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Removal of plant pathogenic bacteria during agricultural managed aquifer recharge to produce irrigation water and protect plant health

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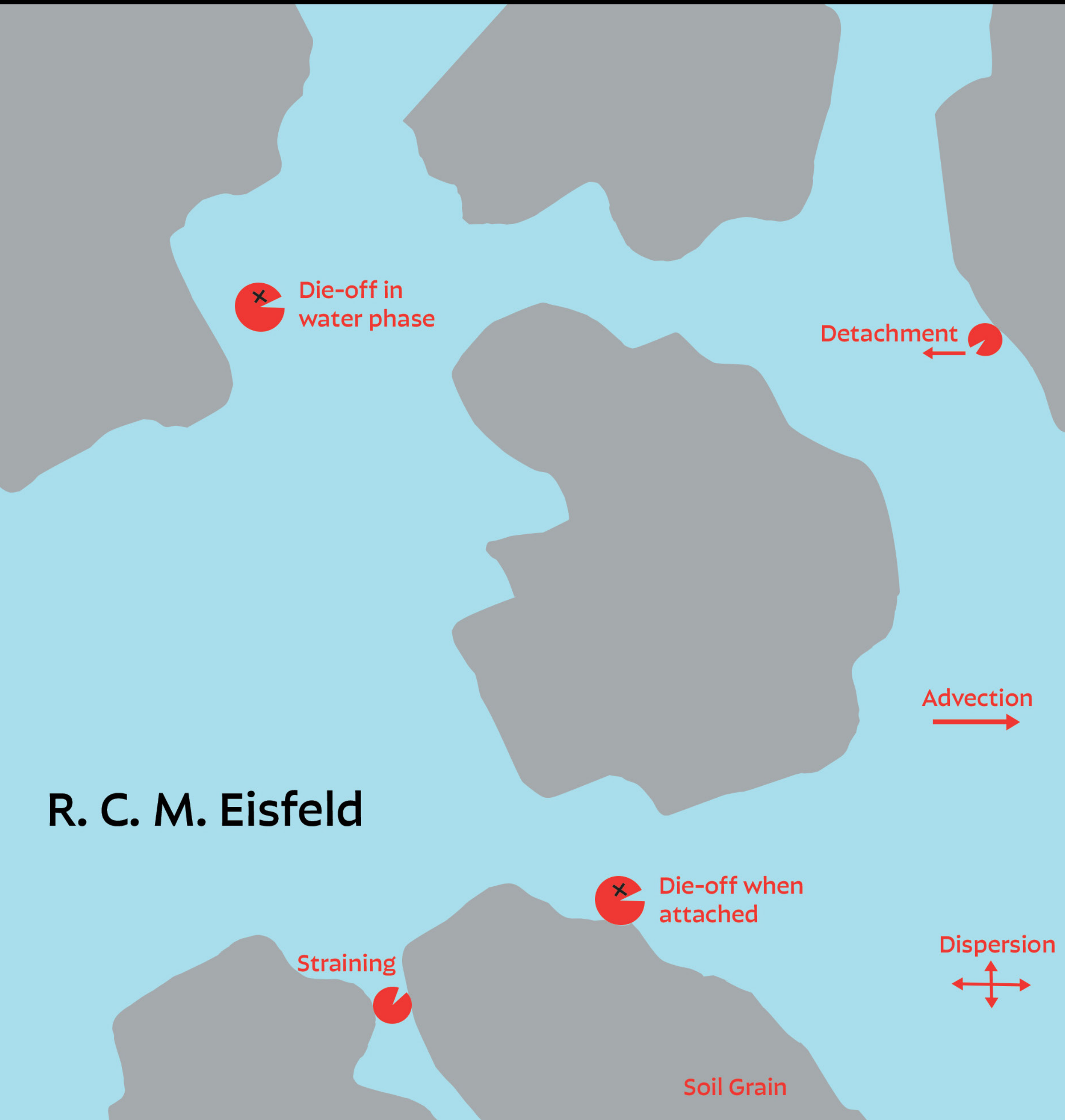
Removal of Plant Pathogenic Bacteria During Agricultural *Managed Aquifer Recharge* to Produce Irrigation Water and Protect Plant Health



R. C. M. Eisfeld

Propositions accompanying the dissertation

Removal of plant pathogenic bacteria during agricultural managed aquifer recharge to produce irrigation water and protect plant health

by

Carina Eisfeld

1. Water is not always where you look for it – intentional aquifer storage has great potential to fight water scarcity while improving water quality (this thesis).
2. Agricultural aquifer storage transfer and recovery (ASTR) removes bacterial plant pathogens satisfactorily to provide irrigation water while protecting plant health (this thesis).
3. Soil passage, the natural treatment during ASTR, is more relevant for bacterial plant pathogen removal than die-off in the water phase (this thesis).
4. The dose makes the poison. High concentrations of *Ralstonia solanacearum* are required to cause visual disease symptoms of bacterial wilt in potato plants while already low concentrations can cause latent infections (this thesis).
5. It is shocking how much science and knowledge is out there about climate change and how little society does to fight it.
6. Preventing surface and groundwater contamination, and improving water quality of polluted waters help to reduce water scarcity.
7. Intensive livestock farming consumes and pollutes enormous amounts of fresh water. Therefore, a shift to a plant-based diet can protect water resources.
8. Interdisciplinarity and collaboration are necessary to solve the world's water problems including water scarcity, floods or water quality issues.
9. Corona times showed that home office is possible and that online meetings facilitate and can improve collaboration.
10. A four day work week increases motivation and keeps an actual healthy work-life balance.
11. Focus on your own progress and growth, rather than comparing yourself to others.

These propositions are regarded as opposable and defendable, and have been approved as such by the promotors.

Removal of plant pathogenic bacteria during agricultural managed aquifer recharge to produce irrigation water and protect plant health

Dissertation

for the purpose of obtaining the degree of doctor
at Delft University of Technology
by the authority of the Rector Magnificus,
Prof.dr.ir. T.H.J.J. van der Hagen,
Chair of the Board for Doctorates
to be defended publicly on

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List of abbreviations

°C	degree Celsius
aGW	artificial groundwater
AIC	Akaike Information Criterion
ASR	aquifer storage and recovery
ASTR	aquifer storage transfer and recovery
b.s.l.	below surface level
BTC	breakthrough curve
CFU	colony forming unit
DL-CVP	double layer crystal violet pectate
DNA	deoxyribonucleic acid
EC	electrical conductivity
EtOH	ethanol
g	gram
ha	hectare
MAR	managed aquifer recharge
mL	millilitre
OD	optical density
pH	power of hydrogen
QMRA	quantitative microbial risk assessment
REV	representative elementary volume
SDG	sustainable development goals
SMSA	semi-selective medium South Africa
SRP	soft rot *Pectobacteriaceae*
TDW	tile drainage water
VBNC	viable but non culturable

Summary

Fresh water is an essential resource for life. Yet, overexploitation of fresh water resources and the decrease of water quality by pollution increase water scarcity worldwide. Furthermore, extreme weather events like droughts and floods will happen more often as consequences of climate change. This further intensifies water scarcity but pushes scientists and practitioners to propose innovative and collaborative solutions. Agricultural production consumes about 70% of the available fresh water resources. As consequence, it is particularly vulnerable for water scarcity as it depends on irrigation to ensure food security. Therefore, this research investigated managed aquifer recharge (MAR) as a nature-based solution to make water available for agriculture. However, one major aspect is the quality of the recovered water used for irrigation. If plant pathogens are present in irrigation water they can cause plant diseases and high crop losses. A MAR designed as an aquifer storage transfer and recovery (ASTR) system has great potential to improve water quality during the recharge process using the natural subsurface treatment. Previous research showed that ASTR can successfully reduce the concentrations of human pathogens in the reclaimed water and thereby risks for human health. However, literature on the removal of plant pathogens in the environment and specifically under aquifer conditions are lacking. An understanding about pathogen die-off and removal under relevant conditions found in aquifers (i.e., natural water-bearing layers in the subsurface) is required to predict changes in water quality and protect plant health.

Hence, for the first time, an agricultural ASTR system was analysed for its potential to remove plant pathogenic bacteria to provide safe irrigation water. In particular, an ASTR pilot site located in North Holland was investigated where tile drainage water (TDW) is collected from a 10-hectare agricultural field and infiltrated into a sandy, anoxic, and originally brackish aquifer at about 10-30 m below surface level. The TDW may contain plant pathogens which pose a threat to crop production. ASTR uses separated wells for infiltration and abstraction of the recharged water which forces the water to flow through the porous medium of the aquifer. It was hypothesized that plant pathogenic bacteria present in the infiltration water will be removed during this filtration process by die-off and by attachment to the sediment grains. The research focussed on three plant pathogenic bacteria which can be found in environmental waters: *Ralstonia solanacearum* and the two Soft Rot *Pectobacteriaceae* (SRP), *Dickeya solani* and *Pectobacterium carotovorum.* They affect a broad variety of crops with hosts ranging from potato to flower bulbs, both being important cash crops worldwide and particularly in

the Netherlands (**Chapter 1**). To analyse removal of the selected pathogens during ASTR, laboratory experiments were executed to characterise die-off in water microcosms and bacterial transport in soil columns. Together with the results of dose-response experiments that studied the effect of contaminated irrigation water on potato plants, all results were ultimately combined in a quantitative microbial risk assessment (QMRA).

In **Chapter 2**, the die-off of the bacterial pathogens in oxic TDW and anoxic aquifer water from the pilot site were analysed. Water microcosms were inoculated with known concentrations (10^4 CFU/mL) of the plant pathogens. The effect of abiotic (temperature, oxygen) and biotic (microbiota) factors on the die-off were investigated. The die-off was monitored until the bacteria were undetectable by media cultivation. A clear effect of temperature and oxic conditions on the die-off of all bacteria was observed. In oxic TDW, the SRP showed 3-log_{10} removal by die-off at 25 °C after 6 days, and at 10 °C after 12 days. The 3-log_{10} removal by die-off of *R. solanacearum* in TDW was similar at both temperatures (about 25 days). In anoxic aquifer water at 10 °C the time to achieve 3-log_{10} removal took up to 60 days. We attributed this longer persistence to a less active microbiota at this low temperature, as well as to anoxic conditions. In most tested conditions, non-linear bacterial die-off could be identified. The shape of the die-off curve was characterized by a initially stable bacterial concentration. This was followed by a fast decline to low concentrations from where the bacteria persisted for a period until the concentration dropped below the detection limit. A non-linear Weibull model with a tail was applied to fit the experimental data. The flexibility of the model to predict log-linear as well as non-linear kinetics allowed to model the bacterial die-off of several species influenced by different environmental conditions.

In **Chapter 3**, soil column experiments analysed the transport and removal of the bacterial pathogens. Less removal was found in clean quartz columns while high removal was observed in natural aquifer sediment (18-40 log_{10}/m). Natural sand has a heterogeneous surface structure and grain size distribution including very fine material. This increased bacterial removal by offering a high surface area for attachment, or by size exclusion due to small pore spaces. Additionally, the attachment of the negatively charged bacteria was expected to be promoted by positively charged metal oxides in the aquifer sediment. It was hypothesized that oxic influent water would increase the presence of metal oxides and enhance pathogen removal. However, this could not be confirmed with the experiments. The bacterial breakthrough curves derived from column experiments were modelled in Hydrus-1D

using the advection-dispersion equation for bacterial transport to obtain attachment and detachment parameters. These results were used to predict pathogen removal in the ASTR pilot site.

Considering the results of die-off and soil column experiments, sufficient removal of the plant pathogenic bacteria during ASTR should be achievable. Nevertheless, a risk remains that some bacteria still persist the aquifer treatment and cause plant disease. Yet, studies that analysed the effect of low pathogens concentrations in irrigation water on plant health are scarce. Therefore, in **Chapter 4** the development of the dose-response relationship of *R. solanacearum* contaminated irrigation water and the risk to cause brown-rot disease in potato is described. In greenhouse experiments, potato plants were irrigated (soil-soak inoculation) with contaminated water containing different concentrations of *R. solanacearum*. The resulting disease symptoms were monitored for about two months after which all plants were analysed for latent infections (no visual symptoms). The lowest inoculation concentration of $5x10^2$ CFU/mL caused one latent infection in 15 tested plants while visual disease symptoms were only observed when irrigating with at least $5x10^5$ CFU/mL. These were, to the best of our knowledge, the first experiments that analysed the dose-response relationship of *R. solanacearum* and potato plants at low inoculum concentrations. The effect of an even lower inoculum was tested in *in vitro* plants where a dose of 0.5 CFU/plant was sufficient to cause wilting after invasive stem-inoculation. The results of the *in vitro* experiments may not represent natural conditions for infections but demonstrated the virulence of *R. solanacearum*. Plus, they allowed to analyse the exact dose necessary to cause an infection under optimal conditions for the pathogen. From the results of greenhouse and *in vitro* experiments, a dose-response model was developed. It describes the quantitative relationship of bacterial dose and the risk of infection or illness and was employed in the risk assessment in **Chapter 5** with the aim to prevent plant infections.

