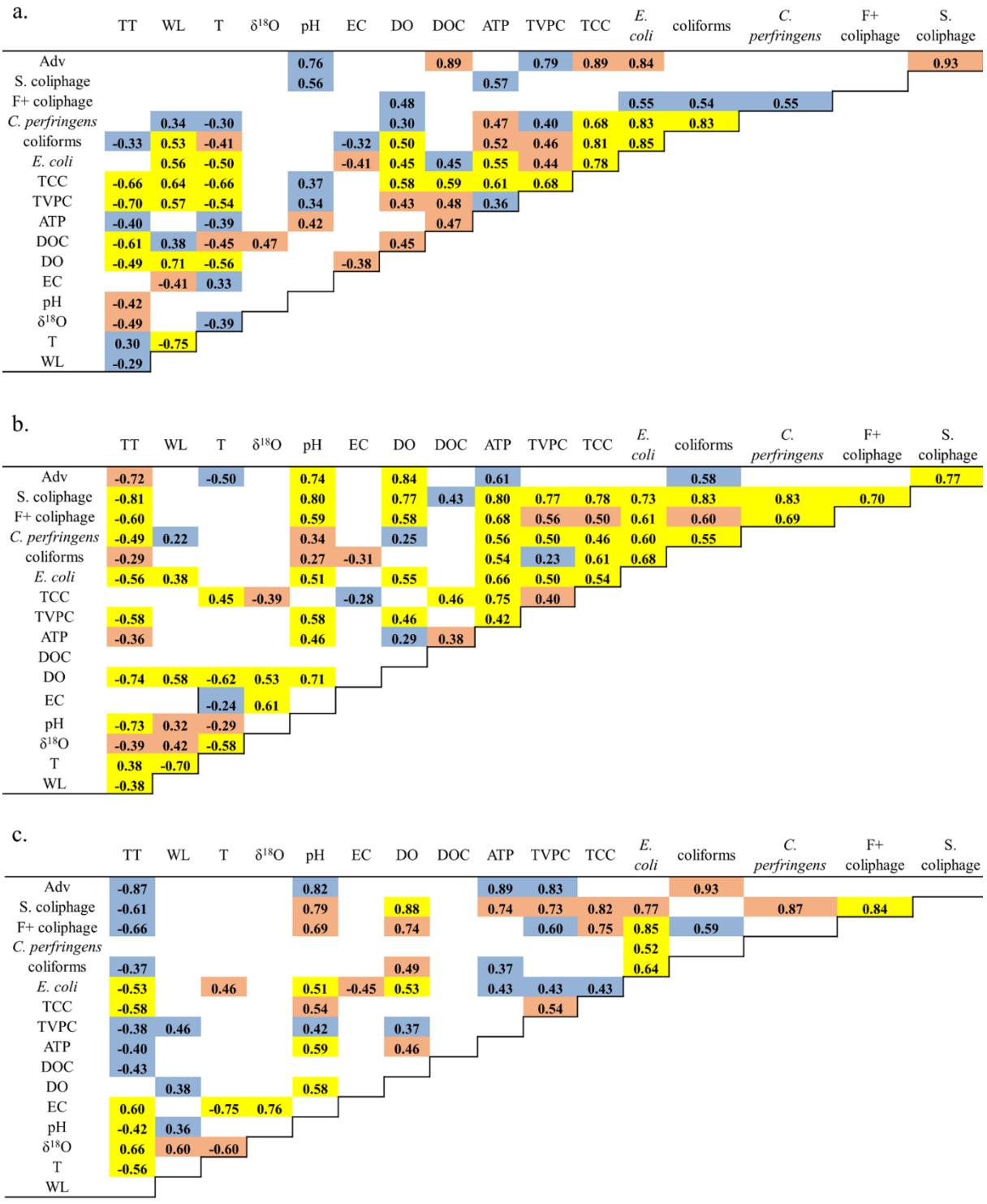

Figure S7 Heat map of spearman rank correlations between biological variables and water physico-chemical parameters during riverbank filtration obtained from water in river, row A, row B and PW5 for different hydrological events: at high river level (a); at medium river level (b); at low river level (c). A statistical correlation is indicated in yellow: P < 0.001; orange: 0.001< P < 0.01; and blue: 0.01 < P < 0.05). TT: travel time; Adv: adenoviruses; S. coliphage: somatic coliphage; T: water temperature; WL: water level.

2.3 The D-A-(C) index: a practical approach towards the microbiological-ecological monitoring of groundwater ecosystems

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Synopsis

Groundwater quality is closely connected to human welfare. It is the most important source for drinking water and irrigation. Because of their low-energy status, groundwater ecosystems are particularly vulnerable to disturbances. Yet, the quality status of groundwater with respect to impacts of disturbances on a biological level have received relatively little attention so far in routine groundwater monitoring. Main reasons for this shortcoming are missing recognition of groundwater ecology in the current legislation and lack of suitable monitoring tools specifically designed for groundwater, which are universally applicable under a range of conditions, and at the same time inexpensive and simple to implement. Based on the omnipresence of prokaryotic microorganisms in groundwater ecosystems, we developed an approach to monitor the microbiological-ecological groundwater quality by combining measurements of prokaryotic cell density (D), microbial activity (A), and (optionally) bioavailable carbon (C). Results of the individual measurements are merged into an easily interpretable index. This index is in essence a multivariate outlier analysis based on robust Mahalanobis distances. The analysis rests on two main assumptions: 1) under natural, undisturbed conditions, cell density, microbial activity, and bioavailable carbon follow a multivariate log-normal distribution; 2) disturbances cause variation in one or more of these parameters that exceeds the natural stochastic variation in the data. The index allows to identify single samples that display larger multivariate variation than would be expected by chance (identification of statistical outliers), which is interpreted as an indication for a disturbance.

To validate this new approach, we tested it in relation to three of the most important threats to groundwater ecosystems, i.e., organic contamination with petroleum hydrocarbons, surface intrusion, and impacts resulting from agricultural land use. A combination of the variables cell density and microbial activity reliably identified all samples affected by hydrocarbon contamination from a pool of samples including data on contaminated groundwater as well as pristine groundwater. Similar results were obtained for the surface water intrusion scenario. Regarding the impacts of agricultural land use, our study showed that affected samples are more reliably detected when concentrations of bioavailable organic carbon were considered in addition to prokaryotic cell densities and microbial activity. Through this study, we found that the amount of bioavailable carbon, especially samples including surface water, needs to be interpreted with caution, because phototrophic cells in surface water get inactivated, die, and decay during lab incubation in the dark. Moreover, this study demonstrates the advantage, in terms of sensitivity and robustness, of the simultaneous multivariate analysis of selected microbiological parameters over separate univariate analyses of each parameter. Furthermore, comparisons of multivariate signatures of the microbiological parameters between non-contaminated aquifers across nine regions in Germany showed significantly different, region-specific patterns. These differences between regions should be taken into account when defining reference sites in ecological monitoring strategies.

Author contributions

Prof. Christian Griebler developed the ecological monitoring approach; Dr. Lucas Fillinger provided the statistical framework for the data analysis. Dr. Lucas Fillinger selected and analyzed the data to present and test the approach in the manuscript, meantime he conceptualized and wrote the manuscript in consultation with Prof. Christian Griebler and other co-authors. Anne Trimbach, He Wang, and Dr. Bernd Bendinger collected the data for disturbance case studies. Concentrations of AOC and ATP, as well as physico-chemical parameters, were determined by Dr. Katrin Hug, He Wang, and Anne Trimbach.

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The D-A-(C) index: A practical approach towards the microbiological-ecological monitoring of groundwater ecosystems

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ABSTRACT

Groundwater is not only a vital resource, but also one of the largest terrestrial aquatic ecosystems on Earth. However, to date, ecological criteria are often not considered in routine groundwater monitoring, mainly because of the lack of suitable ecological assessment tools. Prokaryotic microorganisms are ubiquitous in groundwater ecosystems even under the harshest conditions, making them ideal bio-indicators for ecological monitoring. We have developed a simple, inexpensive approach that enables ecological groundwater monitoring based on three microbiological parameters that can be easily integrated into existing routine monitoring practices: prokaryotic cell density (D) measured by flow cytometry; activity (A) measured as prokaryotic intracellular ATP concentrations using a simple cell-lysis-luminescence assay; and, as an optional parameter, the bioavailable carbon (C) measured as the concentration of assimilable organic carbon in a simple batch growth assay. We analyzed data for three case studies of different disturbances representing some of the main threats to groundwater ecosystems, i.e. organic contamination with hydrocarbons, surface water intrusion, and agricultural land use. For all three disturbances, disturbed samples could be reliably distinguished from undisturbed samples based on a single index value obtained from multivariate outlier analyses of the microbial variables. We could show that this multivariate approach allowed for a significantly more sensitive and reliable detection of disturbed samples compared to separate univariate outlier analyses of the measured variables. Furthermore, a comparison of non-contaminated aquifers from nine different regions across Germany revealed distinct multivariate signatures along the three microbial variables, which should be considered when applying our approach in practice. In essence, our approach offers a practical tool for the detection of disturbances of groundwater ecosystems based on microbial parameters which can be seamlessly extended in the future by additional parameters for higher sensitivity as well as flexibility.

1. Introduction

Groundwater accounts for 95% of all liquid freshwater on Earth, making it the most important source of drinking and irrigation water worldwide. Throughout the globe an estimated 2.5 billion people depend solely on groundwater for their supply of freshwater, while at the same time, this vital resource is increasingly threatened by overexploitation and contamination caused for example by intensive agriculture, industrial activities, or untreated urban runoffs (United Nations World Water Assessment Programme, 2015). However, groundwater aquifers not only serve as a storage of vital freshwater, but, just as important, they support unique ecosystems that accommodate a large diversity of prokaryotic, protozoan and metazoan life (Griebler and Lueders, 2009). The services that societies obtain from groundwater ecosystems, like the provision of clean freshwater, are intimately linked to the activities of these organisms, as they are the driving force behind key biogeochemical processes, the degradation of contaminants, or the elimination of pathogenic microorganisms and viruses

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(Feichtmayer et al., 2017; Griebler and Avramov, 2015).

Over the past decades, largely due to the collective effort of the scientific community, policy makers have become increasingly aware of the strong link between groundwater quality and ecosystem health. As a result, legislative perceptions in parts of the world have started to shift from groundwater environments being first and foremost a commodity, towards their recognition as unique ecosystems that merit protection (Danielopol et al., 2007; Griebler et al., 2014). Examples of legislations that already demand the consideration of ecological criteria in groundwater monitoring can be found in Switzerland (SWPO, 1998) and Australia (EPA, 2003; NSW-SGDEP, 2002), and plans for similar frameworks furthermore exist in the European Union (EU-GWD, 2006). However, the current lack of suitable, universally applicable ecological assessment tools is frequently mentioned to prevent the implementation of these legislations in routine groundwater monitoring, although much progress has recently been made in that direction (e.g. Korbel and Hose, 2011, 2017). While ecological assessment criteria based on sets of indicator organisms have already been firmly integrated into routine monitoring for surface waters, these tools are not compatible with groundwater ecosystems due to the characteristic environmental constrains (e.g. lack of light and primary production, low nutrient levels) and repertoire of organisms (e.g. lack of algae and macrophytes) in these environments (Steube et al., 2009). This calls for new monitoring tools specifically tailored to groundwater ecosystems.

To be integrated into routine monitoring protocols, such tools should fulfill certain criteria: 1) the targeted parameters need to be easy and inexpensive to measure; 2) they need to be sensitive to a wide range of disturbances and globally applicable across different hydrogeological conditions; 3) the data need to be simple to analyze and interpret; 4) ideally, samples for measuring ecological parameters should be obtainable along with samples for typical physicochemical analyses, without the need for additional sampling efforts. Several studies have suggested the use of groundwater fauna as bioindicators for the detection of disturbances of groundwater ecosystems (Bork et al., 2009; Hahn, 2006; Malard et al., 1996; Marmonier et al. 2013, 2018; Mösslacher, 1998; Schmidt et al., 2007; Stein et al., 2012). However, the notoriously sparse and locally often highly heterogeneous distribution of groundwater fauna as well as their absence in anoxic groundwater complicates data collection and interpretation for these types of indicators, and restricts their applicability to oxic aquifers (Korbel and Hose, 2017; Stein et al., 2010).

Prokaryotic microorganisms on the other hand are ubiquitous in aquifers even under harsh conditions (Griebler and Lueders, 2009), making them ideal bioindicators for ecological monitoring under various environmental conditions. The idea of using microorganisms in groundwater monitoring is not new (Brielmann et al., 2009; Foulquier et al., 2011; Griebler et al., 2010; Pronk et al., 2009; Stein et al., 2010; Steube et al., 2009; van Driezum et al., 2018), but so far there are only a few examples of applicable frameworks that integrate microbial parameters into ecological assessment schemes. However, these approaches are either only applicable under certain conditions, for example requiring non-stagnant water in monitoring wells (Mermillod-Blondin et al., 2013), or rely on DNA-based approaches and sophisticated bioinformatic analyses, which probably exceed the current expertise of most local authorities and water suppliers to date, and hence are currently still too advanced to be suitable for routine monitoring practices (Pearce et al., 2011).

To meet the demand for a universally applicable and at the same time user-friendly method for the ecological monitoring of groundwater ecosystems, we present here the D-A-(C) index. D and A stand short for the two microbial (i.e. prokaryotic) variables that form the basis of this concept, i.e. prokaryotic cell density (D) measured by flow cytometry and the activity displayed by these cells (A) measured as intracellular ATP concentrations using a simple cell-lysis-luminescence assay. The bioavailable carbon (C) that is available to the cells can additionally be included as an optional variable as the concentration of assimilable organic carbon (AOC) determined indirectly via a batch growth assay. The usefulness of these variables has already been established for monitoring the stability of drinking water distribution systems (FDHA, 2012; Lautenschlager et al., 2013; Van Nevel et al., 2017; Vang et al., 2014; Vital et al., 2012), and have furthermore been shown to react readily to sudden disturbances in groundwater settings (Foulquier et al., 2011; Herzyk et al., 2017; Mermillod-Blondin et al., 2013), strongly suggesting a great potential for their application in groundwater monitoring. The D-A-(C) index integrates the signals of all these variables in a multivariate analysis and allows for a simple detection of disturbances based on a single value.

In this paper, we will show how disturbances can be detected based on the D-A-(C) index, illustrated by three case studies of different disturbances: 1) organic contamination with hydrocarbons; 2) surface water intrusion; and 3) disturbances related to different types of land use. We will demonstrate how a simultaneous multivariate analysis of the D-A-(C) variables can yield a higher sensitivity and at the same higher robustness compared to separate univariate analyses of the individual variables. Additionally, we will highlight differences in D-A-(C) signatures of non-contaminated groundwater aquifers from nine different regions across Germany and discuss the implications of these differences for groundwater monitoring (a map with the locations of all sampling sites considered in this study is given in Fig. 1).

2. Materials and methods

2.1. Description of the case studies and groundwater sampling

For the first disturbance case study, we simulated a severe organic contamination event by combining data from a non-

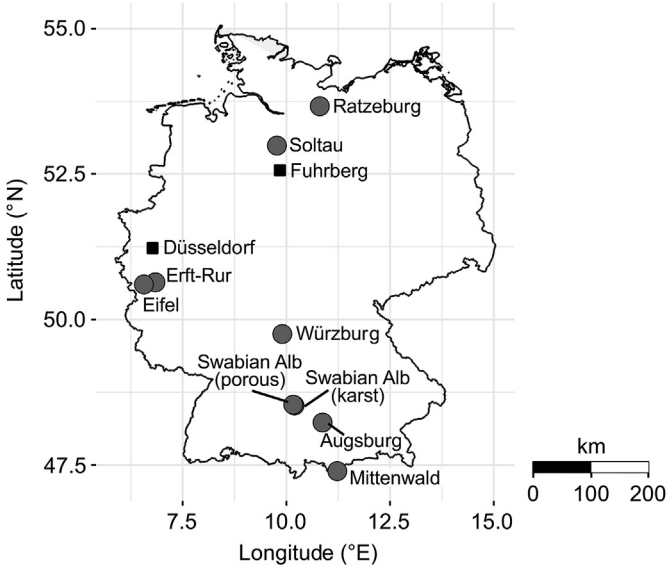

Fig. 1. Schematic map with the locations of the different regions considered in this study. Black squares represent the two regions where data were collected for the disturbance case studies (data for the organic contamination and the surface water intrusion case study were collected in Düsseldorf; data for disturbances related to different types of land use were collected in Fuhrberg). Grey dots represent the nine regions that were analyzed for regional D-A-(C) signatures of non-contaminated aquifers.

contaminated site in the city of Düsseldorf with data from a nearby former coal gasification site in Düsseldorf-Flingern that has a long history of organic contamination with aromatic hydrocarbons (BTEX and polyaromatics). Here, samples were collected across the vertical profile of the contaminant plume using peristaltic pumps connected to the ports of multilevel wells. Out of a total pool of 65 contaminated samples that were collected during different sampling campaigns in 2005, 2006, and 2007 (see Anneser et al., 2008, 2010; Jobelius et al., 2011), data from 30 randomly selected samples were combined with data from 81 non-contaminated samples.

The data for these non-contaminated samples were taken from a total pool of 108 samples that formed the basis for the second case study regarding the impact of surface water intrusion. These samples were collected between January and October 2018 from an alluvial aquifer at the bank of the Rhine River in Düsseldorf at a site where drinking water is produced via river bank filtration. At this site, groundwater monitoring wells were located along a transect perpendicular to the river shore at different distances to the river: sets of three wells each were situated close to the river at distances of about 2 and 20 m, respectively; this area of the aquifer receives river water under normal conditions due to river bank filtration. Another set of three wells and one single well were located approximately 60 and 75 m away from the river, respectively, in an area which usually does not come into contact with the river water but is recharged from landside groundwater. However, high water levels of the Rhine River from January until March 2018 led to an increased intrusion of river water into the aquifer at wells close to the river, as well as surface water intrusion in the more distant areas, which was revealed by water stable isotope signatures (Fig. S2).

For the third case study regarding the impact of different types of land use, we analyzed data collected in Fuhrberg near the city of Hannover where different types of land use can be found close to each other. A total of 29 samples were collected between spring and summer 2018, comprising 9 samples from a forest, and 10 samples from a grassland and an agricultural land, respectively.

For assessing differences in D-A-(C) signatures between regions, we analyzed data from non-contaminated aquifers in nine different regions across Germany (Fig. 1). Non-contaminated groundwater in this context is defined here as shallow groundwater that, although being exposed to a multitude of potential anthropogenic impacts (e.g. waste water, agriculture, or urban run-off), did not display significantly elevated concentrations of concerning chemicals or metals at the time of sampling (mainly based on thresholds set for drinking water). Samples were collected at each region at least during two sampling campaigns between the years 2007 and 2017. An in-depth description of the individual regions would go beyond the scope of this article; instead, a summary of key information is given in Table 1.

Except for the samples from the organically contaminated site as mentioned above, groundwater was collected from fully-screened monitoring wells using a MP1 submersible pump connected to an adjustable frequency converter to control the pumping rate (Eijkelkamp Soil & Water, Giesbeek, The Netherlands). The pump was lowered to half the depth of the water column inside the well. The collection of groundwater was done according to national and international standard protocols (DIN 38402-13; LUBW, 2013; EPA, 2017) with minor modifications. To achieve an initial exchange of the borehole water and water in the direct surrounding of the well, water was pumped at the highest possible rate accepting a maximum drawdown of the groundwater table of 30 cm. As such, wells were purged prior to sample collection, pre-pumping two to three borehole volumes and subsequently waiting until physico-chemical parameters monitored by online measurements (temperature, pH, electrical conductivity, and concentration of dissolved oxygen) were stable. For the collection of groundwater samples, the pumping rate was reduced to one third of the initial rate used for pre-pumping to avoid possible shearing of biofilms due to strong pumping. Samples for cell density measurements (50 mL) were fixed immediately after sampling with glutaraldehyde at a final concentration of 0.5% v/v in sterile Falcon tubes. Samples for ATP measurements were collected in autoclaved glass bottles. Glassware for the collection of AOC samples was baked at 450 °C for 4 h prior to sampling and closed with plastic caps with Teflon inlays that were treated with 10% w/v sodium persulfate at 60 °C for 1 h to remove traces of organic carbon. Samples were stored in the dark at 4 °C for transport to the lab and until analysis (within 48 h after sampling).

Table 1
Overview of sample sizes, aquifer characteristics, and sampling time points for the non-contaminated aquifers that were analyzed for differences in D-A-(C) signatures between regions.

Region	n [a]	Unique wells [a]	Aquifer type	Dominating aquifer material	Dominating redox condition	Approximate span of the sampled area (km)	Groundwater temperature (°C) [b]	pH [b]	Electrical conductivity (µS cm^{-1}) [b]	Sampling campaigns
Ratzeburg	12 (6)	6 (6)	porous	gravel, fine sand	hypoxic-anoxic	3.5	9.6 ± 0.4	7.0 ± 0.6	595 ± 266	spring & autumn 2009
Soltau	33 (16)	17 (16)	porous	gravel, fine sand	hypoxic-anoxic	5	8.7 ± 1.6	6.9 ± 0.9	413 ± 128	spring & autumn 2009
Erft-Rur	20 (10)	10 (10)	porous	gravel, fine sand	oxic	20	10.8 ± 1.6	6.7 ± 0.2	964 ± 278	spring & autumn 2007, 2009
Eifel	22 (11)	11 (11)	fractured	sandstone	oxic	5	10.3 ± 0.5	7.2 ± 0.2	690 ± 236	spring & autumn 2009
Würzburg	40 (29)	16 (15)	porous	gravel, fine sand	oxic	0.5	13.7 ± 2.8	7.2 ± 0.2	972 ± 174	spring & autumn 2016; winter 2017
Swabian Alb	27 (13)	14 (13)	porous	coarse gravel	oxic	20	10.4 ± 0.9	7.1 ± 0.1	768 ± 96	spring & autumn 2007, 2009
Swabian Alb	31 (15)	16 (15)	karst	limestone	oxic	20	10.8 ± 1.0	7.1 ± 0.1	686 ± 47	spring & autumn 2007, 2009
Augsburg	23 (15)	12 (10)	porous	gravel, fine sand	oxic	12	11.2 ± 1.0	7.3 ± 0.2	603 ± 80	spring & autumn 2016
Mittenwald	25 (8)	14 (8)	porous	gravel, coarse sand	oxic	3.5	7.2 ± 0.8	7.8 ± 0.2	344 ± 60	spring & autumn 2009
Total dataset	233	116								

[a] AOC data were not available for all samples; the value within parentheses gives the number of samples and unique wells, respectively, for which AOC data were available.
[b] Mean ± standard deviation calculated across all samples.

2.2. Determination of the D-A-(C) parameters

2.2.1. Prokaryotic cell density (D)

The number of prokaryotic cells was determined by flow cytometry (FC500 CYTOMICS; Beckman Coulter, Brea, CA, USA). To distinguish cells from abiotic particles and background noise, cells in 500 μL sample aliquots were stained with SYBR Green I nucleic acid stain (Invitrogen, Darmstadt, Germany) at a ratio of 1:10,000 v/v followed by incubation at 37 °C for 13 min in the dark. Gates for cell counting were set in dot plots of green fluorescence (recorded at 530 nm; FL1 channel) versus red fluorescence (610 nm; FL3 channel) following protocols from literature (e.g. Hammes and Egli, 2005) and recommendations by government agencies (Kötzsch et al., 2012; Zunabovic-Pichler, 2018). A representative example plot is shown in Fig. S1. Excitation was achieved with a 488 nm argon ion laser. Instrument-specific filter gain settings were as follows: FL1: 569; FL3: 640; trigger threshold (set on FL1): 1. Data acquisition and analysis was done using the CYTOMICS FC500 CXP software (version 2.2; Beckman Coulter). Samples were measured with flow rate set to 'medium' for 60 s. To determine the exact volume of measured sample, a 100 μL suspension with a known number of fluorescent beads (Trucount Tubes; Becton-Dickinson, Franklin Lakes, NJ, USA) was added to each sample as internal standard. Duplicate measurements were performed on biological triplicates for each sample.