In **Chapter 5** all experimental results from die-off, soil-column and dose-response experiments have been used as input data in quantitative microbial risk assessment (QMRA). Based on *R. solanacearum* contaminated source water and its removal (die-off and attachment) during ASTR, infections risks for potato plants were calculated when reusing the ASTR treated water in irrigation. Monte Carlo sampling was used to characterize the distribution of the infection risks given the uncertainty of the input parameters. Different residence times and lengths of soil passages were simulated. This resulted in different pathogen removals as they influence the die-off in the water

phase and removal by soil filtration, respectively. The results of the QMRA showed that a residence time of 30 days alone without soil filtration was insufficient to remove pathogens during ASTR to safe levels. In contrast, soil filtration of 0.7 m length achieved high pathogen removal and will result in negligible infection risk for potatoes when reusing the water in irrigation (infection risk per plant: $5.2x10^{-1}$). Pathogen removals were obtained from column studies and literature indicates overestimation of removals in the field when using lab experiments. Therefore, risks of upscaling from lab to field scale were discussed. Additionally, the use of ASTR treated water (0.7 m aquifer soil passage) on a five hectares potato field was simulated which still resulted in a very low infection risk ($4.7x10^{-6}$ infected plants when irrigating 250 thousand plants). Although 0.7 meters provided sufficient removal, a distance of several meters is recommended in practice (to account for not well known aquifer heterogeneity / scaling effects); the 7 meters at the Breezand pilot site is thus more than sufficient. As indicated by the column results, the QMRA confirmed that the soil passage was the main pathogen removal mechanism in comparison to die-off in the water phase. Furthermore, soil passage adds a predictable treatment step to an ASTR creating a resilient system. The QMRA allowed to compare different operations of an ASTR and formulate recommendations for the safe reuse of drainage water for irrigation. Moreover, the results can be used by policy makers to define an allowable ('safe') bacterial pathogen concentration in irrigation water which currently does not exist. Such concentrations for different pathogens are established in drinking water production to assess if a water treatment yields sufficient pathogen removal where disease burdens remain tolerable.

To conclude, the findings of this PhD research confirmed the initial hypothesis that plant pathogenic bacteria can be removed adequately during aquifer recharge in an ASTR system. The bacteria are mainly removed by attachment to the sediment grains and die-off at the grain surface and to a lesser extent by die-off in the water phase. For an accurate prediction of bacterial removal during ASTR an initial characterization of the aquifer and source water is necessary, or conservative assumptions need be made. Lastly, the scientific findings confirm that the analysed ASTR system pilot site can produce safe irrigation water. Fresh water is stored when available and bacterial pathogens in the source water will be adequately removed during the 7 m long soil passage. The reclaimed water can be used for irrigation without risking plant infections.

Samenvatting

Zoet water is een essentiëel element voor leven. Overexploitatie van zoetwaterbronnen en achteruitgang van de waterkwaliteit door vervuiling vergroten de waterschaarste wereldwijd. Bovendien zullen extreme weersomstandigheden zoals droogte en overstromingen vaker voorkomen als gevolg van klimaatverandering. Dit leidt tot toename van de waterschaarste, maar daagt wetenschappers en mensen uit de praktijk uit om innovatieve en collaboratieve oplossingen te bedenken. 70% van het zoetwatergebruik is de landbouw toe te schrijven. Als gevolg is onze voedselketen bijzonder kwetsbaar voor waterschaarste, aangezien het afhankelijk is van irrigatie. Daarom heb ik onderzoek gedaan naar ondergrondse wateropslag, 'managed aquifer recharge (MAR)', als een op de natuur gebaseerde oplossing voor de zoetwaterbeschikbaarheid in de landbouw. De kwaliteit van het teruggewonnen water dat voor irrigatie wordt gebruikt is een belangrijk aspect in deze systemen. Als er ziekteverwekkers in irrigatiewater aanwezig zijn, kunnen ze plantenziektes en een slechte oogst veroorzaken. Een MAR systeem ontworpen als een aquifer storage, transfer en recovery (ASTR)-systeem gebruikt een injectieput waardoor het grondwater sneller aangevuld wordt dan door natuurlijke bodeminfiltratie. Om het water te onttrekken wordt een tweede put gebruikt. Daardoor vloeit het water door de ondergrond bestaand uit zandlagen met kleinere of grovere zandkorrels. Chemische of biologische verontreinigingen worden tijdens deze natuurlijke zandfiltratie in de ondergrond verwijdert waardoor de waterkwaliteit verbeterd. Eerder onderzoek toonde aan dat ASTR met succes de concentraties van menselijke ziekteverwekkers in het teruggewonnen water kan verminderen, en daarmee ook de risico's voor de menselijke gezondheid. Ook plant pathogene bacteriën kunnen in het water voorkomen, en moeten tijdens de ondergrondse wateropslag verwijdert worden. Literatuur over het verwijderen van plantpathogenen in het milieu en specifiek in grondwater ontbreekt echter. Een goed begrip van het afsterven en verwijderen van ziekteverwekkers onder relevante omstandigheden in de ondergrond is vereist om veranderingen in de waterkwaliteit te voorspellen tijdens ASTR en daarmee de gezondheid van planten te beschermen.

Daarom werd voor het eerst een agrarisch ASTR-systeem geanalyseerd op zijn potentieel om plant pathogene bacteriën te verwijderen met als doel om veilig irrigatiewater te leveren. Een ASTR-pilotlocatie in Noord-Holland is onderzocht waar drainagewater werd opgevangen van een perceel van 10 hectare. Dit water werd geïnfiltreerd in een zuurstofvrije en oorspronkelijk

brakke watervoerende laag van vooral zand op ongeveer 10-30 m onder maaiveld. Het opgevangen drainagewater kan plant pathogenen bevatten die een bedreiging vormen voor de gewasproductie. ASTR gebruikt aparte putten voor infiltratie en onttrekking, waardoor het water gedwongen wordt door het poreuze medium van de watervoerende laag te stromen. Mijn hypothese was dat plant pathogene bacteriën aanwezig in het infiltratiewater tijdens dit filtratieproces zullen worden verwijderd door afsterving en door aanhechting aan de zandkorrels. Drie plant pathogene bacteriën zijn onderzocht die ook regelmatig in het water voorkomen: *Ralstonia solanacearum* en de twee Soft Rot *Pectobacteriaceae* (SRP), *Dickeya solani* en *Pectobacterium carotovorum*. Ze tasten een breed scala aan gewassen aan, met waardplanten variërend van aardappel tot bloembollen: Beide belangrijke marktgewassen wereldwijd en in Nederland (**Hoofdstuk 1**). Om de verwijdering van de geselecteerde pathogenen tijdens ASTR te analyseren, werden laboratoriumexperimenten uitgevoerd om de afsterving in water ('water microcosm' experimenten) en bacterietransport in kolommen gevuld met zand te karakteriseren. Samen met de resultaten van dosis-responsexperimenten waarin het effect van verontreinigd irrigatiewater op aardappelplanten bestudeerd werd, werden alle resultaten uiteindelijk gecombineerd in een kwantitatieve microbiële risicobeoordeling ('quantitative microbial risk assessment – QMRA').

In **Hoofdstuk 2** werd de afsterving van bacteriële plantpathogenen in zuurstofrijk (oxisch) drainagewater en zuurstofvrij (anoxisch) aquiferwater van de proeflocatie onderzocht. 'Water microcosm' werden geïnoculeerd met bekende concentraties (10^4 CFU/ml) van de bacteriële plantpathogenen. Ik onderzocht de effecten van abiotische (temperatuur, zuurstof) en biotische (microbiota) factoren op de afsterving van de plantpathogenen. De afsterving werd bestudeerd totdat de bacteriën niet meer detecteerbaar waren door kweken of specifieke groeimedia. Een duidelijk effect van temperatuur en oxische omstandigheden werd waargenomen op de afsterving van alle bacteriën. SRP vertoonde een 3-log_{10} verwijdering door afsterven na 6 dagen bij 25 °C en na 12 dagen bij 10 °C. De 3-log_{10} verwijdering door afsterving van *R. solanacearum* in drainagewater was vergelijkbaar bij beide temperaturen (ongeveer 25 dagen). In anoxisch aquifer water van 10 °C duurde het tot 60 dagen voor een 3-log_{10} verwijdering. Deze langere persistentie komt waarschijnlijk door een minder actieve microbiota bij deze lage temperatuur en/of anoxische omstandigheden. In de meeste geteste omstandigheden werd niet-lineaire bacteriële afsterving vastgesteld. De vorm van de afstervingscurve werd gekenmerkt door een stabiele bacterieconcentratie aan het begin. Daarna volgde een snelle afname naar lage concentraties, waarna de bacteriën een periode bleven bestaan totdat de concentratie

onder de detectiegrens zakte. Een niet-lineair Weibull model met een staart werd toegepast om de experimentele gegevens te simuleren. De flexibiliteit van het model om zowel log-lineaire als niet-lineaire kinetiek te voorspellen, maakte het mogelijk om de bacteriële afsterving van verschillende soorten, beïnvloed door verschillende omgevingsomstandigheden, te modelleren.

In **Hoofdstuk 3** analyseerde ik de transport en verwijdering van de bacteriële pathogenen in kolom experimenten. Een kolom is een circa 25 cm lange plastikbuis gevuld met zand waardoor water verontreinigd met bacteriën doorgepompt werd. Er werd minder verwijdering gezien in kolommen gevuld met kwartszand, terwijl er een hoge verwijdering werd waargenomen in natuurlijk sediment uit de ondergrond van de pilot locatie (18-40 log_{10}/m). Natuurlijk zand heeft een heterogene oppervlaktestructuur en korrelgrootteverdeling inclusief zeer fijn materiaal. De bacteriën worden onder andere door aanhechting aan zandkorrels verwijdert. Natuurlijke zand heeft meer gunstige locaties voor aanhechting van de bacteriën gegeven door de heterogene oppervlakte structuur, of door grootte-uitsluiting vanwege kleine porieruimten. Bijvoorbeeld werd verwacht dat de aanhechting van de negatief geladen bacteriën zou worden bevorderd door positief geladen metaaloxiden in het aquifer sediment. Er werd verwacht dat zuurstofrijk injectiewater de aanwezigheid van metaaloxiden zou verhogen en de verwijdering van ziekteverwekkers zou verbeteren. Dit kon echter niet worden bevestigd met de experimenten. De bacteriële doorbraakcurven verkregen tijdens de kolomexperimenten werden gemodelleerd in Hydrus-1D met behulp van de advectie-dispersievergelijking voor bacterietransport om hechtings- en onthechtingsparameters te verkrijgen. Deze resultaten werden gebruikt om de verwijdering van pathogenen in de ASTR-pilotsite te voorspellen.

Voldoende verwijdering van plant pathogene bacteriën tijdens ASTR lijkt haalbaar, gezien de resultaten van de water afstervings- en bodemkolomexperimenten Desalniettemin blijft het risico bestaan dat sommige bacteriën de ondergrondse opslag overleven en in het irrigatiewater terecht komen. Er zijn weinig studies die het effect van lage concentraties ziekteverwekkers in irrigatiewater op de gezondheid van planten analyseren. Daarom wordt in **Hoofdstuk 4** de dosis-response relatie van irrigatiewater verontreinigd met *R. solanacearum* en het risico van bruinrotziekte in aardappelen beschreven. Dit waren, voor zover ons bekend, de eerste experimenten die de dosis-responsrelatie van *R. solanacearum* en aardappelplanten bij lage inoculum concentraties analyseerden. In kasexperimenten werden aardappelplanten geïrrigeerd met verontreinigd

water dat verschillende concentraties *R. solanacearum* bevatte ('soil-soak' inoculatie). De resulterende ziektesymptomen werden ongeveer twee maanden gevolgd, waarna alle planten werden geanalyseerd op latente infecties (zonder visuele symptomen). De laagste inoculatieconcentratie van $5x10^2$ CFU/mL veroorzaakte één latente infectie in 15 geteste planten, terwijl visuele ziektesymptomen alleen werden waargenomen bij irrigatie met minimaal $5x10^5$ CFU/mL. Het effect van een nog lager inoculum werd getest in *in vitro* planten. De planten werden eerst met een naald verwond en daarna werd de inoculatie suspensie op de wond aangebracht (invasieve stengel inoculatie). Een dosis van 0.5 CFU per plant voldoende was om verwelking te veroorzaken. De resultaten van de *in vitro* experimenten zijn mogelijk niet representatief voor natuurlijke omstandigheden omdat de planten in een afgesloten buisje groeiden en voor de inoculatie gewond werden. Maar deze *in vitro* experimenten toonden de virulentie van *R. solanacearum* aan en konden de exacte dosis analyseren die nodig is om een infectie te veroorzaken onder optimale omstandigheden voor de ziekteverwekker. Op basis van de resultaten van experimenten in de kas en *in vitro* werd een dosis-responsmodel ontwikkeld. Het beschrijft de kwantitatieve relatie tussen bacteriële dosis en het risico op infectie of ziekte en werd gebruikt bij de risicobeoordeling in **Hoofdstuk 5** met als doel plantinfecties te voorkomen.

In **Hoofdstuk 5** zijn alle resultaten van de afstervings-, bodemkolom- en dosis-response experimenten gebruikt als invoergegevens voor een kwantitatieve microbiële risicobeoordeling (QMRA). Daarin worden infectierisico's voor aardappelplanten berekend bij gebruik van irrigatie water uit een ASTR system. De verwijdering van *R. solanacearum* verontreinigd bronwater werd voor verschillende verblijftijden en lengtes werden gesimuleerd van bodempassages van injectieput naar onttrekkingsput. Een langere verblijf in der ondergrond en een langere bodempassage van het water door de ondergrond verhogen de verwijdering van chemische en biologische contaminanten inclusief de bacteriën. Monte Carlo statistiek werd gebruikt om de verdeling van de infectierisico's te karakteriseren gezien de onzekerheid van de invoerparameters. De QMRA toonde aan dat een verblijftijd van 30 dagen zonder bodempassage onvoldoende was om ziekteverwekkers tijdens ASTR tot veilige niveaus te verwijderen. Een bodempassage van 0.7 m lengte zorgde voor een hoge verwijdering van ziekteverwekkers en zal resulteren in een miniem infectierisico voor aardappelen bij hergebruik van het water in irrigatie (infectierisico per plant: $5.2x10^{-11}$). De snelheid van verwijdering van pathogenen was verkregen uit kolomstudies maar de literatuur laat zien dat kolomexperimenten zorgen voor een overschatting van verwijderingen in het veld. Daarom werden de risico's van opschaling van laboratorium

naar veldschaal besproken: gebruik van irrigatie water uit een ASTR system (0.7 m aquifer bodempassage) werd voor een aardappelveld van vijf hectare gesimuleerd. Dit resulteerde nog steeds in een zeer laag infectierisico en voorspelde 4.7×10^{-6} geïnfecteerde planten bij irrigatie van 250 duizend planten. Hoewel 0.7 meter voldoende verwijdering opleverde, wordt in de praktijk een afstand van enkele meters aanbevolen (om rekening te houden met niet goed bekende heterogeniteit / schaaleffecten van watervoerende lagen); de 7 meter op de pilotlocatie Breezand is dus ruim voldoende. De QMRA bevestigde dat de bodempassage het belangrijkste mechanisme was om ziekteverwekkers te verwijderen in vergelijking met afsterven in de waterfase. Bovendien voegt bodempassage met bekende lengte een berekenbare behandelingsstap toe aan een ASTR systeem. In de QMRA zijn verschillende werkingen van een ASTR met elkaar vergeleken, aan de hand daarvan kunnenaanbevelingen worden geformuleerd voor veilig hergebruik van drainagewater voor irrigatie. Bovendien kunnen de resultaten door beleidsmakers worden gebruikt om een toelaatbare ('veilige') pathogenen concentratie van bacteriën in irrigatiewater te definiëren.

De bevindingen van dit promotieonderzoek bevestigen de initiële hypothese dat plant pathogene bacteriën adequaat kunnen worden verwijderd tijdens de ondergrondse wateropslag in een ASTR-systeem. De bacteriën worden voornamelijk verwijderd door aanhechting aan de sedimentkorrels en afsterven aan het korreloppervlak, en in mindere mate door afsterven in de waterfase. Voor een nauwkeurige voorspelling van de verwijdering van bacteriën tijdens ASTR is een initiële karakterisering van het bronwater en de watervoerende laag noodzakelijk, of er moeten conservatieve aannames worden gemaakt. Ten slotte bevestigen de bevindingen dat de geanalyseerde proeflocatie van het ASTR-systeem veilig irrigatiewater kan produceren.

Chapter 1 **Introduction**

1.1 Fresh water scarcity

The United Nations sustainable development goals (SDG) aim to achieve access to clean water and sanitation for people worldwide by 2030 (SDG 6). Yet, more than two billion people currently do not have access to safe drinking water. Furthermore, about half of the world population still lacks safely managed sanitation and the disposal of untreated wastewater into the environment depicts a risk to human health and pollutes water resources. This leaves a vulnerable population behind, depriving them from economic growth as water availability and poverty is strongly linked (UN et al., 2022). Moreover, agriculture depends on the availability of irrigation water of sufficient quality to secure crop yields. This is in line with SDG 2 that aims to ensure food production by adapting to climate change and supporting ecosystem functioning. The lack of rainfall especially impacts poor and marginalized groups whose livelihoods often depend on the agricultural output produced on their small-scale farms (FAO, 2022). Besides, even farmers in 'water-rich' countries like the Netherlands are suffering from water scarcity due to prolonged drought periods (Mens et al., 2022).

Water scarcity is a result of the pollution and overexploitation of fresh water resources combined with the devastating effects of climate change. On the one hand, extreme floods of the past years demonstrated globally their disastrous effects, e.g., in Germany (Fekete and Sandholz, 2021), Bangladesh (UN, 2022) or in West/Central Africa (UNHCR, 2022). Thirty-three million people were affected by the floods in Pakistan in 2022, of which 6.4 million needed humanitarian help. The floods led to a collapse of health services and destroyed harvests and agricultural fields with negative consequences on food security (Devi, 2022; Pradhan et al., 2022). A low microbiological water quality can have adverse effects on human health caused by waterborne pathogens. Equally, plant pathogens are a threat to crop health if they are present in waters used for irrigation. On the other hand, severe and prolonged droughts threaten people's lives especially in arid and semi-arid regions. Furthermore, droughts in temperate regions are intensifying as consequence of climate change. The existing water stress will worsen worldwide as climate models predict an increase in rare extreme rainfall

events as well as 'megadroughts' (Cook et al., 2022; Gründemann et al., 2022). Farmers are compensating shortages of rainfall by using irrigation water, if it is available. Thereby, agriculture accounts for the highest withdrawal of fresh water (about 70%) although regional differences in water use exist. In Europe, about 30% of available fresh water is used for irrigation whereas South Asia consumes about 90%. A third of this fresh water is derived from groundwater extraction which is depleting over time as rain fall is often insufficient to replenish the aquifers (FAO, 2022). Coastal and Delta regions are particularly vulnerable to groundwater extraction as these densely populated and urbanized areas often sustain intensive agriculture and require large water resources. However, available groundwater is mostly brackish in coastal areas where salt water intrusion results in mixing of fresh and salty groundwater. This process is further accelerated by sea level rise and continuous groundwater extraction also causes land subsidence (Minderhoud et al., 2020).

Water quality is an integral part of water resource management as the improvement of water quality in surface and groundwater will reduce water scarcity (van Vliet et al., 2017). For example, the 'fast fashion' industry uses and pollutes enormous volumes of water considering their whole supply chain (about 79 trillion litres of water per year) (Niinimäki et al., 2020). In agriculture, fertilizers or manure used to maximise crop yields leach through the soil into surface water or groundwater and contribute to the eutrophication of water sources (Basu et al., 2022). Nature-based solutions offer great potential to tackle problems of water availability while improving water quality and reducing risks caused by waterborne diseases. One of their advantages is that they can be applied for different urban or agricultural settings. For example, the reestablishment of wetlands can help to manage floods by trapping rain water run-off. They act like a sponge which increases aquifer recharge and mitigates land erosion (UN-Water, 2018). A nature-based solution that involves a more advanced infrastructure is managed aquifer recharge which includes river-bank filtration or aquifer storage and recovery (Dillon, 2005). This study focussed on the potential of aquifer storage transfer and recovery to intentionally recharge an aquifer while improving water quality during the storage. The focus lies on the removal of plant pathogenic bacteria in order to safely reuse the recovered water for agricultural irrigation.

1.2 Agricultural aquifer recharge, transfer and recovery (ASTR)

Managed aquifer recharge

Water scarcity is the imbalance between water supply and demand. Groundwater extraction may satisfy the demand of water in times of need but overuse leads to the depletion of this natural resource as natural recharge by rain events is insufficient. The intentional recharge of aquifers with different source waters is called managed aquifer recharge (MAR) which accelerates aquifer recharge while the natural subsurface can improve water quality (Pyne, 1995; Dillon et al., 2010). Thereby, water is stored in the subsurface when excess water is available from where it can be recovered in times of need. By storing the water in the subsurface it is protected from external contamination or losses due to evaporation in comparison to other open water storage systems. Additionally, a smaller surface area is required for MAR in comparison to water stored in surface basins, dams, or storage tanks (Alam et al., 2021). The first systems of intentional water recharge have been recorded in China about 475-221 B.C. where surface water infiltration was increased by digging channels (Wang et al., 2014). Technological development over the centuries allowed to develop highly engineered MAR systems which are adapted to the local hydrogeochemical conditions and the specific needs of the water users. Especially in the past 60 years, an increase in theoretical and practical knowledge about MAR has led to a 5% yearly growth of MAR installations (Dillon et al., 2019). The term 'MAR' describes different water recharge systems such as riverbank filtration, dune filtration, underground dams, rainwater harvesting, or soil aquifer treatment. These systems differ in their technological setup which depends on the location and the overall purpose of the recharge or usage of the recovered water. Nevertheless, they all have in common to use the subsurface for water storage while enhancing groundwater quality when the water moves through the porous media of the aquifer where the subsurface acts as a natural filtration. Consequently, even source waters of lower water quality can be considered for the recharge as water quality will improve during storage (Dillon, 2005). For example, bank filtration is an established MAR type which is used in many countries as a low-cost pre-treatment in drinking water production. To do so, groundwater is extracted from a well located at a certain setback distance from a lake or river which induces water flow from the surface water bed towards the groundwater well. During this process, pollutants in the surface water are removed by physical filtration and microbial degradation which depend on geochemical interactions with the aquifer material (ion exchange, precipitation and sorption) (Ray et al., 2002).