2.2.2. Activity (A; intracellular ATP)

ATP was measured using the BacTiter-Glo Microbial Cell Viability Assay kit (Promega, Madison, WI, USA) based on the method by Hammes et al. (2010) with slight modifications. The measurement is a luminescence assay based on the ATP-dependent oxidation of luciferin catalyzed by luciferase. The kit offers a single-step sample preparation protocol by combining the bacterial cell lysis buffer and the luciferase enzymes in a single reagent. Samples and the BacTiter-Glo reagent, prepared according to the manufacturer's instructions, were pre-warmed separately at 38 °C for at least 2 min before mixing 1 mL sample with 50 μL reagent. The mixture was incubated at 38 °C for 1 min and the luminescence was measured on a GloMax 20/20 Luminometer (Promega). Concentrations were determined by comparison with external ATP standards dissolved in ATP-free water (both Fisher Scientific, Waltham, MA, USA), using ATP-free water as blank. To correct for the contribution of extracellular ATP in the samples to the measured luminescence signal, the measurements were performed on an unfiltered sample fraction, representing the total ATP concentration in the sample, and additionally on a fraction of the same sample that was passed through a 0.1 μm polyvinylidene fluoride Millex syringe filter (Merck, Kenilworth, NJ, USA) before sample preparation to remove cells, thus containing only extracellular ATP. The concentration of intracellular ATP was then calculated by subtracting the extracellular concentration from the total (Hammes et al., 2010). All measurements were carried out in triplicate.

2.2.3. Carbon (C; assimilable organic carbon (AOC))

AOC concentrations were determined indirectly with a batch growth assay based on the method by Hammes and Egli (2005). The assay quantifies prokaryotic cell growth of a natural microbial community in a water sample on the bioavailable carbon present in that sample during an incubation under *in situ* conditions until stationary phase (in our case over a period of 30 days). To this end, a sample was diluted 10:1 with filter-sterilized groundwater from the same site. The filtered groundwater served as carbon source to initiate batch growth of the cells derived from the unfiltered sample. Samples for the determination of total prokaryotic cell numbers were taken immediately at the beginning of the incubation and at the end. The cells that were produced during the incubation were used as a measure for the amount organic carbon that was assimilated into microbial biomass. Thus, the AOC concentration was calculated from the average net growth determined from triplicate incubations converting the number of produced cells into carbon with a constant factor of 20 fg organic C $cell^{-1}$ (Griebler et al., 2002). For the incubation, 80 mL diluted groundwater was incubated in 100 mL glass bottles at *in situ* groundwater temperature in the dark with shaking at 100 rpm. Cell numbers were measured as total prokaryotic cell counts by flow cytometry as described above. All glassware used for the incubations were treated to remove traces of organic carbon as above.

2.3. Determination of hydrochemical parameters and isotope ratios

Groundwater temperature, pH, electrical conductivity, and oxygen concentrations were measured on-site using field sensors (WTW, Weilheim, Germany). Concentrations of dissolved organic carbon (DOC) were measured in samples passed through a 0.45 μm filter (VWR, Radnor, PA, USA) that was rinsed three times with ultra-pure water prior to sample filtration to remove residual carbon. Samples were filtered on-site and subsequently acidified with 20 mM HCl (final concentration). DOC was determined by high-temperature (680 °C) catalytic oxidation on a Shimadzu TOC-LCPH Analyzer equipped with a non-dispersive infrared (NDIR) detector (Shimadzu, Kyoto, Japan). Nitrate concentrations were measured by ion chromatography at GBA Group laboratories (GBA Group, Gesellschaft für Bioanalytik mbH, Hamburg, Germany). Stable isotope ratios of oxygen ($^{18}O/^{16}O$) were determined using a PICARRO L2130-i Isotopic Water Analyzer (PICARRO, Santa Clara, CA, USA). $\delta^{18}O$ ratios were measured in samples via equilibration with CO_2 at 18 °C for 5 h under constant shaking. $\delta^{18}O$ ratios were determined relative to internal standards that were calibrated against V-SMOW, V-GESP, and V-SLAP standards (International Atomic Energy Agency, Vienna, Austria). All measurements were performed at least in duplicate.

2.4. Data analysis

In order to detect disturbances in the three case studies, we combined the signals of the D-A-(C) variables in a multivariate analysis as schematically shown in Fig. 2. Under normal undisturbed conditions, samples are expected to display a certain distribution in a multivariate space along these variables. For multivariate normally distributed data, a cloud of samples arrange in an elliptical form which is defined by the mean values of the variables forming the center of the ellipse, the variances of the individual variables, and the covariance between them, determining the shape and slope of the ellipse. Disturbances that cause severe changes in one or more of these variables increase the distance of affected samples to the center of the ellipse beyond the range of distances expected due to random variation. In other words, samples affected by a disturbance can be considered outliers. The distance of a single sample to the center is calculated as the Mahalanobis distance according to Equation (1):

$$MD_i = \sqrt{(X_i - \mu)' \times S^{-1} \times (X_i - \mu)} \quad (1)$$

where X_i is a vector with the values of the individual variables for a single sample, and μ and S^{-1} are the vector with the variable means and the inverse of the covariance matrix, respectively, calculated from the full data. This distance is unitless and indicates how far a given sample lies away from the center of a multivariate distribution. Hence it can be considered a multidimensional extension of

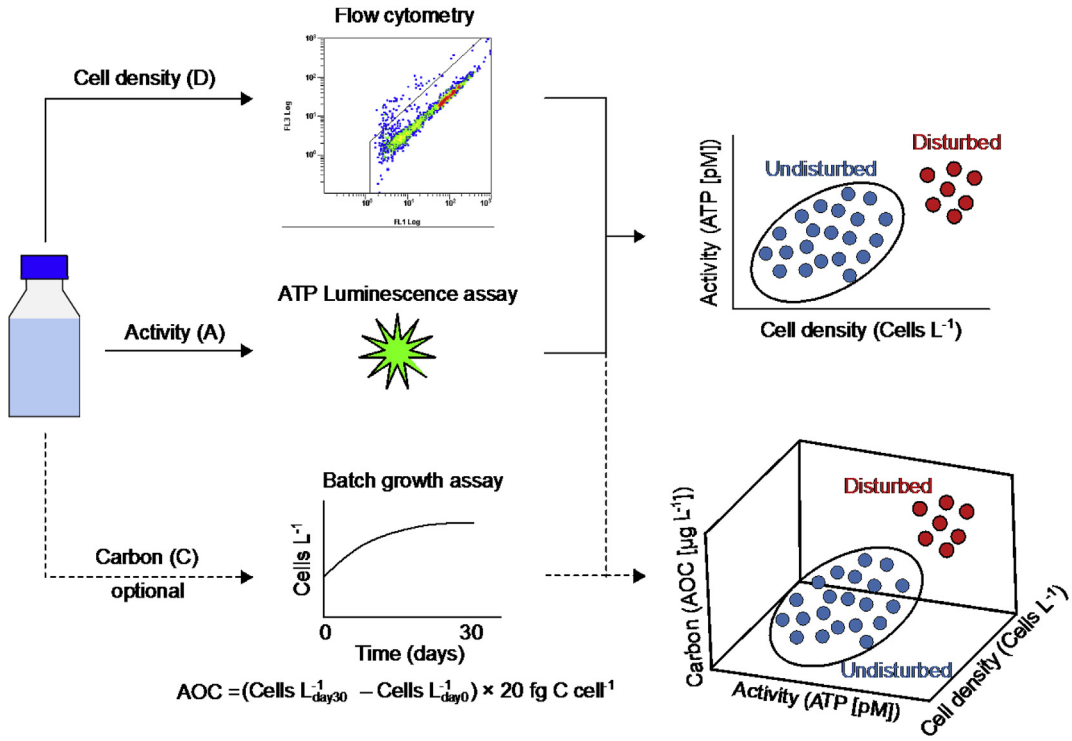

Fig. 2. Schematic overview of the D-A-(C) workflow. The measured variables are combined in a multivariate analysis to detect disturbances. Samples affected by a disturbance are identified as multivariate outliers that deviate from the distribution of undisturbed samples along the measured variables. The D-A-(C) index is used to distinguish significant multivariate outliers from random variation in the data as explained in the main text.

univariate z-scores (Manly, 1994). The Mahalanobis distance takes values of the square root of a chi-squared distribution with as many degrees of freedom as there are variables in the analysis (e.g. df = 3 when all three D-A-C variables are considered in the analysis). This information is used to set thresholds to distinguish significant outliers from random variation in the data. For example, 97.5% of the values of a chi-squared distribution with three degrees of freedom are <9.35. Thus, a sample with a Mahalanobis distance above the critical value of $\sqrt{9.35}$ would be declared an outlier at a 0.975 confidence level and consequently be interpreted as a sign of a disturbance (a simple numerical example is given in the Supplementary Material along with Fig. S5). Problems arise, however, if Mahalanobis distances are calculated based on the raw data directly, since the presence of already a small fraction of outliers in the dataset can severely distort the estimates of μ and S. Therefore, we used the Fast-MCD algorithm, which can provide reliable robust estimates of μ and S in datasets contaminated with outliers, and calculated robust Mahalanobis distances based on these estimates (Hubert and Debruyne, 2010; Rousseeuw and van Driessen, 1999). Briefly, the algorithm searches for a subset of samples of size h (Eq. (2): $h = \frac{n+p+1}{2}$, where n is the number of samples in the dataset and p the number of variables) with the smallest determinant of S, i.e. with the smallest dispersion in the multivariate space, and subsequently calculates μ and S based on these h samples. We will refer to the final robust Mahalanobis distances as the D-A or D-A-C index, respectively, depending on the variables included in the analysis. A sample was declared an outlier at a 0.975 confidence level if its calculated index value exceeded the square root of the 0.975 quantile of a chi-squared distribution with the according degrees of freedom as described above (Rousseeuw and van Driessen, 1999).

The strength of this multivariate approach is that it not only takes into account the variance of each variable, but also possible correlations (in the form of covariance) between these variables, which are not taken into account in separate univariate analyses of the individual variables (Manly, 1994). To illustrate this strength, we compared the multivariate D-A-(C) approach to a univariate method for outlier detection. For this univariate approach, separate thresholds were set for each variable based on the median and the median absolute deviation (MAD). This approach was chosen because the median and the MAD provide more robust estimates of the center and the dispersion of data, respectively, even in the presence of outliers, compared to the mean and standard deviation similar to what has been explained above. Using this approach, a sample was considered an outlier if it fell outside the range of median $\pm 2.5 \times$ MAD as recommended by Leys et al. (2013).

To assess differences in D-A-(C) signatures between regions, i.e. differences between the covariance matrices and variable means, we again applied the Fast-MCD algorithm to the data from the non-contaminated aquifers sampled at the different regions across Germany (Fig. 1). Box's M test was used to test for differences in covariance matrices (i.e. robust estimates of S) between regions. Differences in means for each variable were assessed using Welch's ANOVA followed by the corresponding Games-Howell post-hoc test for pairwise comparisons to assess differences between individual regions. Outliers for a given region that were detected based on the robust Mahalanobis distances were not considered for the analysis of differences in means.

All analyses were done in R (version 3.5.0; R Core Team, 2018) and were performed on logarithmically ($\log_{10}$) transformed data to achieve normality. Normality was confirmed using the Shapiro-Wilk test implemented in the 'stats' package and its multivariate extension to test for multivariate normality implemented in the 'mvnormtest' package (Jarek, 2012). Robust estimates of μ and S and robust Mahalanobis distances with additional adjustment for sample size according to Pison et al. (2002) were obtained using the

'covMcd' function of the 'robustbase' package (Maechler et al., 2018). Confidence ellipses (two-dimensional) and ellipsoids (three-dimensional) were calculated at a 0.975 confidence level based on the robust estimates of μ and S using the 'ellipse' function of the 'ellipse' package (Murdoch and Chow, 2018) and the 'ellipse3d' function of the 'rgl' package (Adler and Murdoch, 2018), respectively. Box's M tests were performed using a modified version of the according function from the 'biotools' package (da Silva et al., 2017) with the modification that the robust estimates of S were used as input instead of the raw data. Welch's ANOVA was carried out using the 'oneway.test' function ('stats' package); Games-Howell tests were performed using the 'userfriendlyscience' package (Peters et al., 2018). An example of the D-A-(C) analysis workflow in R to detect disturbances is provided as Supplementary Material.