Agricultural MAR

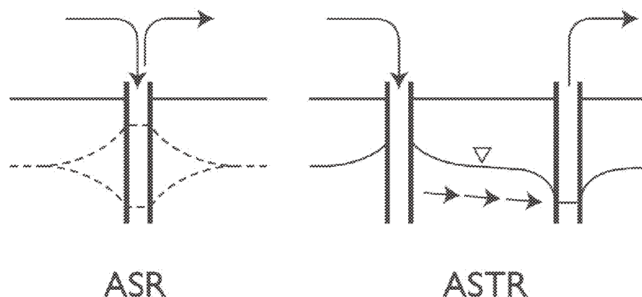
Since 40% of the available land surface is used for agricultural production (Foley et al., 2005) these areas have great potential to be used for MAR. Agricultural land can be used as infiltration basins if the upper soil layer is permeable and the underlying aquifer unconfined. During the non-cropping season, or if the respective crop withstands wet conditions, the agricultural land is flooded with surface water which increases groundwater recharge and makes water available for different purposes. This method of agricultural MAR (Ag-MAR) is applied in many areas of California in the United States and has the capacity for widespread global implementation (Levintal et al., 2022). Infiltration techniques for agricultural MAR are also applied in Australia and in their review, Page et al. (2023) identified a total of thirteen schemes of agricultural MAR. The majority (seven) used infiltration and four schemes were using well-injection techniques. Two systems were currently undergoing feasibility testing. Agricultural MAR in the Netherlands is also under development since the water demand for agriculture is increasing due to the consequences of climate change. For example, treated wastewater effluent from a sugar beet factory is infiltrated via an aquifer storage and recovery (ASR) system to provide high-quality irrigation water for horticultural greenhouse production (Zuurbier et al., 2018). The pre-treatment of the effluent water is costly but necessary to avoid contamination of the groundwater and guarantee the required water quality of the recovered water used in irrigation. In this thesis, an agricultural MAR designed as an aquifer storage, transfer and recovery (ASTR) system to produce irrigation water is investigated. Infiltration into the subsurface is done via pumping wells as the water is stored below a confining clay layer. The source water used for infiltration is excess rain water collected via the tile drainage system. Although the water quality of the source water is expected to be sufficient for irrigation, there may be chemical pollutants or pathogens present. We hypothesize that these pollutants (not exceeding a certain concentration) will be removed during the natural soil passage of the ASTR system and water quality improves during the storage. To conclude, different solutions for agricultural MAR exist in order to increase groundwater recharge and provide irrigation water. These solutions have to be adapted to the local conditions including quality of the source water, and geological and climatic conditions.

Aquifer storage, (transfer) and recovery – AS(T)R

Improvement of water quality can also be expected in a MAR type called aquifer storage and recovery (ASR, Figure 1A). In ASR, water is infiltrated into the subsurface and abstracted from the same well. During infiltration, the entering water replaces the native groundwater of the aquifer and travels

radially away from the infiltration well. The travel distance depends on the infiltrated water volume and the porosity of the aquifer which may vary along its depth profile. Having one well for abstraction and infiltration, recently infiltrated water is the first to be abstracted again. A longer storage or residence time increases improvement of water quality as chemical or biological pollutants will degrade or die-off over time. Water from earlier infiltrations travelled further away from the well and had a longer residence time. Consequently, the quality of the recovered from an ASR can be more heterogeneous because it can be composed of waters of different residence times. In contrast, aquifer storage transfer and recovery (ASTR, Figure 1B) uses spatially separated infiltration and abstraction wells. The water has to flow from infiltration to abstraction well for recovery and is thereby naturally filtrated. Pathogenic organisms present in the infiltrated water will die-off in the water phase or will be removed by interaction (e.g., attachment) with the soil grain phase. A greater distance between the wells will result in a higher treatment. The soil passage also implies a minimum residence time in an ASTR based on water flow velocity. Although ASR has great potential to increase water quantity through recharge, the improvement of water quality is less well predictable. In ASR, degradation of pollutants only relies on the residence time and less on sorption (e.g., attachment) to the aquifer sediment.

This study focussed on an ASTR system to provide safe irrigation water as the recovered water may contain zero or very low concentrations of bacterial pathogens. Their specific removal mechanisms during recharge are discussed in Chapter 1.5. However, changes in water quality during storage in the ASTR system will be compared with an ASR system.



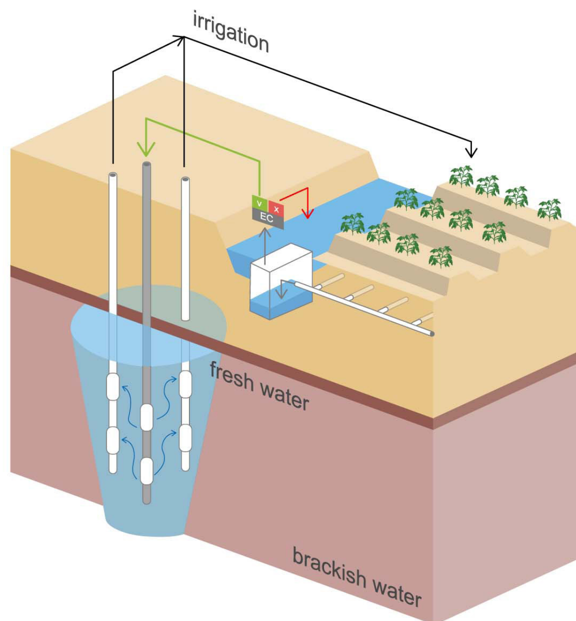
▲ **Figure 1** Comparison of two managed aquifer recharge systems: aquifer recharge and recovery (ASR) where the same well is used for infiltration and abstraction of water, and aquifer storage transfer and recovery (ASTR) where the water needs to flow from infiltration to abstraction well for recovery. The soil passage in ASTR set by the distance between both wells improves water quality. Scheme adapted from (Dillon, 2005).

Study site

Figure 2 shows a scheme of the pilot ASTR system where the water is stored and recovered for agricultural irrigation. The ASTR is situated in an agricultural area in a polder in the North-Western part of the Netherlands (coordinates: 52.8883, 4.8221). After rain events, tile drainage water (TDW) is collected from a 10 hectares (ha) agricultural land and stored in the underlying confined aquifer. The rain water reaches the drains (located about 0.7 m below surface level (b.s.l.)) as it percolates through the top soil from where chemical and biological agropollutants may be released and carried along. All the tile drains end up in a collection drain, from which TDW is discharged to a storage tank (ca. 1 m^3). As the water level exceeds a threshold, a pump within the storage tank is activated. First, disc filters (pore size: 40 μm) treat the pumped TDW to remove larger suspended solids to avoid clogging of the screens of the infiltration wells. Then, a vertical well infiltrates the TDW below a confining Holocene clay/peat layer over a depth of 11.5-33.0 m b.s.l. The aquifer consists of sand layers of late Holocene and Pleistocene origin, is anoxic, originally brackish and has a constant temperature of about 10 °C. A freshwater storage is created through the infiltration of the oxic, fresh TDW which replaces the native groundwater. Maliva et al. (2006) suggests that about 70% of fresh water can be recovered from a brackish aquifer. The stored water can be reused in dry periods for irrigation but needs to meet quality requirements as the infiltrated TDW can contain pollutants originating from different sources. Excess fertilizers and pesticides applied during agricultural production will trickle into the ground after rainfall until they reach the TDW. Moreover, surface water may mix with the TDW when agricultural fields are flooded during storm events by overflowing ditch water. In this context, plant pathogenic bacteria like *Ralstonia solanacearum* which are present in Dutch surface water may contaminate the TDW and thereafter the aquifer (Janse, 1996). Plus, the practice of level controlled drainage which pumps ditch water into the drains can contaminate the TDW and pose a risk when the increased soil water level reaches plant roots (de Wit et al., 2022). This method will be safer if reclaimed water from the ASTR system is used for the controlled drainage as it should contain less or no pollutants.

The infiltrated water will undergo different biochemical reactions. For example, oxygen is reduced within two days and nitrate within four to seven days using push-pull tests to assess aquifer reactivity (Kruisdijk and van Breukelen, 2021). Changes in water quality will depend on the aquifer hydrogeochemistry and the composition of the microbiota present in the water phase and attached to the sand grains (Bekele et al., 2018). As changes in water quality will depend on the residence time (time between infiltration

and abstraction event) of the water and the soil passage (distance between infiltration and abstraction well) this study aims to find the optimal ASTR configuration that removes selected bacterial pathogens sufficiently to produce safe irrigation. This requires quantitative data on the removal of the selected pathogens under aquifer conditions which are not yet available. This research is done within the AGRIMAR project to investigate water quality changes of an agricultural MAR system and is a follow-up project of the Spaarwater by Acacia B.V. (Acacia Water, 2019). Of course, chemical water quality (pH, salinity, etc.) is an equally important parameter in irrigation. Therefore, a second PhD research within the AGRIMAR project conducted by Emiel Kruisdijk dealt with the fate of agrochemicals. Plus, he analysed the consequences of well clogging during aquifer storage and recovery (Kruisdijk, 2022).



▲ **Figure 2** Schematic representation of an agricultural field connected to a managed aquifer recharge site. The site is designed as an aquifer storage transfer and recovery (ASTR) system. Excess rain water reaches the tile drainage system buried at about 70 cm depth. The collection drain terminates into a concrete reservoir where the electrical conductivity (EC) and turbidity of the tile drainage water is measured. If the EC or turbidity is below a set threshold value, the water is infiltrated via the injection well (depicted in grey). From there, the water travels through the sandy aquifer to the abstraction wells (depicted in white) and can be recovered for irrigation.

1.3 Ornamental and potato cultivation in the Netherlands

The Netherlands are the second biggest exporter of agricultural goods (measured in value) after the United States. Of an estimated export volume of 104.7 billion euros in 2021, 75.7 billion euros were generated by Dutch production while 32 billion euros were generated through re-exports. Dutch goods are mostly (67%) exported to the European Union (Jukema and Ramaekers, 2021). Ornamental products (indoor plants, flower bulbs, tree nursery, cut flowers) achieved the highest export volume of 12 billion euros. Potato products ranked on the 9th position of Dutch agricultural export goods which includes harvested tubers (seed, starch or consumption potatoes) as well as processed potato products (e.g., fries production). In the Netherlands, potatoes are grown on about 166 000 ha. Seed potatoes make up about a third of the Dutch potato market which are 65% of the globally traded seed potatoes, making them one of the global players in seed potato production. As seed potatoes are the starting material for the following cropping season they have to be of high quality and pest-free. These quality standards increase their economic value (Berkhout et al., 2022). Consequently, high hygiene standards are required in potato production to maintain this position and avoid economical losses (Breukers et al., 2005).

Altogether, potato as well as ornamental production are of high economic importance in the Netherlands whereas potatoes also have an important function as staple food worldwide. Originating from the Andes, today, potatoes are farmed worldwide: "from China's Yunnan plateau and the subtropical lowlands of India to Java's equatorial highlands and the steppes of the Ukraine" (FAO, 2009). After wheat, maize and rice they rank as the fourth most important food crop. Comparing to the other staple foods potatoes show the highest 'nutritional productivity' in relation to their water use. The resulting tuber is full of carbohydrates, rich in micronutrients and contains high amounts of Vitamin C (FAO, 2008). Furthermore, a smaller surface area is necessary to produce potatoes in comparison to other major crops (FAO, 2009). Nevertheless, there are great differences in crop yields worldwide which can range between 2-44 tons of potatoes per hectare. The low yields may be caused by insufficient irrigation or fertilizers, or as a result of plant diseases. Billions of people are relying on potatoes as a staple crop. Therefore, the increase in yields can play an important role in promoting food security and reducing poverty.

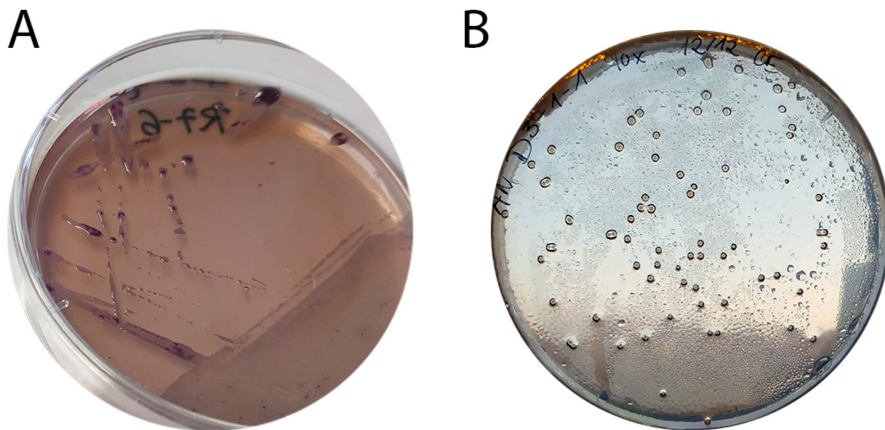
Richard et al. (2022) estimated that biotic stresses (weeds, pests, pathogens) cause global potato losses of about 14.8 million tons yearly (38.4 billion dollars), of which about one third (5.4 million tons, 13.9 billion dollars) could be attributed to pathogens (fungi or bacteria) although crop protection was applied. An outbreak of late blight in 1845 caused by the water mould *Phytophthora infestans* went down in history as the 'Irish Potato Famine' causing death to about one million people. A similar longstanding history in causing plant diseases have the soft rot *Pectobacteriaceae* (SRP). They affect many crops including potatoes and their wide host range also comprises ornamental plants, vegetable crops and even fruit crops (van Gijsegem et al., 2021). Due to severe losses caused by these bacterial pathogens, they have been ranked in the top ten list of plant pathogenic bacteria in 2010 (Mansfield et al., 2012). This list also includes *Ralstonia solanacearum* which causes potato brown rot and bacterial wilt to many plants worldwide. Yield reductions of 30-90% caused by *R. solanacearum* have been reported in Bolivia, together with losses of up to 98% during tuber storage (Guchi, 2015). High hygiene standards during the production process are required to avoid crop losses. This includes the use of certified pest-free seed and plant material for propagation as well as clean production processes. Machinery has to be cleaned regularly and water used for irrigation may not contain pathogenic organisms.