3. Results

3.1. Using the D-A-(C) index to detect disturbances

3.1.1. Case study 1: organic contamination

For simplicity, and for reasons that will be discussed below, the analysis was based only on the variables cell density and activity to introduce how disturbances can be detected based on the identification of outliers. All contaminated samples were correctly identified as outliers by the multivariate approach using the D-A index, which exceeded the critical value of the chi-squared distribution for all contaminated samples (Fig. 3). In general, the contaminated samples showed higher activity levels as well as higher cell densities compared to the non-contaminated samples. However, if both variables were considered separately in univariate analyses, only 83% of the contaminated samples would have been correctly identified as outliers based on cell density; based on activity, the fraction would have been even lower with only 53%. Even if the outcomes of the two separate univariate analyses were to be considered together, still 17% of the contaminated samples would not have been detected as outliers. This is apparent from the bivariate plot where a group of contaminated samples clearly deviates from the distribution of non-contaminated samples indicated by the confidence ellipse, but still falls within the univariate thresholds for cell density and activity (Fig. 3). In fact, by looking at the bivariate plot it is becomes clear that, in principle, any sample that would lie to the top left or the bottom right of the confidence ellipse would not be detected as an outlier using the univariate approach if it still fell within the range defined by the univariate thresholds, even though it would clearly deviate from the distribution of the rest of the data along the two variables. This distribution is not only shaped by the variances of the variables but also their mutual covariance, which is ignored with the univariate approach. Thus, by making use of the additional information about covariance, the multivariate approach fully exploits the information gained from the two variables. Thereby it is able to provide a more accurate picture about the available data and accordingly allows for a more accurate identification of outliers.

To assess the impact of the fraction of contaminated samples in the dataset on the ability of the two approaches to detect those contaminated samples as outliers, we repeated the analysis from above with datasets containing different fractions of contaminated samples, ranging from 10 to 60%. For each fraction, we constructed 100 datasets with a total of 70 samples that were randomly selected from the non-contaminated site and the hydrocarbon-contaminated site, respectively. For each dataset, we calculated the average fraction of contaminated samples that were correctly identified as outliers using the univariate approach, where a sample was declared an outlier if it fell outside the median $\pm 2.5 \times$ MAD thresholds of either of the two variables, and the multivariate approach, respectively (Fig. 4). At a fraction of 10% of contaminated samples in the dataset both approaches were equally reliable, detecting >99% of the contaminated samples as outliers (Welch's t-test, $t_{99} = 1.42$, $p = 0.16$). However, already at 20% contaminated samples, the multivariate approach detected the contaminated samples significantly more reliably (98.5%) compared to the univariate approach (93.7%) (Welch's t-test, $t_{140.2} = 6.21$, $p \ll 0.0001$), and was able to correctly identify >96% of the contaminated samples as outliers up to a fraction of 30% contaminated samples in the dataset, above which also the multivariate approach started to noticeably lose its reliability.

3.1.2. Case study 2: surface water intrusion

As for the previous example, the analysis was based only on the variables cell density and activity. Out of the total of 108 samples, nine samples were identified as outliers using the multivariate approach as indicated by the D-A index for these nine samples which exceeded the critical value of the chi-squared distribution (Fig. 5). The presence of these outliers coincided with the period of the increased surface water intrusion and seemed to reflect the

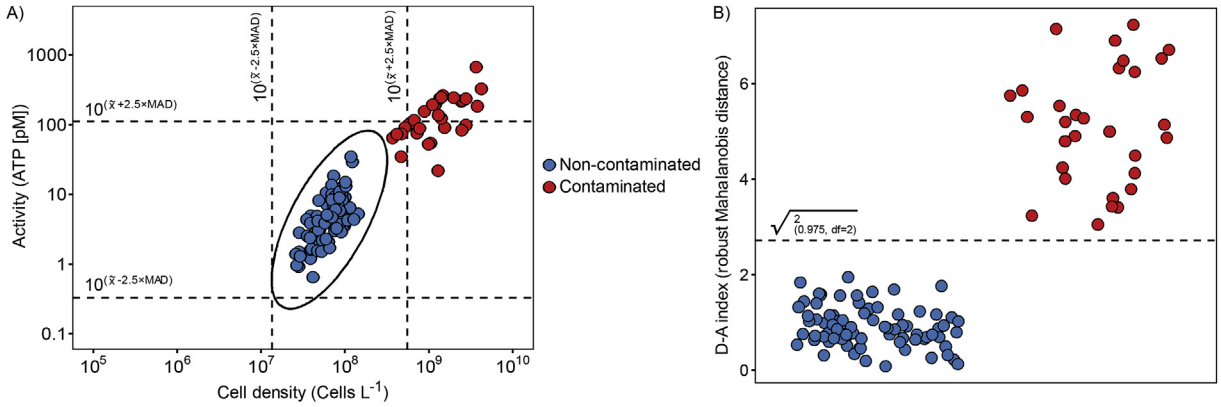

Fig. 3. (A) Bivariate plot showing the distribution of samples along the variables cell density (measured as prokaryotic Cells L^{-1}) and activity (measured as prokaryotic intracellular ATP concentrations) for the organic contamination data. Dashed lines represent the univariate thresholds calculated as median $\pm 2.5 \times$ MAD for each variable. The ellipse shows the 0.975 confidence ellipse calculated from the robust estimates of the center and covariance matrix of the data (note: all calculations were done on $\log_{10}$-transformed data; values were converted back to the original scale to allow for a more intuitive representation of the data). (B) D-A index values (robust Mahalanobis distances) for the samples shown in A. The dashed line represents the critical value of the chi-squared distribution at a 0.975 confidence level with two degrees of freedom.

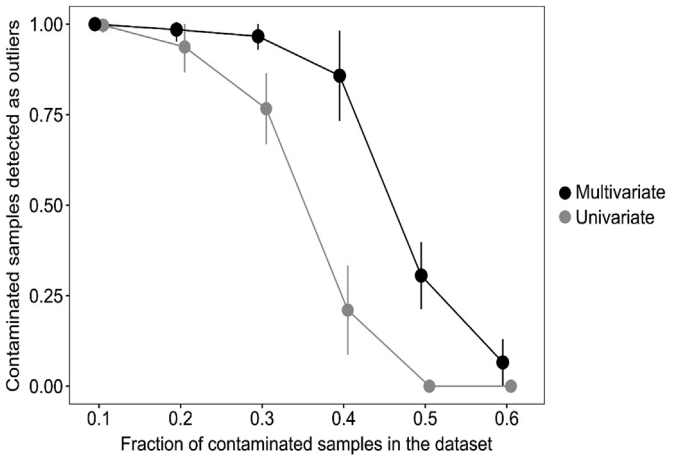

Fig. 4. Comparison of the ability to identify contaminated samples as outliers depending on the fraction of contaminated samples in the dataset between the multivariate approach using the D-A index (robust Mahalanobis distances) and the univariate approach based on separate thresholds of median $\pm 2.5 \times$ MAD for cell density and activity, respectively. Each data point shows the average fraction of contaminated samples correctly identified as outliers calculated from 100 randomly assembled datasets each containing 70 samples in total with different fractions of contaminated samples. Error bars show the standard deviation of the averages.

spatiotemporal impact of this event: in January, at the early stage of the event, the effect of the surface water intrusion was only apparent close to the river as indicated by the outlier from a well at a 2 m distance from the river. However, at the later stage in March, when the surface water had reached the areas more distant from the river, outliers were also detected 60 and 75 m away from the river. After the surface water had receded, the system appeared to have recovered from the disturbance. The only exception was a single well 60 m away from the river that was still identified as an outlier in April, June, and October, which, however, did not seem to be directly related to the surface water intrusion (Fig. S2).

Similar to the results for the first disturbance case study, the multivariate approach detected outliers more sensitively compared to the univariate approach. Only four samples that were identified as outliers in the multivariate analysis also fell outside the range defined by the median $\pm 2.5 \times$ MAD for cell density as well as activity if both were considered separately. If the information for both variables were to be combined, still one outlier would not have been detected which was detected by the multivariate approach.

3.1.3. Case study 3: impact of different types of land use

Considering only cell density and activity in the multivariate analysis, no significant outliers were detected for any of the three types of land use, although the D-A index value of a single sample from the agricultural land was close to the critical value of the chi-squared distribution (Fig. 6). However, if the AOC concentration was additionally included in the analysis, five outliers could be clearly identified based on the D-A-C index, three of which derived from the agricultural land, showing lower AOC concentrations than the majority of the samples, and two from the grassland, which showed higher AOC concentrations. Similar to the first two disturbance case studies, these outliers would not have been detected based on separate univariate analyses (except for one sample from the agricultural land, which fell below the lower threshold for activity; Fig. S3). The hydrochemical data revealed that the three outlier samples from the agricultural land displayed exceptionally high nitrate concentrations ($>100\ mg\ L^{-1}$) as well as unusually high dissolved oxygen concentrations for the region, and were among the samples with the lowest pH in the entire dataset (Fig. S4). For the two outliers from the grassland, the chemical data revealed unusually high concentrations of dissolved organic carbon, corresponding to the high AOC concentrations for these samples, as well as relatively low pH values (Fig. S4).

3.2. Regional differences in D-A-(C) signatures

For simplicity of display, the signatures of the nine different regions are shown in Fig. 7 only based on the two variables cell density and activity. The signature of each region is defined by the center (i.e. mean values of each variable) and the covariance matrix, which both are graphically captured by the confidence ellipse. Already based on visual comparison of the confidence ellipses, clear differences were apparent between each region and the full dataset as well as between the individual regions, both in terms of variable means and covariances. Significant differences in covariance matrices between regions were confirmed using Box's M test on the robust estimates of the covariance matrices ($\chi^2_{24} = 76.5$, $p \ll 0.0001$). For the comparisons of variable means, only samples were considered that were not indicated to be outliers for a given region based on their robust Mahalanobis distances at a confidence level of 0.975. As seen for the covariance matrices, significant

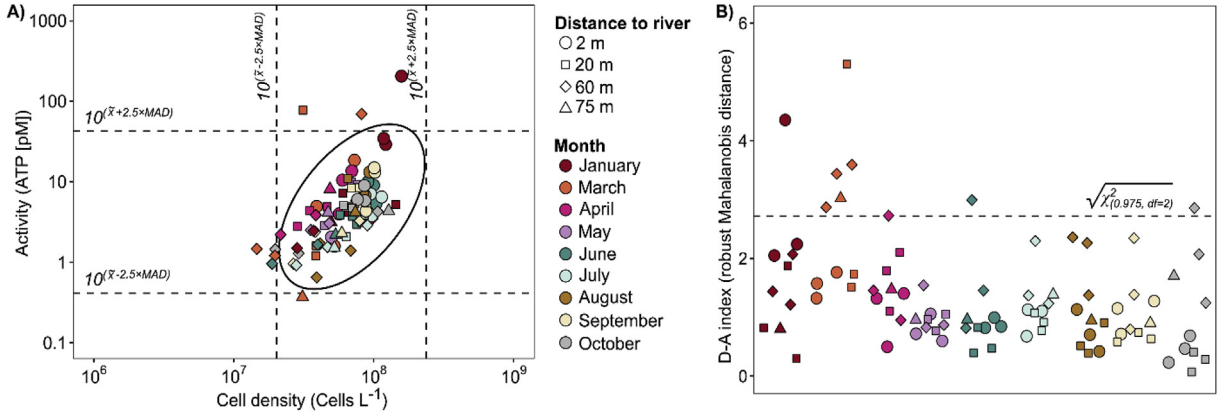

Fig. 5. (A) Bivariate plot showing the distribution of samples along the variables cell density (measured as prokaryotic Cells L^{-1}) and activity (measured as prokaryotic intracellular ATP concentrations) for the surface water intrusion data. Dashed lines represent the univariate thresholds calculated as median $\pm 2.5 \times$ MAD for each variable. The ellipse shows the 0.975 confidence ellipse calculated from the robust estimates of the center and covariance matrix of the data (note: all calculations were done on $\log_{10}$-transformed data; values were converted back to the original scale to allow for a more intuitive representation of the data). (B) D-A index values (robust Mahalanobis distances) for the samples shown in A. The dashed line represents the critical value of the chi-squared distribution at a 0.975 confidence level with two degrees of freedom.

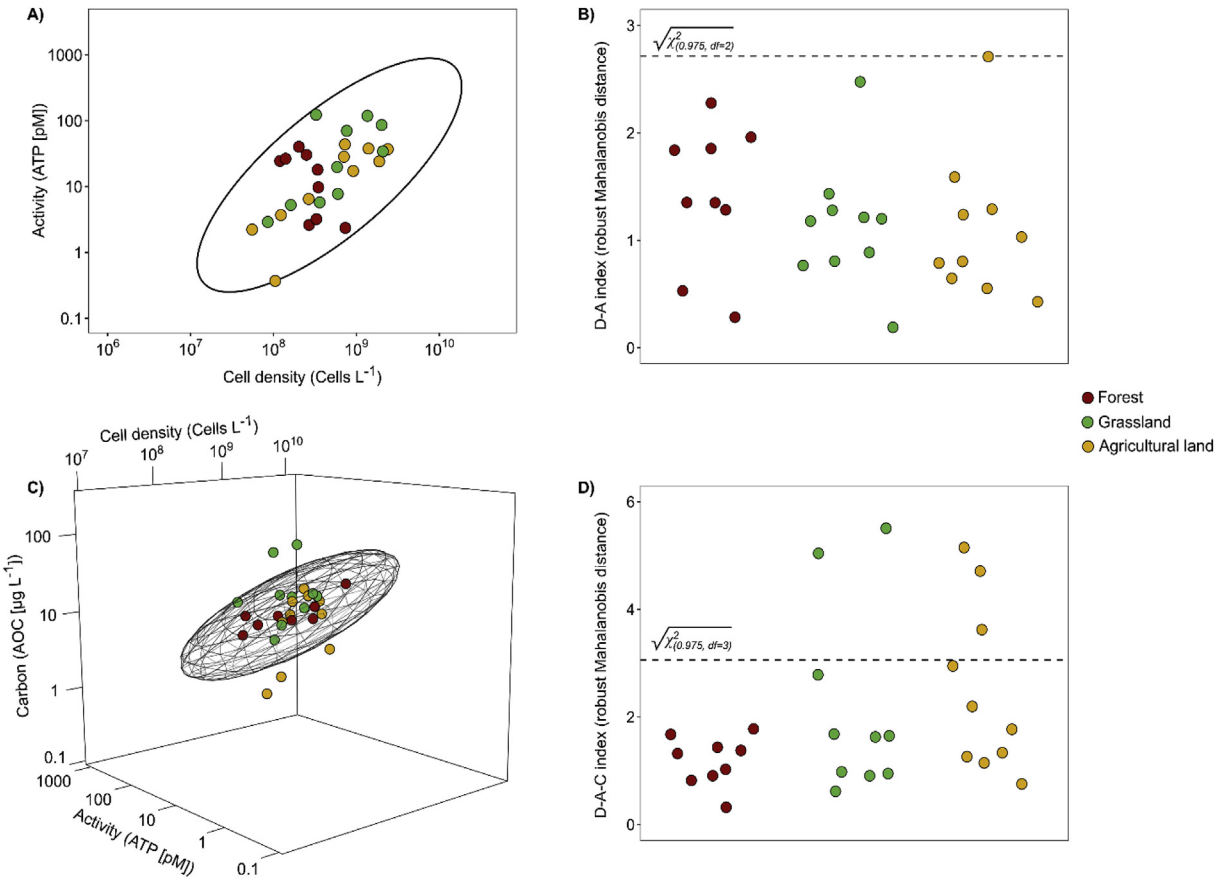

Fig. 6. (A) Distribution of samples along the variables cell density (measured as prokaryotic Cells L^{-1}) and activity (measured as prokaryotic intracellular ATP concentrations), and additionally carbon (measured as AOC concentrations) (C), for the different types of land use with 0.975 confidence ellipse (A)/ellipsoid (C) calculated from the robust estimates of the center and covariance matrix of the data. (B) and (D) D-A-(C) index values (robust Mahalanobis distances) of the samples shown in (A) and (C), respectively. Dashed lines represent the critical values of the chi-squared distribution at a 0.975 confidence level with two and three degrees of freedom in (B) and (D), respectively.

differences were also found for the means of cell density (Welch's ANOVA, $F_{8;\ 75.5} = 44.3$, $p \ll 0.0001$) and activity (Welch's ANOVA, $F_{8;\ 75.8} = 14.0$, $p \ll 0.0001$). Unfortunately, AOC concentrations were not available for all samples. We repeated the analyses only considering regions for which at least 10 samples were available with data for all three D-A-C variables (Table 1). The results were in agreement with the previous analysis showing significant differences between covariance matrices (Box's M, $\chi^2_{36} = 133.8$, $p \ll 0.0001$) and average AOC levels (Welch's ANOVA, $F_{6;\ 27.5} = 12.9$, $p \ll 0.0001$). Additional pairwise comparisons of means for each variable revealed that although the overall differences were significant, differences between pairs of regions were relatively small, mainly occurring within one order of magnitude for each variable (Fig. 8; summary statistics for each region are given in Table S1). Moreover, even though there were significant correlations between the D-A-C variables considering the full dataset (Pearson's $r_{\text{cell density-activity}} = 0.463$, $p \ll 0.0001$; $r_{\text{activity-carbon}} = -0.285$, $p = 0.008$; not significant: $r_{\text{cell density-carbon}} = 0.191$, $p = 0.08$), the pairwise comparisons further showed that these variables are not mutually redundant as significant differences between two regions in one variable did not necessarily coincide with significant differences in another variable for a given pair of regions.

4. Discussion

Despite the fact that policy makers in different parts of the world have started to acknowledge the ecosystem status of groundwater aquifers, and therefore no longer consider them as lifeless storages of freshwater, ecological aspects still receive little attention in groundwater monitoring to date. However, several advantages could be gained from the incorporation ecological parameters into routine groundwater monitoring. Not only would it allow for a better protection of unique groundwater ecosystems, it would also enable a more sustainable and informed ecosystem management, thereby helping maintain important ecosystem services such as the provision of clean freshwater (Griebler and Avramov, 2015; Griebler et al., 2014). Moreover, given that the indigenous organisms have adapted to the characteristically stable environmental conditions of groundwater aquifers, they can be expected to react sensitively to various disturbances, some of which might not be detected by conventional monitoring of physico-chemical parameters alone. Therefore, the sensitivity of routine groundwater monitoring could be increased by considering ecological parameters (Danielopol et al., 2007; Griebler and Avramov, 2015; Griebler et al., 2014). However, one of the main reasons for the currently still lacking implementation of ecological criteria is certainly the lack of suitable tools that can be easily incorporated into already existing routine monitoring practices. The integrated analysis using the D-A-(C) index presented here offers an easily implementable approach to meet this demand.

Previous studies have analyzed responses of prokaryotic microorganisms to different disturbances. For instance, increased microbial activity and growth have been reported in response to increased concentrations and fluxes of carbon caused by

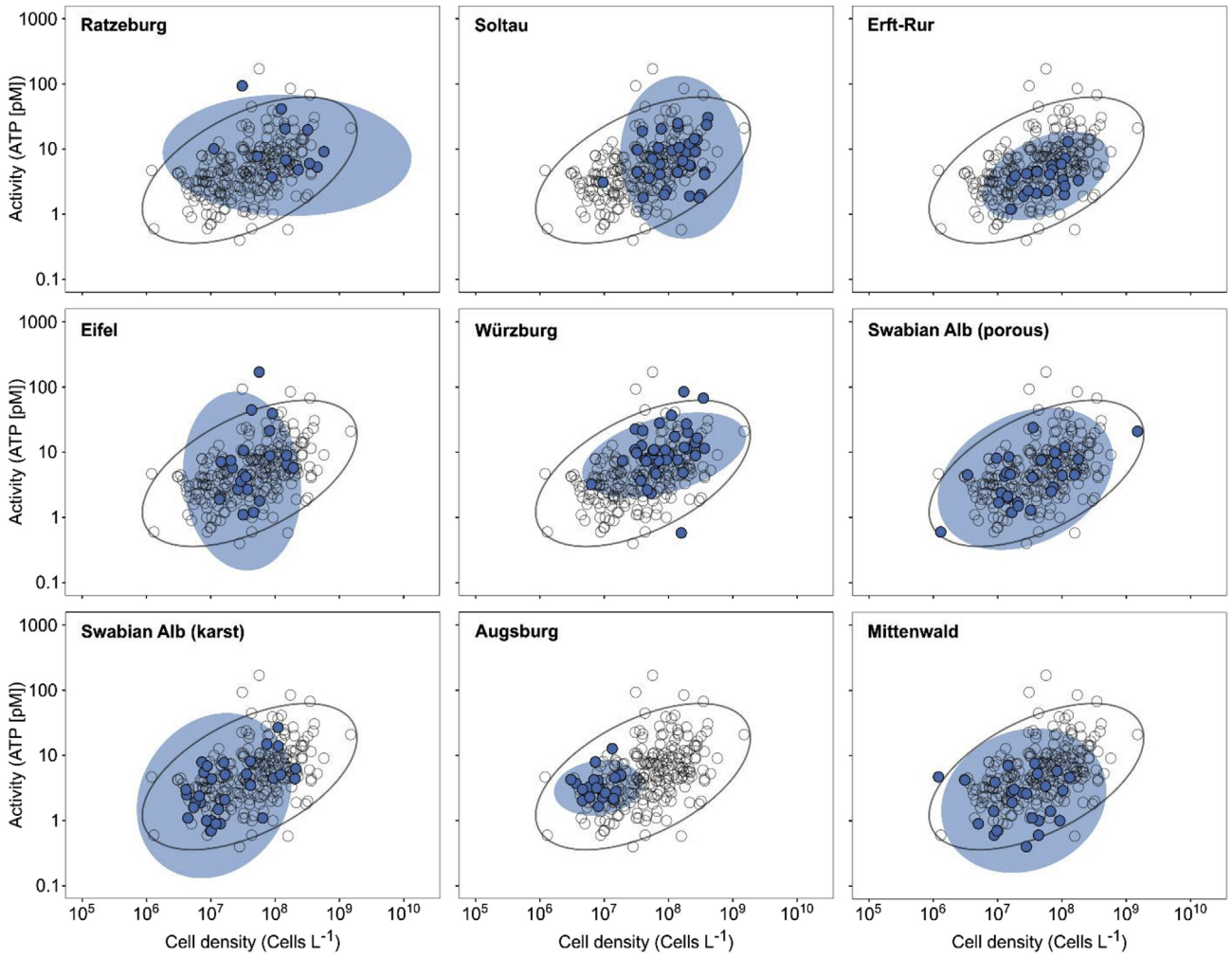

Fig. 7. Signatures along the variables cell density and activity of the nine non-contaminated aquifers at the regions indicated in the upper left corner of each plot. Open symbols represent the full dataset comprising all nine regions and the corresponding 0.975 confidence ellipse calculated from the robust estimates of the center and covariance matrix of the data; colored symbols represent data for an individual region (note: all calculations were done on $\log_{10}$-transformed data; values were converted back to the original scale to allow for a more intuitive representation of the data).

groundwater recharge with surface water (Foulquier et al., 2011; Mermillod-Blondin et al., 2013; van Driezum et al., 2018) or suddenly occurring contamination with aromatic hydrocarbons (Herzyk et al., 2017). These general trends are in line with our observations for the first two disturbance case studies. However, in contrast to the monitoring of drinking water distribution systems (FDHA, 2012; Van Nevel et al., 2017), most of the studies that have investigated these parameters in relation to different disturbances of groundwater ecosystems so far have mainly been descriptive and did not provide a practical framework of how these parameters could be integrated into monitoring protocols.

Our approach uses an integrated analysis of multiple microbiological variables to detect disturbances. Although it has been shown that for example microbial activity correlates positively with microbial cell numbers (Eydal and Pedersen, 2007; Foulquier et al., 2011; van der Wielen and van der Kooij, 2010; Van Nevel et al., 2017), a pattern that was also reflected in our data, these variables should not be regarded as mutually redundant. Our analyses showed that disturbances were not equally mirrored by these two variables, and furthermore, that significant differences between regions in one variable did not necessarily coincide with significant differences in another variable. It is crucial to realize that total prokaryotic cell counts are a measure of the total number of cells in a sample, while only the active fraction of these cells contributes to the activity measured as intracellular ATP concentrations. These two variables can be affected differently by different disturbances or over different time scales. For instance, it could be shown that microbial cell density can exhibit a delayed response to increased nutrient inputs (Foulquier et al., 2011), while intracellular ATP concentrations can react almost immediately (Hofmann and Griebler, 2018). Moreover, although such a pattern was not observed in our particular example of the surface water intrusion event, it is easy to imagine how surface water intrusion could cause an increase in microbial cell numbers without changes in activity. This would be the case if cells from the surface water were transported into the groundwater, but were inactivated by the characteristically lower nutrient levels in the groundwater compared to surface water. Hence, considering multiple variables can allow for a more nuanced approach to detect different types of disturbances.