1.4 The plant pathogens

1.4.1 *Ralstonia solanacearum*

Bacterial wilt has been recorded for the first time by Smith in 1896 (Kelman, 1998). It is caused by the plant pathogen *Ralstonia solanacearum* which is a Gram-negative, soil-borne bacterium which can be found worldwide due to its host range of about 200 plant species (Hayward, 1991). Hosts include *Solanaceous* crops, groundnut, banana and plantain, weeds, some tree species and ornamental plants (Hayward, 1994). Figure 3 shows the colony morphology of *R. solanacearum* on semi-selective South Africa medium which is an irregularly shaped, white slimy colony with a pink center. The disease symptoms of bacterial wilt on a tomato plants and of potato tuber soft rot are shown in Figure 4. As the species is very heterogeneous it has undergone several taxonomic changes in the last hundred years. Earlier described as *Pseudomonas solanacearum,* the bacterium was categorized into different races according to its host range (Buddgenhagen, 1962). Hayward (1964) further characterized a collection of 185 isolated *P. solanacearum* into biotypes according to their ability to oxidize three different disaccharides

or three hexose alcohols. With the development of DNA sequencing based on 16S rRNA genes, Yabuuchi et al. (1995) proposed the new genus *Ralstonia* which included the phenotypic characterization and fatty acid analysis of the isolates. Fegan and Prior (2005) described *R. solanacearum* as a species complex consisting of "a cluster of closely related isolates whose individual members may represent more than one species". They categorized the bacterium into four phylotypes based on their geographical origin. *R. solanacearum* was originally described as a tropical pathogen originating from South America and adapted to warmer temperatures. However, global trading of agricultural goods has resulted in the establishment of a cold adapted variant of *R. solanacearum* (phylotype II, race 3 biovar 2) in Europe where it threatens potato production causing brown rot (Janse, 1996). The most recent taxonomical revision was done by Safni et al. (2014) who divided the species complex into three 'genospecies': *R. solanacearum* which only contains phylotype II, *R. pseudosolanacearum* which includes phylotypes I and III, and *R. syzgii* represents phylotype IV.



▲ **Figure 3** Colony morphology of *Ralstonia solanacearum* on SMSA medium (A) showing typical white slimy colonies or irregular shape and with a pink center. The right pictures (B) show *Dickeya solani* on DL-CVP medium where it forms distinct cavities in the upper layer which consist of pectin which the bacteria can degrade with its pectinolytic enzymes.

Since the early 1990's, many brown rot disease outbreaks caused by *R. solanacearum* have been observed in European potato production. One of the main contributors to disease dissemination is (latently) infected seed material. To restrict further pathogen spread, strict hygiene measures have been introduced in potato production including the certification of seed material (Janse, 1996). The detection of brown rot on a farm in the Netherlands has severe consequences as the complete harvest needs to

be destroyed, followed by a ban on seed potato production on the infested agricultural field for 4-5 years (NVWA, 2021). Import of seed tubers into the European Union from outside member states is forbidden and testing of tuber lots designated for seed tuber production is required. Additionally, *R. solanacearum* has been put under quarantine status in the European Union (Directive, 1998). In the US and Croatia, it is described as a potential bioterrorism agent (Bokan et al., 2001; USDA, 2002).

▲**Figure 4 A:** Bacterial wilt on tomato plants caused by *Ralstonia solanacearum*. The plant on the right is a control plant inoculated with sterile water. The two plants on the left have been stem-inoculated (10^8 CFU/mL) with a suspension of *R. solanacearum* and show different stages of bacterial wilt. The middle plant is completely wilted and the left plant shows 100% wilting in the lower leaves while the upper leaves show beginning wilting symptoms, curling of the leaf edges. **B:** Brown rot in potato tubers caused by *R. solanacearum*. The mother plant has been inoculated with $5x10^9$ CFU via soil-soak inoculation and the pathogens systemically spread inside the plant and into the progeny tubers. The stolon ends have been removed for analysis. The tubers show different stages of infection indicated by the arrows: the vascular ring discolours **(a)** and white bacterial ooze emerges **(b)** followed by browning of the vascular ring **(c)** and rotting of tuber tissue **(d)**.

As part of the pathogen's life cycle, *R. solanacearum* can persist outside the host environment in soils within plant residues or can be found in surface waters (EFSA et al., 2019). The bacteria's persistence in surface water is facilitated by alternative hosts such as *Solanum dulcamara* which grow along water canals. These plants get infected with *R. solanacearum* without showing symptoms and release bacteria when the environmental conditions are favourable, for example when the water temperature rises after the winter season (Wenneker et al., 1999). As the use of *R. solanacearum* contaminated surface water in irrigation has been linked with disease outbreaks, the irrigation with surface water has been prohibited in the European Union for seed tuber production. Moreover, fields where starch and consumption potatoes are produced cannot be irrigated if the bacterium is detected in surface water near cropping fields (Directive, 1998). Together with seed tuber testing, these measures have successfully decreased potato brown rot outbreaks almost to zero (Janse, 2012). Nevertheless, *R. solanacearum* is still found in surface waters and treatment of the water is required if irrigation is needed due to drought periods.

1.4.2 Soft rot *Pectobacteriaceae*

A similar long-standing history as *R. solanacearum* have bacteria that cause soft rot on vegetables which have first been described in early 1900 (Jones, 1900). Bacterial soft rot is caused by many bacterial genera but species of *Dickeya* and *Pectobacterium* are the most studied as they cause the highest economical damage (van Gijsegem et al., 2021). They belong to the Soft Rot *Pectobacteriaceae* (SRP) and are considered the causative agent of potato blackleg and tuber soft rot (Pérombelon, 1992). The bacteria are Gram-negative, non-sporing and facultative anaerobes and were formerly summarized under the name 'soft rot *Erwinias*'. They produce large quantities of cell wall degrading enzymes (e.g., pectin lyases, cellulases) during plant invasion which also helps them to outcompete other bacteria (Collmer and Keen, 1986). When cultured on double layer crystal violet pectate (DL-CVP) they cause distinctive cavities in the medium as shown in Figure 3 where the upper layer consist of pectin. During the last century, soft-rot bacterial species have undergone a lot of nomenclature changes and the development of molecular methods allowed to identify and differentiate many new species (Czajkowski et al., 2015; Garlant, 2015; Toth et al., 2021). For example, *Erwinia carotovora* sp. *atroseptica* has been isolated from potato black leg and was later re-classified within the genus *Pectobacterium* (Hauben et al., 1998) while *Erwinia chrysanthemi* isolated from chrysanthemum had been placed in the genus *Dickeya* (Samson et al., 2005). The high disease potential of *Dickeya sp*. and *Pectobacterium sp*. is also attributed to their very broad host-range towards crops, vegetables, fruit crops and ornamentals (Ma et

al., 2007). A recent study isolated 161 pectinolytic strains of *Pectobacterium* and four *Dickeya* strains from symptomless vegetables and ornamental plants (potato, fennel, leek, iris etc.) purchased on local Polish food markets (Smoktunowicz et al., 2022). The isolates were resilient to a wide range of pH, salt concentrations or levels of water availability. Plus, they macerated plant tissues from different plant species demonstrating their adaptability to different environments and plant hosts. Moreover, the detected species corresponded with bacteria isolated from symptomatic plants from different countries (e.g., Brazil, USA, Israel, Morocco) or with bacteria found in French and Finish waterways (Laurila et al., 2008; Ben Moussa et al., 2022).

This study analyses two species of the SRP namely *P. carotovorum* and *D. solani*. *P. carotovorum* causes rotting especially during storage of potatoes but can also cause slow wilt in the field which is favoured by higher temperatures (Haan et al., 2008). *P. carotovorum* also poses a threat to ornamental monocot production such as *Zantedeschia* sp. (calla lilly) and also infects vegetable crops like onion and banana (Yedidia et al., 2011). *D. solani* is a new emerging pathogen that has been isolated from diseased potato plants in 2005 in Poland (Sławiak et al., 2009) and was established as a new species in 2014 (van der Wolf et al., 2014). It was probably introduced from hyacinth cultivation where it crossed into potato production (Parkinson et al., 2015). Since then, it is fast spreading in Europe through latently infected seed tubers and first incidents outside Europe have been reported as for example in Turkey or Brazil (Cardoza et al., 2016; Ozturk and Aksoy, 2017). *Dickeya* spp. was considered to be mainly located in tropical and subtropical regions, but *D. solani* is emerging in cooler climates because of a broader temperature spectrum (Toth et al., 2011).

1.4.3 Plant pathogens: environment to disease development

Every crop production system has to cope with plant pathogens and pests in form of insects, fungi, nematodes, protozoa, bacteria or viruses and wild plants have developed various direct and indirect mechanisms to defend plant pathogens. In contrast, breeding for cultivars for agricultural production results in high-yielding crops but reduced the genetic range. During the 20th century breeding for resistance was not prioritized, instead, pest problems were resolved through the application of pesticides (Stenberg, 2017). Intensified agriculture using monoculture systems together with a loss in biodiversity further facilitate the establishment of plant pathogens (Zhan et al., 2015). Anderson et al. (2004) identified the introduction of pathogens through anthropogenic actions as the main driver of "pathogen pollution", the emergence of plant diseases. Thereby, worldwide trading of plant material

and insufficient inspection of symptomless seed material contribute to the long distance dissemination of plant pathogens. Once established, plant pathogens can persist outside the host within the environment from where they are further distributed locally. Hence, surface water contaminated with plant pathogens poses a risk to crop production if the surface water is used without prior treatment for irrigation (Hong and Moorman, 2005; Lamichhane and Bartoli, 2015). *R. solanacearum* is regularly detected in surface waters in the European Union (Commission, 2017) but has also been isolated in Egypt from canal water next to potato growing areas (Tomlinson et al., 2009). Irrigation with *R. solanacearum* contaminated water has been recognized as inoculum source for potato brown rot. Bacterial pathogens such as *R. solanacearum* or SRP enter the plant through natural openings like stomata or newly emerging root zones (Charkowski et al., 2012; Siddiqui et al., 2014). *R. solanacearum* has been reported to survive in natural drainage water for about 60 days where the presence of other microorganisms and a low temperature (4 °C) reduced the survival (Elsas et al., 2001). Equally, SRP are found worldwide in waterways (McCarter-Zorner et al., 1984) and a survey of a river stream in France detected the bacteria over a distance of hundreds of kilometres (Ben Moussa et al., 2022). After a plant infection, plant pathogens may be released into the soil where they can re-infect neighbouring plants. Moreover, the pathogens may persist over prolonged periods in infested soils. *R. solanacearum* has been detected from several months to up to two years in the Netherlands and plant debris contributed to the survival of the pathogen (van Elsas et al., 2000). Soil has also been identified as a reservoir for *P. carotovorum* where it was detectable for about 40 days (Perombelon and Hyman, 1989). As *D. solani* has been described more recently, no studies are available that evaluate this species regarding its environmental survival. Only one study investigated the fate of *Dickeya dianthicola* IPO1991 in different water types and on different materials (van Doorn et al., 2008). In tap water at 15 °C, the pathogen died within a few hours while it remained detectable for 45 days in sterile buffer solution . Next to transmission of plant pathogens via contaminated water or soil, dissemination via infected insects has been recognized as an infection pathway of SRP contributing to the pathogen's dissemination over long distances (Rossmann et al., 2018). Although (latently) infected seed material poses the highest risk for disease transmission, it is essential to know about a plant pathogen's epidemiology to predict its potential for dissemination outside the host. The saprophytic life cycle of a pathogen increases to chances of dispersal and re-infection of a new host after being transported through the environment.