We additionally demonstrated the advantages of a simultaneous, multivariate analysis of the considered microbiological variables compared to separate univariate analyses of each variable. The main advantage of the multivariate approach is that correlations between the variables are also taken into account, thereby capturing the actual distribution of samples along the measured variables, unlike univariate approaches where each variable is

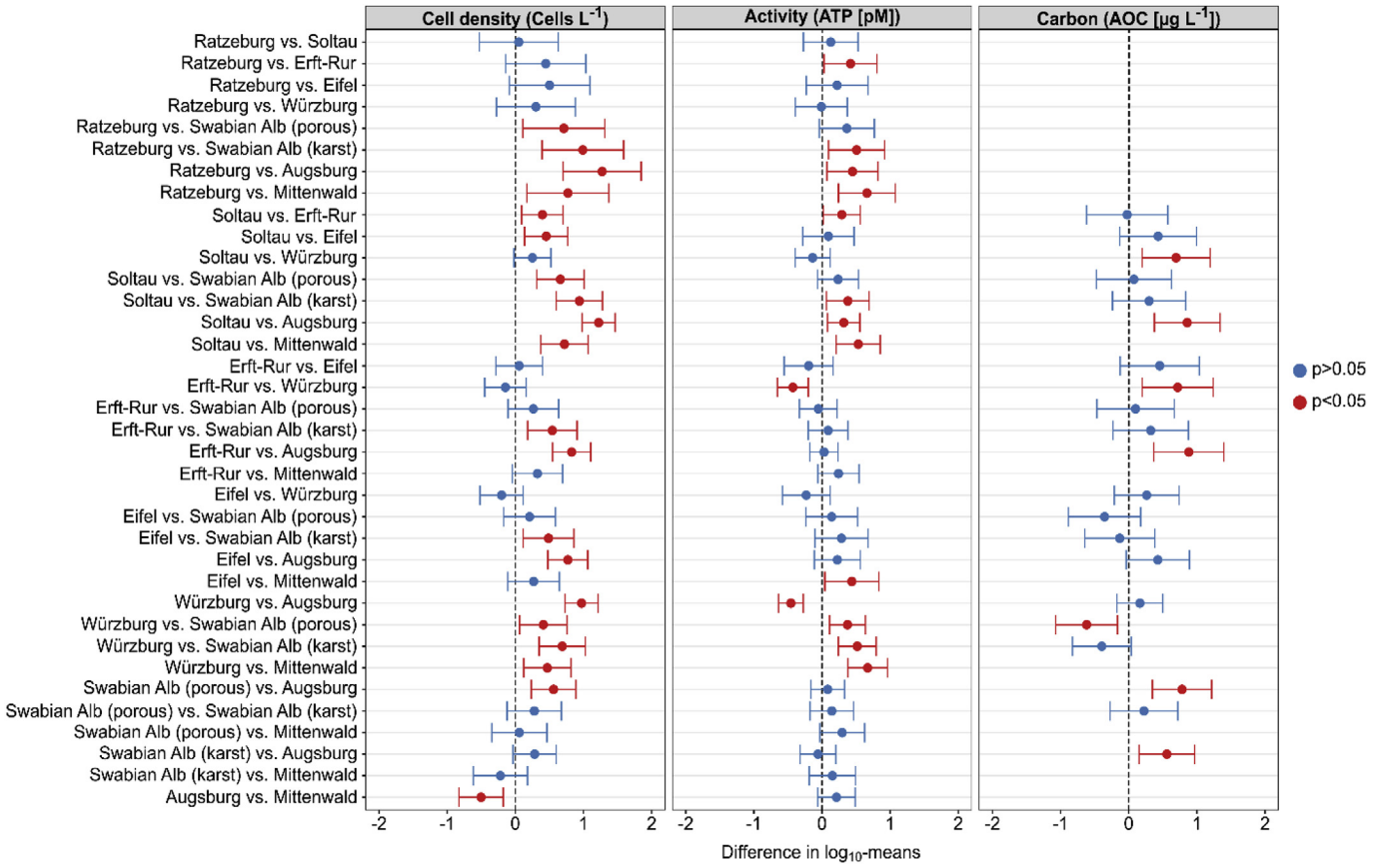

Fig. 8. Multiple pairwise comparisons using Games-Howell post-hoc tests for differences in each D-A-C variable between non-contaminated aquifers at different regions studied across Germany. Points show the size of the difference in $\log_{10}$-means between two regions (a difference of 1 shown here represents a difference of one order of magnitude on the original scale); error bars represent 0.95 confidence intervals of the differences. Red symbols indicate significant differences ($p < 0.05$) between two regions. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).

analyzed separately and thereby possible correlations are ignored (Manly, 1994). Our analyses showed that disturbances were not only detected more sensitively by the multivariate approach, but also that it was more robust by being able to detect disturbances more reliably in datasets with higher fractions of contaminated samples compared to the univariate approach. Moreover, using the D-A-(C) index (i.e. robust Mahalanobis distances) as indicator of disturbances offers an easy interpretation of the data based on a single value which integrates the signals from multiple variables. The power of this approach to detect anomalies (i.e. outliers) has already been successfully demonstrated in various contexts, ranging from finance to astronomy and biotechnology as well as quality monitoring of industrial manufacturing processes (Cohen Freue et al., 2007; Hubert et al., 2017; Rousseeuw and van Driessen, 1999), which allows for a seamless transfer of this approach into the context of ecological monitoring. Moreover, the approach can be easily extended by additional variables that may be identified in the future as sensitive indicators of disturbances without compromising the simplicity of the analysis, since the interpretation would still be based on a single index value regardless of the number of variables considered in the analysis. For example, additional information such as cell size distributions, numbers of phototrophic cells, ratios of live over dead cells, or ratios of cells with high nucleic acid content over cells with low nucleic acid content, can be directly derived from flow cytometry measurements used to determine cell densities (Hammes et al., 2011; Hammes and Egli, 2010; Van Nevel et al., 2017; Wang et al., 2010). This enables a straightforward amendment of the analysis which may further increase the sensitivity of detecting disturbances.

We have illustrated how the analysis can be extended, and its sensitivity can be increased, by including additional variables based on the example of the third disturbance case study related to the impacts of different types of land use. In this example, disturbances related to agricultural land use that were not identified by only looking at cell density and activity could be identified by additionally including AOC concentrations in the analysis. Although a higher sensitivity was achieved in this particular example by amending the analysis with AOC, there are certain pitfalls associated with this variable that need to be considered. It is important to remember that the AOC concentration is derived indirectly from the increase in cell numbers in a sample over a certain amount of time during batch incubation under *in situ* groundwater conditions (i.e. in the dark and the according temperature) (Hammes and Egli, 2005). However, from our own experience, this measure is only applicable under certain conditions. For instance, we have frequently noticed that the cell density decreases over the course of the incubation resulting in calculated negative AOC concentrations. This is mainly the case for surface water samples, where phototrophic cells die and decay during the incubation in the dark, or for groundwater samples that are strongly affected by surface water and may have contained phototrophic cells. Moreover, we can think of cases where the presence of toxic compounds like heavy metals can lead to growth inhibition (Şengör et al., 2009). In such a case, the cell density in a sample would not increase, or only increase slightly, during the incubation, which would be falsely interpreted

as low AOC concentrations. Similar situations would arise in cases where carbon is not the limiting resource, but the cell growth is constrained by the availability of other essential nutrients like nitrogen or phosphate (Hofmann and Griebler, 2018), or electron acceptors, as is often the case for organically contaminated groundwater (Meckenstock et al., 2015). In the light of these pitfalls, we recommend the use of AOC as an additional variable only for groundwater samples in absence of strong surface water impacts. Furthermore, protocols to measure AOC could be adjusted by amending samples with additional nitrogen and phosphate sources to overcome potential biases of AOC estimates caused by nutrient limitations (Trimbach et al., in prep.).

Certain shortcomings may also be encountered if only cell density and activity are considered. The disturbance case studies that we investigated to test our approach could all be roughly categorized as disturbances that affect organic carbon and nutrient levels in the groundwater. Even though we could clearly demonstrate that these disturbances can be detected by the multivariate analysis of prokaryotic cell density and activity, there are other potential impacts which might not be detected using this approach. One example could be groundwater contamination with micropollutants like pharmaceuticals or pesticides, which has become a growing concern over the past years (Fenner et al., 2013; Lapworth et al., 2012). Since these compounds typically occur in groundwater only in concentrations of a few nanograms to micrograms per liter, often much lower than ambient AOC concentrations, they usually do not serve as substrate for microbial growth (Helbling, 2015) and, from a critical point of view, might not be expected to have toxic effects on microorganisms that would cause noticeable changes in intracellular ATP concentrations. Whether impacts caused by micropollutants can be detected at all based on microbiological parameters needs to be assessed in future research. However, in general, certain other drawbacks of our approach could be alleviated by incorporating additional variables in the analysis as discussed above.

Looking at the regional D-A-(C) signatures of non-contaminated aquifers across Germany, we noticed significant differences in terms of average levels of prokaryotic cell density, activity, and AOC, respectively, as well as covariance matrices. A detailed discussion of differences between individual regions would exceed the scope of this paper. However, the main implication of these findings for groundwater monitoring is that these differences between regions need to be taken into account for data collection and interpretation by analyzing data on a suitable regional scale. Furthermore, our analysis revealed that disturbances can only be reliably detected up to a certain fraction of disturbed samples in the dataset. Therefore, in practice, a number of known reference monitoring wells should be routinely included in the analysis that are well protected against disturbances and provide a representative estimate of D-A-(C) signatures for a given region. A step towards the biogeographical classification of groundwater ecosystems has been made by Stein et al. (2012), who showed that the distribution of metazoan groundwater fauna across Germany does not agree with conventional classifications based on hydrogeology, geochemistry, or bioregions defined based on surface water fauna. In the light of these findings, we suggest that also the definition of suitable biogeographical boundaries for microbiological parameters should be guided by ecological criteria rather than being solely based on hydrogeology or geochemistry.

5. Conclusion

We have shown that prokaryotic cell density and activity can be used as reliable and sensitive indicators of disturbances of groundwater ecosystems. These parameters can be easily integrated into existing groundwater monitoring schemes as they are simple and inexpensive to measure, and furthermore can be analyzed in samples that can be directly collected along with samples for the routine monitoring of conventional physicochemical parameters. Furthermore, we have demonstrated that the multivariate analysis of these variables using the D-A-(C) index not only allows for a higher sensitivity, but also increases the robustness of the analysis compared to separate univariate analyses of the individual variables. An even higher sensitivity might be achieved by including additional parameters in the analysis such as AOC concentrations under certain conditions. Moreover, the presented approach can be seamlessly adapted to detect various types of disturbances by including additional parameters in the future, while still allowing for an easy data interpretation based on a single index value (i.e. robust Mahalanobis distances) that integrates the signals of all variables included in the analysis. Significant differences in the multivariate D-A-(C) signatures of the microbial variables between regions stress the importance of analyzing data and selecting reference wells at a suitable regional scale, the definition of which should not only be guided by hydrogeology or geochemistry, but primarily by ecological criteria.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.watres.2019.114902.

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Supplementary material

The D-A-(C) index: a practical approach towards the microbiological-ecological monitoring of groundwater ecosystems

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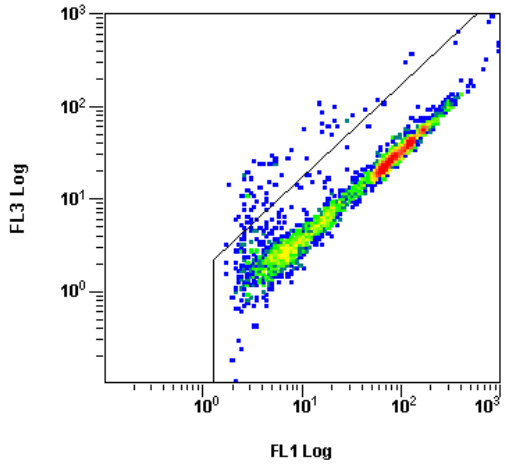

Figure S1: Representative example of a flow cytometry dot plot of red fluorescence (FL3) versus green fluorescence (FL1) used in this study. Each dot represents a single event; events within the gate (to the right of the solid line) were counted as prokaryotic cells. The color gradient indicates the density of events (blue=low number of events; red=high number of events).