1.5 Pathogen removal during MAR to improve water quality

Pathogens can be removed from water by physical, chemical and biological mechanisms. These treatments have been analysed especially in greenhouse cropping systems where excess irrigation water is captured and treated to reuse costly fresh water (Raudales et al., 2017). Physical methods to remove pathogens from water resources include heat treatment, UV radiation or membrane filtration while chemical methods consist of chlorine or ozone application, amongst others (Raudales et al., 2014). Biological control methods where antagonistic microorganisms or host-specific bacteriophages are added to the water depict a promising method to tackle plant pathogens (Rivas-García et al., 2020). For example, bacteriophages were recognized as sustainable biocontrol method against *R. solanacearum* or *D. solani* (Czajkowski et al., 2017; Álvarez et al., 2019). Nevertheless, studies on the applicability of bacteriophages in the field or within water treatment are scarce. Furthermore, research analysing the mechanisms of the interactions between bacteria and phages are lacking (Álvarez and Biosca, 2017).

This study focusses on the removal of plant pathogenic bacteria during ASTR to recover the water for agricultural irrigation. As introduced in Chapter 1.2, pathogen removal will depend on the residence time in the aquifer and the length of the soil passage given by the distance between infiltration and abstraction well. We hypothesize that a certain residence time combined with a certain soil passage length will remove the bacterial plant pathogens sufficiently. The required storage time and distance needs to be found out experimentally and depends on the pathogens. To predict pathogen removal during aquifer storage an understanding of bacterial transport in the subsurface is necessary which can be investigated in column experiments. A bacterial solution of known pathogen concentration is pumped through a soil column of known length and the bacteria are enumerated in the effluent solution. The bacteria are transported with the water flow through the porous sand medium and get inactivated by die-off in the water phase or attachment to the sand surface, die-off at the grain surface, or are retained within small sand pores by size exclusion. The difference in concentrations of in- and outflowing water allows to draw a breakthrough curve from which the pathogen removal within the analysed soil can be calculated (Schijven, 2017). An extended version of the advection-dispersion equation describes water flow and the transport of the bacterial colloids through the saturated soil. However, it is not possible to predict which of the mechanisms caused the removal. To distinct between die-off in the water phase and removal

by soil passage, the die-off of the bacterial pathogens has to be studied in separate water microcosm experiments. Waters from the aquifer location are inoculated with a known concentration of bacteria and the influence of different abiotic (e.g., pH, temperature) and biotic (e.g., competing microorganisms) factors analysed (Roszak and Colwell, 1987). The change in concentration is monitored over a period until the bacteria cannot be detected anymore and from which a die-off model can be developed. The different die-off kinetics (e.g., log-linear vs. non-linear die-off) should be considered to predict the removal by die-off (Peleg and Cole, 1998). Removal by soil filtration depends mostly on the physicochemical composition of the aquifer matrix, but also its microbial community (Bekele et al., 2018). Natural sediments usually contain positively charged metal-oxide coatings on the grain surface. They represent sites for favourable attachment of negatively charged microorganisms. The process of metal oxidation can increase when oxic TDW is infiltrated into the aquifer which may lead to a higher pathogen removal by attachment (Johnson et al., 1996; Bradford et al., 2013).

The removal of the bacteria is impacted by heterogenic processes during aquifer storage and the aquifer biogeochemistry. Hence, an initial characterization of the aquifer (pH, ionic strength, grain size, soil type, flow velocity, redox conditions) is required as they strongly influence the transport of microorganisms (Bradford et al., 2013). Several studies analysed the removal efficiency of MAR systems focusing on human pathogens and related human health risks after irrigation of crops or recreational areas (e.g., (Toze, 2004; Page et al., 2010; Ayuso-Gabella et al., 2011). In contrast, no study investigated the transport and removal of plant pathogens during MAR, although MAR treated water is often intended for irrigation and related consequences for plant health have high ecological and economic value. So far, only slow sand filtration (SSF) has been evaluated as efficient water treatment for the removal of different plant pathogens (Ferreira et al., 2012; Lee and Oki, 2013; Raudales et al., 2014). There, pathogens are removed by size exclusion (physical), interaction with the soil surface (chemical) or through interaction with the biological active layer ('*Schmutzdecke*') (biological). The soil aquifer treatment of an ASTR relies on similar processes occurring at the soil-water interphase (Ginn et al., 2002). However, SSF and ASTR differ in hydrological and geochemical conditions in terms of lithology, water saturation, redox conditions, and biological activity. This makes a direct comparison of both systems regarding pathogen removal difficult. Still, they both can improve water quality through filtration processes.

Lastly, field instead of column experiments could give a more realistic picture about pathogen removal. In field experiments, the bacteria are exposed to the complex processes within an aquifer which cannot all be simulated in a column study. However, biosafety regulations restrict or forbid (*R. solanacearum* is a quarantine organism) the use of field experiments. Therefore, column studies are a good alternative that also allow the control of critical parameters (pH, velocity, temperature, etc.).

Effect of contaminated irrigation water

This research hypothesizes that bacterial pathogens present in the source water will be removed sufficiently during ASTR. However, if the bacterial die-off in the water phase results in a persisting population due to non-linear die-off, there exists the risk that bacteria will be present in the recovered water. This may also be caused by detaching bacteria during soil filtration that get re-released into the water. The effect of these low pathogen concentrations present in irrigation water is not well studied. The virulence of plant pathogens is mostly analysed under favourable conditions for infection (e.g., invasive injection of bacteria), or by using very high inoculation concentrations (e.g., Lebeau et al. (2010)). Nevertheless, the lowest concentration (biological threshold) necessary to infect a plant is not known for *R. solanacearum*. Therefore, this research also analysed the effect of *R. solanacearum* contaminated irrigation water on potato plants. The relationship between the number of cells in irrigation water and the risk to cause disease can be expressed by a dose-response model. For *D. solani* and the SRP *P. parmentieri* the colonization of potato after spray-inoculation was studied but the authors did not develop a dose-response model from their results. Dose-response models are well established for human pathogens, for example to analyse the infectivity of *E. coli* after the consumption of contaminated food (Teunis et al., 2004). Thereafter, dose-response model are an integral part of quantitative microbial risk assessment (QMRA) (Schijven et al., 2011).

1.6 Quantitative microbial risk assessment (QMRA)

This study analyses the potential of an ASTR system to store TDW, improve water quality and recover it for irrigation. Plant pathogenic bacteria may be present in the infiltrated water and need to be removed during aquifer storage in order to minimize risks to disseminate plant diseases. Different risk management strategies exist to analyse a MAR system. The use of quantitative microbial risk assessment (QMRA) allows a prediction of the changes in water quality and the analysis of different pathogen removal mechanisms during ASTR (Imig et al., 2022). QMRA can calculate risk probabilities of different ASTR scenarios by analysing the following steps (Haas et al., 2014):

(i) **Hazard identification**: the biological agent causing harm is identified.

(ii) **Exposure assessment**: at first, the concentration of the plant pathogens in the source water is determined. Second, the quantitative removal of the pathogens during ASTR is assessed. The pathogen concentration will be reduced by attachment to aquifer sediment and by dieoff in the water phase and on solid surfaces . Both removal mechanisms have to be quantified experimentally or data should be obtained from literature. Lastly, the final exposure concentration is calculated using the source water concentration and pathogen reduction by the aquifer treatment.

(iii) **Dose-response analysis**: given a certain exposure concentration, the probability of infection or illness can be evaluated when the host (plant) gets in contact with the pathogen (bacterium). This requires knowledge about the dose-response relationship of the host-pathogen interaction which is also determined experimentally (Teunis and Havelaar, 2000).

(iv) **Risk characterization:** following steps (i)-(iii), the risk of causing plant infections after irrigation with ASTR treated surface water is calculated. It is estimated for a specific ASTR configuration (soil passage length, residence time) but can be compared with different ASTR scenarios. For example, the residence time can be adapted by the farmer, by only abstracting water a certain period after the last infiltration event, which influences the bacterial die-off in the water phase. Furthermore, the distance between infiltration and abstraction well determines the soil passage and consequently the bacterial removal by attachment (Dillon et al., 2009). A longer filtration distance may be necessary to reach higher pathogen removal if a high pathogen load is expected in the source water.

Lastly, ASTR can be compared with an ASR system that uses the same infiltration and abstraction well. Although water will pass through the porous medium of the aquifer during ASR which will cause pathogen removal by attachment, reverse flow during abstraction may also promote detachment, and the minimum residence time is less than with ASTR. Therefore, ASR can only rely on the residence time of the water in the aquifer and the related pathogen die-off in the water phase. This treatment may be sufficient when the pathogen die-off is expected to be high; or the time between infiltration and abstraction is long enough; or the source water is known to contain zero or very low pathogen concentration.

QMRA is a tool which is already successfully implemented in water reclamation projects that evaluated the removal of human pathogenic organisms to produce drinking water (WHO, 2011; Schijven et al., 2019). It has also been employed to analyse different MAR systems which were used to produce irrigation water for recreational areas or crop irrigation (Ayuso-Gabella et al., 2011; Page et al., 2015; Masciopinto et al., 2020). The related human health risks may not exceed a certain target concentration. For example, the acceptable concentration of *Campylobacter* in drinking water is 10^4 cells/L (WHO, 2011). Up until now, QMRA has not been used to analyse the removal of plant pathogens using MAR and the related risks for plant health. Water scarcity is increasing the need for water reclamation technologies to produce fresh water. QMRA can be a valuable tool to evaluate improvement in water quality and reveal weak points of a water treatment scheme. Additionally, it can help to define acceptable ('safe') concentrations of plant pathogens in irrigation water. Consequently, the reclaimed water will avoid drought related crop losses while protecting plant health while. To summarize, the goal for QMRA "is to have a matrix of conditions that allow proponents of MAR to predict or anticipate rates of natural attenuation for their site-specific conditions" as stated by Bekele et al. (2018).

1.7 Research questions

Main research questions

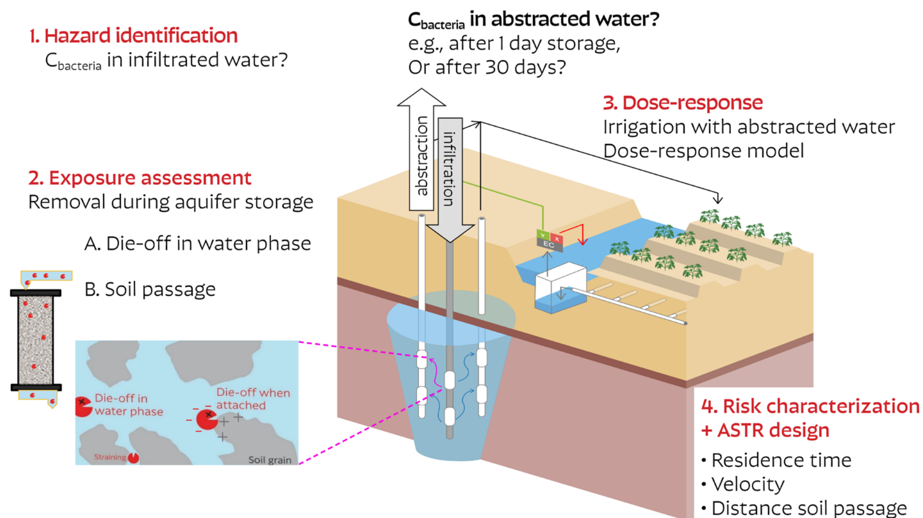
Can AS(T)R adequately reduce the number of bacterial pathogens if present in the tile drainage water that is used for infiltration and storage in the aquifer? Is the extracted water safe and of sufficient water quality (no or very low pathogen concentrations) to use it for crop irrigation?

Specific research questions

1. What are the die-off kinetics of *R. solanacearum*, *D. solani* and *P. carotovorum* in TDW and anoxic aquifer water using water microcosms?
    a. How does the presence of microbiota influence the bacterial die-off?
    b. How does temperature influence the die-off?
    c. How does oxygen influence the die-off?
2. How are the bacteria removed during the soil passage with ASTR as simulated with column experiments?
    a. What are the attachment and detachment rates of the bacteria in saturated porous media, reflecting aquifer conditions?
    b. How does porous media type or the grain size influence the bacterial transport?
    c. How do oxic or anoxic conditions influence the bacterial transport?
3. What is the dose-response relationship of the bacterial pathogen *R. solanacearum* and potato plants when contaminated irrigation water is applied?
    a. Is there an acceptable concentration of plant pathogens in irrigation water which does not cause disease?
    b. How does the inoculation method influence the dose-response relationship? Is one cell per plant enough to cause disease?
4. Can AS(T)R improve microbiological water quality and remove bacterial pathogens to reduce infection risks if treated irrigation water is used?
    a. What is the main pathogen removal mechanisms during ASTR?
    b. What is the required ASTR design and operation to reduce the risk of plant pathogens?
    c. Is ASR an alternative to ASTR to remove pathogen sufficiently and provide safe irrigation water?

1.8 Research approach and outline

To answer the research questions raised in Chapter 1.7 the following research approach is used and visualized in Figure 5. Steps 1-4 in Figure 5 represent the quantitative information which is required for the QMRA which simultaneously analyses the ASTR system and its potential to remove plant pathogenic bacteria. At first, the bacterial concentration in the infiltration water is assessed. Second, the removal of the bacterial pathogens is analysed in lab experiments where the environmental conditions of the aquifer are simulated. The die-off in the water phase is studied in water microcosms using either oxic TDW or anoxic aquifer water. Next to the effect of oxygen, the effect of temperature and microbiota on pathogen die-off are investigated. In soil column experiments, the transport of the bacterial pathogens in saturated porous media is studied. The research questions in 1.7 are answered using (i) the results from different lab experiments,(ii) the modelling of these results to predict removal of the bacteria during aquifer recharge, and (iii) the risk estimation of reusing ASTR treated water for irrigation.



▲ **Figure 5** Overview of the research approach to study the fate of plant pathogenic bacteria during aquifer storage transfer and recovery (ASTR) to produce irrigation water for crop production.

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Chapter 2
Die-off of plant pathogenic bacteria in tile drainage and anoxic water from a managed aquifer recharge site

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Highlights

- First study to investigate the water die-off of *D. solani* and *P. carotovorum* in natural waters, and of *R. solanacearum* in anoxic aquifer water
- Faster die-off in oxic tile drainage water at 10 °C and 25 °C than in anoxic aquifer water (10 °C)
- Longest die-off (about 3-log_{10}) in anoxic aquifer water took about 60 days
- A flexible, non-linear Weibull model with tail describes the bacterial die-off
- Results can be used to determine residence times in MAR systems

Abstract

Managed aquifer recharge (MAR) can provide irrigation water and overcome water scarcity in agriculture. Removal of potentially present plant pathogens during MAR is essential to prevent crop diseases. We studied the die-off of three plant pathogenic bacteria in water microcosms with natural or filtered tile drainage water (TDW) at 10 and 25 °C and with natural anoxic aquifer water (AW) at 10 °C from a MAR site. These bacteria were: *Ralstonia solanacearum* (bacterial wilt), and the soft rot Pectobacteriaceae (SRP) *Dickeya solani* and *Pectobacterium carotovorum* sp. *carotovorum* (soft rot, blackleg). They are present in surface waters and cause destructive crop diseases worldwide which have been linked to contaminated irrigation water. Nevertheless, little is known about the survival of the SRP in aqueous environments and no study has investigated the persistence of *R. solanacearum* under natural anoxic conditions. We found that all bacteria were undetectable in 0.1 mL samples within 19 days under oxic conditions in natural TDW at 10 °C, using viable cell counting, corresponding to 3-$\log_{10}$ reduction by die-off. The SRP were no longer detected within 6 days at 25 °C, whereas *R. solanacearum* was detectable for 25 days. Whereas in anoxic natural aquifer water at 10 °C, the bacterial concentrations declined slower and the detection limit was reached within 56 days. Finally, we modelled the inactivation curves with a modified Weibull model that can simulate different curve shapes such as shoulder phenomena in the beginning and long tails reflecting persistent bacterial populations. The non-linear model was shown to be a reliable tool to predict the die-off of the analysed plant pathogenic bacteria, suggesting its further application to other pathogenic microorganisms in the context of microbial risk assessment.

2.1 Introduction

Agricultural production requires intensification to satisfy the demands of the expanding world population. Nevertheless, crop production is often impaired by water shortages caused by droughts or the availability of fresh water (Coulter, 2004). For example, groundwater in coastal regions is not an option as irrigation source because it is often brackish (Oude Essink et al., 2010). Moreover, surface water can be unsuitable for irrigation as it may carry plant pathogens causing diseases such as brown rot to (seed) potatoes and ornamentals (Hong and Moorman, 2005). The implementation of water management strategies can reduce stress on water resources. Thereby, the potential of water reuse in agriculture gains attention with focus on the quality of the recycled water (Alcalde-Sanz and Gawlik, 2017). A nature based solution for water reuse is managed aquifer recharge (MAR) that collects excess tile drainage water after heavy rain events to store it in the subsurface. The stored water remains protected from evaporation and is available for irrigation or the management of the groundwater table in the field throughout the year. In this study, we investigate the dieoff of bacterial plant pathogens in different water types from a MAR site to mimic the injection of contaminated water into the MAR system.

The *Ralstonia solanacearum* species complex (RSSC) comprises three different species, namely *R. solanacearum*, *R. pseudosolanacearum* and *R. syzigii*. They can cause bacterial wilt also known as brown rot, a highly destructive disease that affects more than 200 plant species (Hayward, 1991; Fegan and Prior, 2005). This pathogen complex has a quarantine status in the European Union (Directive, 1998). In temperate climates, potato production is affected by *R. solanacearum* race 3 biovar 2 (phylotype II), a species adapted to cooler regions (Janse, 1996). As a reaction to a high number of potato brown rot outbreaks in Europe in 1995, several measures were taken to control the disease, including seed testing. Although these regulations have shown some success, they still could not eradicate the disease completely (Janse, 2012). In addition to latent infected seeds, other inoculum sources play a role in the sporadic infections, as the bacteria can survive in the environment, outside the potato plant. In the Netherlands, *R. solanacearum* has been detected in surface water over the past 20 years and brown rot disease outbreaks were directly linked to the use of contaminated surface water as irrigation source (Wenneker et al., 1999; NVWA, 2018). As a result, an irrigation ban with surface water for seed potato production has been set in place which effectively reduced brown rot disease outbreaks (Janse, 2012). Nevertheless, the measure reduced freshwater sources for irrigation.Similar to bacterial

wilt, blackleg and soft rot diseases caused by *Dickeya* and *Pectobacterium* species within the family of soft rot Pectobacteriaceae (SRP) have a negative impact on potato production (van der Wolf and De Boer, 2007). The host range of the pectinolytic bacteria *Dickeya solani* and *Pectobacterium carotovorum* sp. *carotovorum* is not restricted to potato, they also affect other food crops and ornamental plants. For example, soft rot diseases pose a threat to the highly profitable flower bulb industry, whereas bacterial wilt caused by *R. solanacearum* has caused high damage in glasshouse ornamental crops (Ma et al., 2007). Although SRP are well studied pathogens, measures to control the pathogens in the field are only partially effective. Latently infected seed material is the main cause for the dissemination of the pathogens. Still, soft rot and blackleg appear during the cropping season even if pathogen-free seed material grown via micropropagation was used (Charkowski, 2018). The entry points are diverse as the bacteria have been observed in the environment, including plant debris, soil or waterways (Perombelon and Kelman, 1980; Laurila et al., 2008). Moreover, insects, nematodes, or aerosols emitted during field work contribute to the spread of SRP. In some studies, disease outbreaks were related to the usage of contaminated irrigation water (Cappaert et al., 1988). Kastelein et al. (2020) reported that spray inoculation of potato leaves with 10^2 CFU/mL *D. solani* resulted in diseased plants in greenhouse experiments.

A solution to provide irrigation water free of pathogens can be MAR, where excess freshwater is stored in the subsurface and serves as an irrigation reservoir (Dillon et al., 2019). In this research, we investigate a MAR system which uses aquifer storage, transfer and recovery (ASTR) technology that injects collected fresh tile drainage water (TDW) into an anoxic brackish aquifer. The TDW is excess rain water, that percolates through the upper soil layer into drains, buried below the agricultural field. Depending on the agricultural practices and period of the year, the chemical and biological composition of the TDW may change. For example, during the cropping season fertilizers are applied which can lead to increased nitrate concentrations in the TDW. In ASTR the injected water travels through the soil towards the extraction well. We expect that this natural soil filtration step removes agropollutants, including bacterial pathogens, in different removal processes. The major processes are die-off in the water phase, irreversible attachment to sediment particles, straining, as well as die-off through predation and competition with other microorganisms (Bradford et al., 2013). Very little information is available about the removal efficiency of pathogens during MAR as the potential of subsurface treatment has been poorly recognized (Page et al., 2010). The irrigation water can be considered safe, if the pathogen load of the recycled water is below the threshold

inoculum level which does not lead to the infection of crop plants. As part of the risk estimation of reusing TDW for agricultural irrigation, we need to determine the removal of the pathogens from the system.

Few studies investigated the die-off of *R. solanacearum* in natural water types such as river water (Álvarez et al., 2007), agricultural drainage water (Elsas et al., 2001), or drainage water leaching from rock wool in a rose-growing greenhouse (Stevens et al., 2017). The results of these studies showed that temperature and the natural microbiota have a significant effect on the die-off of *R. solanacearum* in non-sterile microcosms compared with sterile conditions. However, no study was found that describes the die-off of the bacterium under anoxic conditions in natural water, which is highly relevant for their fate in saturated soil conditions, as found in the aquifer or deeper soil layers where oxygen is limited. To our knowledge, only one publication examined the inactivation of *D. solani* and *P. carotovorum* in sterile waters, but not in waters under natural conditions (van Doorn et al., 2008).

The aim of this study was to investigate the die-off of *R. solanacearum*, *D. solani,* and *P. carotovorum* sp. *carotovorum* in natural TDW and anoxic aquifer water from an agricultural field and to develop a die-off model. Knowledge about the survival of these plant pathogens in the agro-ecosystem is relevant for a successful management of the disease. The model can be used in microbial risk assessment to predict the die-off of the pathogens in natural waters to minimize the risks of irrigation related disease outbreaks. For a safe MAR application, the input for the risk estimation requires the precise description of the bacteria's die-off. So far, the die-off of *R. solanacearum* has been described using log-linear kinetics. Linear models may describe the die-off of the bacteria insufficiently by over- or underestimating their persistence, especially over prolonged time periods. A bacterial population may vary in resistance to environmental stresses and, therefore, die-off follows a non-linear pattern instead of a log-linear (first-order rate) (van Boekel, 2002). It has been shown for *R. solanacearum* that different subpopulations are present during the die-off in microcosms. Moreover, the bacteria underwent morphological changes or entered into the viable but non-culturable (VBNC) state (Elsas et al., 2001; Álvarez et al., 2008). For a better prediction of the bacterial die-off, we applied a non-linear Weibull + tail, a Weibull, and a log-linear model, modified after Albert and Mafart (2005), and selected the best model. It takes into account persistent bacterial populations that may pose a threat to crop production. Finally, the selected die-off models are a crucial component in risk assessments that aim to develop guidelines for the safe application of recycling water systems such as MAR.