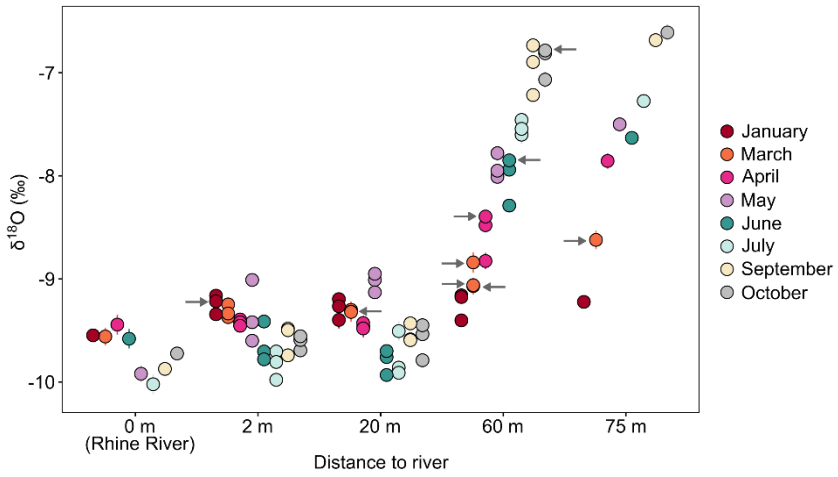

Figure S2: Stable oxygen isotope ratios at different time points for the surface water intrusion data (disturbance case study 2, see main text) measured in the Rhine River and groundwater monitoring wells located at different distances to the river. Grey arrows indicate samples identified as outliers based on the D-A index (see main text). No corresponding isotope data was available for data presented in the main text for August.

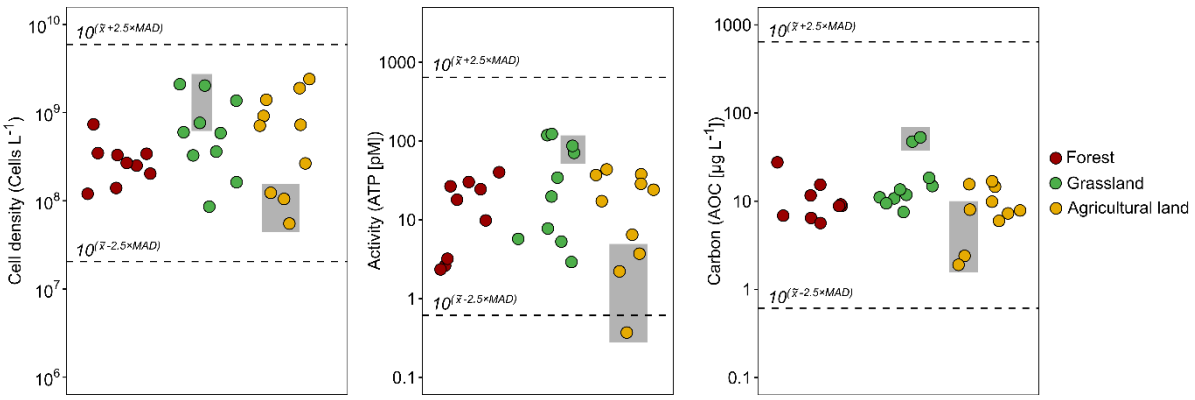

Figure S3: Separate univariate analyses of cell density (measured as prokaryotic Cells L^{-1}), activity (measured as prokaryotic intracellular ATP concentrations), and carbon (measured as AOC concentrations) for different types of land use (disturbance case study 3, see main text). Dashed lines represent the univariate thresholds calculated as median ± 2.5×MAD for each variable. Samples identified as outliers based on the D-A-C index are highlighted with grey rectangles (see main text; note: all calculations were done on $\log_{10}$-transformed data; values were converted back to the original scale to allow for a more intuitive representation of the data).

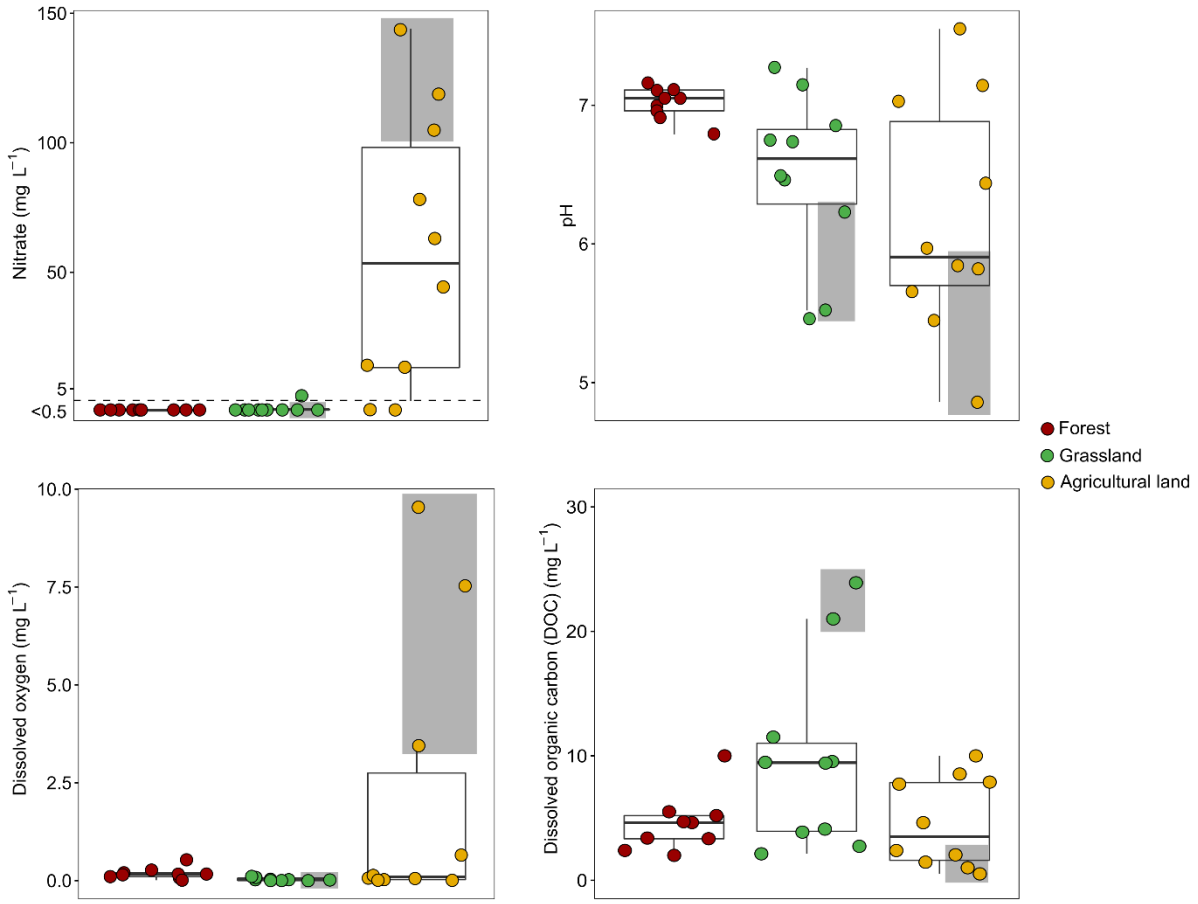

Figure S4: Boxplots of hydrochemical variables for different types of land use (disturbance case study 3, see main text). Samples identified as outliers based on the D-A-C index are highlighted with grey rectangles (see main text). For nitrate, the majority of samples exhibited concentrations below the detection limit (0.5 mg L^{-1}) represented by the dashed line.

Numerical example of the calculation of Mahalanobis distances

The analysis shown in Figure S5 is a fictive example of a dataset containing 20 undisturbed samples and a single disturbed sample. For simplicity, the example only deals with two variables representing $\log_{10}$-transformed prokaryotic cell density (D) and activity (A) similar to the data shown in the main text.

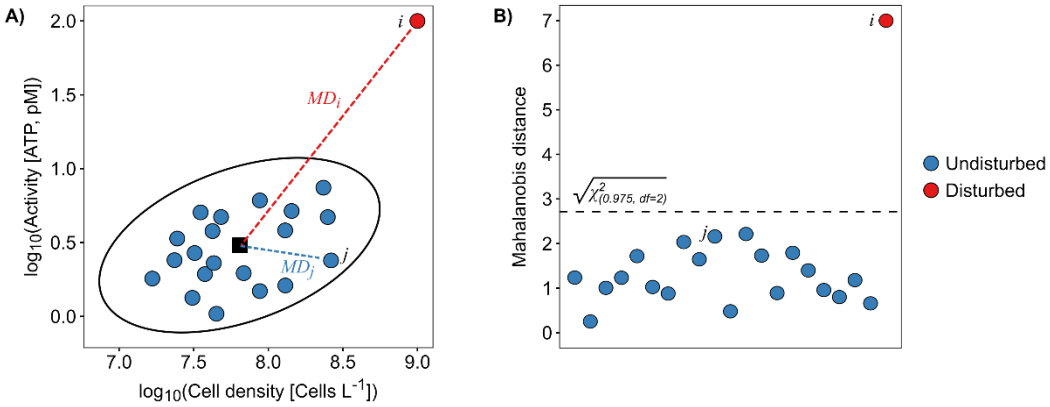

Figure S5: (A) Distribution of samples along the variables cell density and activity. The center defined by the variable means is represented by the black square. The distances to the center for samples *i* and *j* are indicated by the dashed lines. (B) Mahalanobis distances calculated for the samples shown in (A). The dashed line represents the critical value of the chi-squared distribution at a 0.975 confidence level with two degrees of freedom.

Cell density and activity have mean values of 7.81 and 0.48 and variances of 0.12 and 0.05, respectively. The covariance between cell density and activity is 0.03. Hence, the vector μ defining the center of the group of samples and the covariance matrix S are as follows:

$$\mu = \begin{bmatrix} \bar{D} \\ \bar{A} \end{bmatrix} = \begin{bmatrix} 7.81 \\ 0.48 \end{bmatrix}$$

$$S = \begin{bmatrix} var(D) & cov(D,A) \\ cov(D,A) & var(A) \end{bmatrix} = \begin{bmatrix} 0.12 & 0.03 \\ 0.03 & 0.05 \end{bmatrix}.$$

Thus, the inverse of S is:

$$S^{-1} = \begin{bmatrix} 10.08 & -6.64 \\ -6.64 & 25.47 \end{bmatrix}.$$

The values of cell density and activity for the disturbed sample *i* are 9.00 and 2.00, respectively. Hence the vector X_i giving the coordinates for that sample in the bivariate plot is:

$$X_i = \begin{bmatrix} D_i \\ A_i \end{bmatrix} = \begin{bmatrix} 9.00 \\ 2.00 \end{bmatrix}.$$

Based on this the Mahalanobis distance for sample *i* is calculated as:

$$MD_i = \sqrt{(X_i - \mu)' \times S^{-1} \times (X_i - \mu)}$$

$$= \sqrt{[(9.00 - 7.81) \quad (2.00 - 0.48)] \times \begin{bmatrix} 10.08 & -6.64 \\ -6.64 & 25.47 \end{bmatrix} \times \begin{bmatrix} (9.00 - 7.81) \\ (2.00 - 0.48) \end{bmatrix}}$$

$$= 7.01.$$

Because the analysis comprised two variables, the critical value derived from the chi-squared distribution with two degrees of freedom at a 0.975 confidence level is:

$$\sqrt{\chi^2_{(0.975,\ df=2)}} = 2.72.$$

Since the Mahalanobis distance of sample *i* exceeds this critical value, that sample is considered an outlier. For comparison, doing the same calculation for the undisturbed sample *j* with

$$X_j = \begin{bmatrix} D_j \\ A_j \end{bmatrix} = \begin{bmatrix} 8.42 \\ 0.38 \end{bmatrix}$$

gives

$$MD_j = 2.19,$$

which is smaller than the critical value of the chi-squared distribution and therefore sample *j* is not considered an outlier at a 0.975 confidence level.

Table S1: Summary statistics of prokaryotic cell density, activity, and AOC concentration for non-contaminated aquifers sampled at the different regions across Germany (see Fig. 1, main text) given as geometric mean, minimum, maximum, and geometric standard deviation for each variable (all calculations were performed on $\log_{10}$-transformed data and values were converted back to the original scale). Only samples were considered that were not identified as outliers for the individual regions (or within the full dataset) based on the D-A-(C) index (see main text).

Region	*n*[a]	*Cell density (Cells L^{-1})*		*Activity (ATP [pM])*		*Carbon (AOC [µg L^{-}]^{1})*	
		Mean (Min.; Max.) ×10^{8}	*SD*	*Mean (Min.; Max.)*	*SD*	*Mean (Min.; Max.)*	*SD*
Ratzeburg	11	1.51 (0.11; 5.75)	3.07	9.31 (3.70; 41.40)	2.08	NA	NA
Soltau	32 (11)	1.34 (0.33; 4.09)	2.17	6.93 (1.80; 30.60)	2.33	12.97 (2.80; 63.50)	2.63
Erft-Rur	20 (7)	0.54 (0.16; 1.80)	2.08	3.56 (1.20; 13.00)	1.72	13.69 (5.80; 54.30)	2.12
Eifel	21 (7)	0.47 (0.14; 1.92)	2.23	5.59 (1.10; 44.70)	2.70	4.81 (2.40; 13.40)	1.96
Würzburg	37 (27)	0.75 (0.06; 3.62)	2.32	9.52 (2.34; 37.02)	1.90	2.62 (0.36; 10.67)	2.75
Swabian Alb (porous)	25 (10)	0.29 (0.03; 1.80)	2.77	4.03 (1.20; 24.00)	2.16	10.89 (2.50; 35.00)	2.23
Swabian Alb (karst)	28 (13)	0.15 (0.04; 1.22)	2.84	2.90 (0.70; 15.00)	2.42	6.52 (1.70; 26.50)	2.40
Augsburg	22 (11)	0.08 (0.03; 0.18)	1.62	3.33 (1.66; 7.98)	1.43	1.80 (0.79; 3.39)	1.66
Mittenwald	24	0.26 (0.03; 1.32)	2.69	2.04 (0.40; 7.60)	2.35	NA	NA
Total dataset	225 (116)	0.41 (0.01; 15.00)	3.61	4.73 (0.60; 41.40)	2.44	5.73 (0.18; 202.60)	3.38

[a] AOC data were not available for all samples; the value within parentheses indicates the number of samples for which AOC data were available.

3. Conclusions and outlook

In this thesis, I set out to provide a better understanding of individual processes involved in the attenuation of bacteriophage MS2, a surrogate for pathogenic viruses, during bank filtration. Moreover, the attenuation efficiencies of bank filtration for different pathogens and fecal indicators are investigated. And an ecological monitoring tool based on simple microbial parameters is developed to meet the demand of scientific water management.

In the first section, we identified attenuation processes involved in the transport and reduction of pathogenic viruses during underground passage including adsorption, inactivation, and decay. Among these processes, inactivation turned out to be the most important. Upon comparison with a sterile incubation pre-experiment, key factor controlling inactivation and decay in our first field experiment was mainly attributed to the activity of autochthonous microorganisms. In addition, adsorption was an important attenuation process albeit only a small portion of MS2 adsorbed on the sediment/matrix in our experiment. The comparably low adsorption of MS2 to the sediment was explained by many factors, such as the low adsorption capacity of MS2; high flow velocity applied during the experiments; and the assumed aggregation of phages due to the high concentration. We found that the high initial viral load directly affected the attenuation efficiency. We hypothesized that this phenomenon occurs due to lower relative mass transfer to the solid phase and the enhanced aggregation at high initial viral load. Unfortunately, in our pre-experiment where we applied a moderate viral load, we did not monitor the variation of total MS2 in pore-water during underground passage nor the adsorbed fraction of total and infectious MS2. Thus, we cannot distinguish which attenuation process was mainly influenced by the difference in viral load. Another blind spot of this study was the aggregation of viruses. Aggregation may play a key role in influencing adsorption, especially at a high initial viral load. We assumed that aggregation rarely occurs in the natural environment. Therefore, we recommend for future studies on virus transport through bank sediments to consider aggregation of viruses. When dissecting the individual attenuation processes during virus transport, total as well as infectious phages need to be followed in numbers not only in pore water but also adsorbed onto the sediment matrix.

Furthermore, we found that hydrological extremes pose a risk to bank filtration efficiency, especially in cases where the bank sediments were contaminated with viruses before. We also

discovered that viruses adsorbed to the sediment are sheltered and keep their infectivity. Thus, in case of heavy rains and the recharge of water with a low ionic strength, infectious viruses are desorbed from the sediment. In consequence, there is a risk of a second viral contamination pulse in the aftermath of a storm. In our study, we simulated a simple rainfall using deionized water. However, different rainwater qualities and amounts may have different effects on the mobilization of adsorbed viruses. For example, acid rain may still plague a few countries until the last century. Facing the serious topic of climate change, we have to do our best to protect the environment on one hand and to develop science-based solutions to meet the worst-case scenarios on the other hand. I think it is necessary to further investigate the impact of different types of hydrological extremes and different water qualities, such as acid rain, on virus transport through bank sediments in the near future.

In the second part of my thesis work, first, we tried to find out if there are suitable indicators for virus attenuation during riverbank filtration, especially during periods of varying hydrological conditions. The adequacy of fecal bacteria for indicating the presence and concentration of human viruses has been questioned in recent years (Albinana-Gimenez *et al.*, 2009; Formiga-Cruz et al, 2003). The main difference in the attenuation processes of bacteria and viruses during underground transport is that viruses, which are much smaller in size, are less subject to straining in sandy aquifer sediments and soils. With coliphages, despite having the same size as pathogenic viruses (e.g., adenoviruses, enteroviruses), the adsorption and persistence also vary depending on particle specificity, i.e., their surface charge and hydrophobicity. The same is true for other viruses. Thus, coliphages alone may not be a sufficient indicator for the overall virus transport during bank filtration. In our study, we found that adenovirus abundances correlated with total prokaryotic cell counts (TCC), total viral-like particle counts (TVPC), *E. coli*, and somatic coliphages at high river levels, while adenovirus numbers correlated with microbial activity, coliforms, and somatic coliphages at medium river levels and microbial activity, TVPC, and coliforms at low river levels. Thus, compared with either fecal bacteria or coliphages alone, the combination of *E. coli*, coliforms, and somatic phage could best predict adenovirus transport during riverbank filtration, even under different river levels. The occurrence of pathogenic viruses in raw drinking water is a serious human health concern, and due to the limitations of current quantitative detection methods and the extremely low concentration of pathogenic viruses in natural water, we desperately search for indicators to replace them. In a future study, a pre-concentration of >100 L of raw drinking

water should be done to achieve a lower limit of detection for the pathogenic viruses. In addition, vegetable viruses, such as pepper mild mottle virus, could also be a new candidate to be explored as indicator for the occurrence of different pathogenic viruses. The advantage of using vegetable viruses as an agent of pathogenic viruses over fecal bacteria and coliphages is that they are not only of the same size as pathogenic viruses, but also excreted in considerable concentrations by human beings.

In addition, we investigated the influence of varying hydrological conditions on the attenuation of pathogenic viruses, fecal and viral indicators during riverbank filtration. Generally, the biological indicators were attenuated most effective in terms of log reduction during high river levels rather than at low and medium river levels. This can be explained mainly by the high abundance of respective agents in source surface water during high water levels. On the other hand, we observed that at high river levels a full sediment passage (i.e., from the river to the production well) was needed to fully attenuate the various biological indicators, while at low and medium river levels, the initial sediment passage (i.e., from the river to wells A) was sufficient. A previous study conducting investigations at the same riverbank filtration site provided evidence that high coliform and coliphage numbers in surface water can pose a serious threat to water at the production well at high river levels, because the capacity of riverbank filtration was exceeded (Rohns *et al.*, 2006). At high river levels water at the production well was composed exclusively of surface water. Moreover, the adsorption of particles was decreased by a shortened travel time, lower pore water ionic strength, and elevated pore water dissolved organic carbon concentration. Compared with high river levels, the low river levels posed a rare risk to the efficiency of riverbank filtration in our study, except for the occasional occurrence of coliforms and *C. perfringens* at the production well. The potential threat of low river levels to the efficiency of riverbank filtration mentioned in the previous studies was mainly because of clogging including excessive biomass accumulation, precipitation of Mn^{4+} or Fe^{3+} after consumption of DO and denitrification, and sedimentation of accumulated suspended particles (Diem *et al.*, 2013; Engesgaard *et al.*, 2006; Sprenger *et al.*, 2011). In our study we did not observe signs of denitrification although the concentration of dissolved oxygen dropped to 0 many times in the riverbank filtrate during the summer drought in 2018. Viruses and bacteria proved to be less inactivated and adsorbed under anoxic conditions (Klitzke *et al.*, 2015; Frohnert *et al.*, 2014). In summary, although the performance of riverbank filtration appeared to be sufficient in our study with the different river levels, studies on the attenuation efficiency

of riverbank filtration for pathogenic viruses are still needed to cover hydrological extremes, such as very high water levels and extended flood situation as well as long-term droughts.

In the third section of this thesis, a suitable and applicable method for the ecologically monitoring of groundwater microbiological-ecological status based on prokaryotic cell density, microbial activity, and/or bioavailable carbon is introduced. This assay allows detecting the changes in water quality of groundwater via different types of disturbances. Testing the newly developed assessment tool at different locations and situations demonstrated that the prokaryotic cell density and the microbial activity based on ATP concentration are robust indicators, while the assimilable organic carbon (AOC) is only applicable as an indicator under certain conditions. The AOC measurement requires an incubation of the original water sample for 14-30 days in the dark. Because surface water also contains phototrophic cells that die and decay during dark incubation, new dissolved carbon is produced biasing the exact qualification of heterotrophic concentration of dissolved organic carbon during incubation. In consequence, while AOC is a reliable indicator for the energy available to prokaryotes in groundwater, it needs to be applied with cautions in surface waters or the groundwater intruded strongly by surface water. Moreover, the presence of toxic compounds such as heavy metals can act as a growth inhibitor, thus leading to erroneous measurements of bioavailable organic carbon using the proposed batch incubation growth assay.

To better explore possible causes of disturbances and to determine the natural state of groundwaters within each sub-region, we recommend replacing the AOC measurement in this routine monitoring toolbox with one or more parameters that are more robust in the future. Here, we introduce fluorescence spectroscopy of dissolved organic matter (DOM), one simple and inexpensive measurement, to be involved in the future development of ecological monitoring tool (Retter *et al.*, 2021). Fluorescence spectroscopy of DOM has already been applied to examine shifts in DOM composition in surface water systems with varying environmental conditions, and used as an indicator to study the fecal contamination in groundwater and drinking water system (Benk *et al.*, 2019; Harjung *et al.*, 2020; Nowicki *et al.*, 2019; Sorensen *et al.*, 2020). We focus on two indices that characterize well the fluorescent DOM in groundwater, i.e., HIX (humification index) and BIX (freshness index). Because groundwater environment typically contains low nutrient and low biological productivity, most of the DOM in groundwater is usually composed of recalcitrant humic compounds from the surface (i.e.,

HIX), while the contribution of DOM produced freshly by microorganisms is relatively small (i.e., BIX), (Shen *et al.*, 2015; Hofmann *et al.*, 2020). Furthermore, considering disturbances such as intensive agriculture or heavy rain events, the increased input of organic matter from the surface may directly cause changes in groundwater DOM or indirectly stimulate/inhibit groundwater biological productivity.

In summary, the results presented in this thesis contribute to a better understanding of the transport of allochthonous viruses during bank filtration and the effect of different hydrological events on the attenuation efficacy of bank filtration. Furthermore, a practical tool was introduced to assess the ecological groundwater status which meets the urgent need of modern environmental laws in Europe and worldwide.

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