2.2 Material and Methods

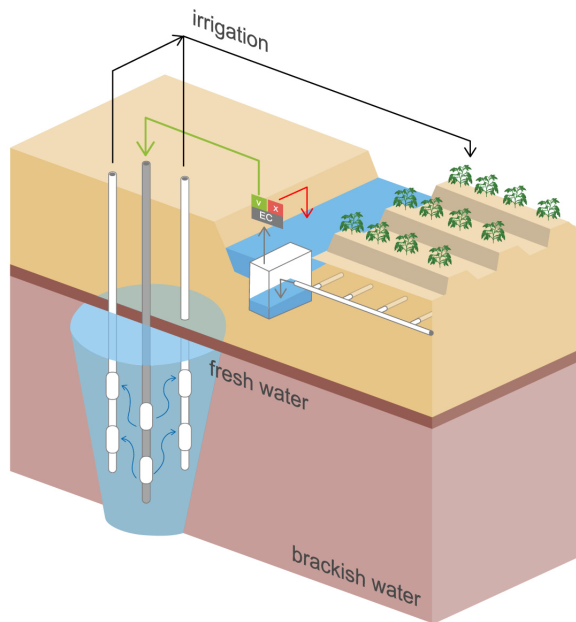
2.2.1 Bacterial strains and growth conditions

R. solanacearum race 3 biovar 2 (phylotype II) strain IPO-1828, *D. solani* IPO-2266 and *P. carotovorum* sp. *carotovorum* IPO-1990 were used in this study. They were received from the working collection at Wageningen UR. Strains were kept at -80 °C using the multi-purpose protect cryobeads system (Technical Services Ltd). The strain of *R. solanacearum* used in this study is naturally resistant to rifampicin and was re-isolated from tomato plants infected with the Dutch strain IPO-1609, originally isolated from potato. It was grown on non-selective Yeast Peptone Glucose Agar (YPGA) (EPPO, 2004), prepared with 5 g/L yeast extract, 5 g/L peptone, 10 g/L glucose, 15 g/L agar, and supplemented with rifampicin (50 mg/L). Liquid cultures were prepared in Casamino acid Peptone Glucose (CPG) broth (Hendrick and Sequeira, 1984) composed of 1 g/L casamino acids, 10 g/L peptone, and 5 g/L glucose. *D. solani* and *P. carotovorum* sp. *carotovorum* were both naturally resistant to streptomycin and were grown on non-selective Tryptone Soya Agar (TSA; Oxoid; Thermo Fisher Scientific) supplemented with streptomycin (100 mg/L). Liquid cultures of these bacteria were prepared in LB (Luria Bertani) medium. Duchefa Biochemie (Haarlem, The Netherlands), Sigma-Aldrich (St. Louis, MO), and Fisher Scientific (Hanover Park, IL) were our chemical suppliers.

2.2.2 Water samples

The research site as part of the Spaarwater project (Acacia Water, 2019) initiated by Acacia Water B.V. is located on a 1.5 ha field in Borgsweer, situated in the province of Groningen, the Netherlands, near the Wadden sea (53.2945693 N, 7.0045595 E). Access to the field site was granted by Acacia Water B.V. and the farmer. The marine clay topsoil is laying on a thick aquitard layer of 8 m depth consisting of clay and peat, and is optimal for the cultivation of (seed) potatoes. The irrigation with surface water is forbidden by Dutch law with the risk of spreading the brown rot causing *R. solanacearum*. The ASTR system, as shown in Figure 1, is used to store fresh TDW beneath the aquitard layer, in the underlying, confined unconsolidated sandy aquifer stretching over a depth of 8 m to 30 m below surface level. The aquifer consists of layers originating from different geological formations, resulting in different hydraulic conductivities (4-45 m/day). After infiltration, the fresh water is available for later abstraction and can be used as irrigation water in dry summer periods. Below the parcel, a network of drainage pipes is buried which are connected to a collection drain that discharges into a built concrete reservoir (volume is ca. 1 m^3). In this reservoir, the electrical conductivity (EC) of the TDW is continuously sensed as measure of salinity.

The water is used for infiltration into the aquifer if the EC does not exceed a set value (EC = 1700 μS/cm) needed for the irrigation of the selected crops. Otherwise, the TDW is discharged into the surface water system along the agricultural fields. As the pilot was no longer in operation, the last infiltration event dated back to more than a year ago. The tile drainage system was still operable and collected TDW which was automatically discharged into the surface water canal neighboring the field. The microbial aquifer community in the aquifer was therefore not influenced by any recent TDW injection. Two water samples were collected at this research site for the water microcosm experiments (described later).



▲ **Figure 1** Schematic representation of an agricultural field connected to a managed aquifer recharge site. The site is designed as an aquifer, storage, transfer, and recovery (ASTR) system. Excess rain water reaches the tile drainage system buried at 70 cm depth. The collection drain terminates into a concrete reservoir where the EC of the tile drainage water is measured. If the EC is below a set threshold value, the water is infiltrated via the injection well (depicted in grey). From there, the water travels through the sandy aquifer to the extraction wells (depicted in white) and can be used for irrigation.

Sampling

All water samples were collected in autoclaved 1- or 2-L glass bottles and completely filled before closing. Water quality parameters such as pH, EC, oxygen concentration and water temperature were measured in the field using a portable multimeter with respective probes (Odeon, Aqualabo, France). In the laboratory, Hach Lange kits were used to measure nitrate, phosphate, chemical oxygen demand (COD), and ammonium. The kits are based on colorimetric assays and the concentrations were assessed using a VIS-spectrophotometer (DR3900, Hach Lange, Germany). To be able to collect fresh TDW, we awaited a rain event which would allow the flushing of the drainage system. On a first field visit in April 2018, oxic TDW water was collected from the TDW collection point (concrete reservoir) of the MAR system. During a second visit in December 2018, anoxic water from the aquifer (9-10 m below surface level) was pumped from a monitoring well with a 1 m long filter screen, using a peristaltic pump (Watson Marlow 704U/R) at a flow rate of 12 L/min. The tubing system was cleaned before sampling using a 10 times diluted solution of commercial bleach, followed by pumping for 10 min from the selected well to replace the standing water in the well at least 3 times. The anoxic water was filled into 2 L Duran glass bottles that were priorly flushed with N_2 gas. The tube connected to the pump was placed at the bottom of the bottle. The bottle was then filled from bottom to top, while keeping the tube inside the water to limit the introduction of air. After filling the bottles completely with water, they were first closed with a black rubber stopper, and then closed with a plastic screw cap with aperture, which allows to take a water sample with a needle and syringe while maintaining anoxic conditions.

2.2.3 Die-off experiments with water from a MAR site

The die-off experiments were conducted with naturally oxic tile drainage water and with anoxic aquifer water. As summarized in Table 1, the waters have been treated in four different ways before the bacterial pathogens were added: (i) natural oxic TDW (containing nitrate) or anoxic aquifer water (almost without nitrate) without treatment, maintaining the whole microbiota; (ii) 0.22 μm filtered TDW, to remove most of the microbiota; (iii) autoclaved TDW to inactivate all microbiota as control; (iv) natural aquifer water with the addition of 50 mg/L NO_3^- (added as sodium nitrate); to assess the possible influence of nitrate, present in TDW as a result of agricultural practices. This nitrate concentration was chosen as it is representative for concentrations found in Dutch surface waters receiving TDW. Nitrate will be present in the aquifer until the infiltrated TDW is denitrified. For both the natural microcosms of oxic TDW and anoxic AW a non-inoculated control microcosm was prepared. The oxic TDW microcosms were incubated

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at 10 °C or 25 °C in the dark while shaking (150 rpm). Anoxic experiments were conducted only with natural aquifer water at one temperature (10 °C). Experiments with natural aquifer water where nitrate was added, were only tested with *R. solanacearum* and *D. solani*. The temperatures were chosen as they represent the nearly constant temperature in the aquifer (10 °C); while 25 °C reflects infiltration of rainfall events during the warmer summer months. Moreover, the latter covers a temperature that is more representative of tropical regions, where these bacteria also play an important role as disease causing organisms.

▼ **Table 1** Experimental conditions for the bacterial inactivation study in different water types.

Water type	**Tile Drainage**			**Aquifer**	
Redox	Oxic			Anoxic	
Temperature [°C]	10 or 25			10	
Nitrate*	41 mg/L			< 0.5 mg/L	
Treatment	natural	0.22 µm filtered	autoclaved	natural	natural + 50 mg/L NO_3
Inoculation concentration of bacterial pathogen	10^4 CFU/mL				

* average concentrations at Borgsweer pilot site, 2016-2017 (Acacia Water, 2019)

For experiments under natural conditions, the water samples were used 1-4 days after collection to avoid changes in the microbial composition. For die-off experiments in filtered water, TDW was filtered using a sterile 0.22 µm pore-size cellulose acetate filter membrane (Sartorius, Germany). Autoclaved conditions were achieved by pressure-sterilization at 121 °C for 20 min. For die-off experiments in 0.22 µm filtered or autoclaved conditions, the samples were stored at 4 °C until usage. Note, filtered and autoclaved experiments are artificial conditions not representative of natural conditions during MAR. They served as control experiments to analyze the effect of microbiota on the pathogens as well as the general persistence of pathogens in sterile water.

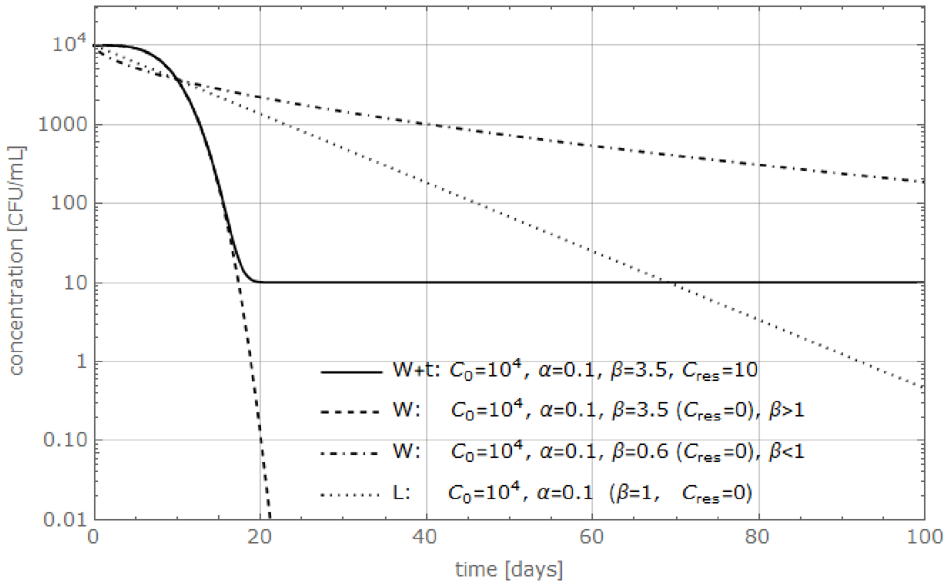
The inoculation suspensions were prepared by growing *R. solanacearum* for 15 h in 5 mL CPG broth and *D. solani* or *P. carotovorum* sp. *carotovorum* for 12 h in 5 mL LB liquid medium. Culture conditions were 25 °C and 150 rpm, after which the grown cultures were harvested by centrifugation (3500 x *g*, 20 min at room temperature) followed by washing and resuspending the pellet in 5 mL a quarter strength Ringer's solution (Sigma-Aldrich; St. Louis, USA). This pelleting and washing step was repeated twice to remove any excess broth. The bacterial suspension was then diluted to reach an optical density of 0.1 at 600 nm representing a concentration of 10^8 CFU/mL which was also confirmed by dilution-plating. For the aerobic experiments, 1 L of each microcosm solution was prepared in a sterile glass flask and 0.1 mL of 10^8 CFU/mL bacterial suspension was added to reach a final concentration of 10^4 CFU/mL. From this suspension, 200 mL were transferred to 250 mL Duran glass bottles. This procedure could not be used in the anoxic experiment as the introduction of oxygen had to be excluded. There, 180 mL serum bottles (Wheaton Scientific, Millville, USA) were flushed with N_2 gas, closed with a butyl rubber stopper and a metal crimp, and autoclaved. With a 50 mL syringe attached to a needle, a total of 100 mL of the aquifer water was transferred to the experimental flask. Before the transfer, the syringe was flushed three times with N_2 gas to avoid oxygen contamination during the transfer. Each flask had to be inoculated individually. The inoculation solution was prepared as described previously; but here, 0.1 mL of 10^7 CFU/mL bacterial suspension was added to the 100 mL microcosm to reach a final concentration of 10^4 CFU/mL. The concentration of 10^4 CFU/mL as inoculum was chosen as the pathogen concentrations in natural water systems are often found to be very low. Wenneker et al. (1999) detected *R. solanacearum* in ditches next to agricultural fields at concentrations ranging between 10^2 – 10^5 CFU/mL. Higher inoculum density may give the introduced pathogenic bacteria a competitive advantage against other microorganisms. Furthermore, mimicking more realistic concentrations with lower starting concentrations in the laboratory is not convenient as high volumes of water are necessary for the detection. The chosen starting concentration is a compromise and allows to follow the inactivation over a sufficiently long time period and the collection of sufficient data points for establishing the type of die-off kinetics by modelling. All microcosms were prepared in duplicates and the bacterial numbers were evaluated by dilution plating in duplicates per time point.

2.2.4 Enumeration of bacteria from water microcosms

Sampling from the microcosms was performed regularly after inoculation until the last sampling point was reached, which was at the detection limit of 3-10 CFU/mL or below the detection limit when no colonies were culturable anymore. *R. solanacearum* was enumerated on semi-selective medium South Africa (SMSA) (Elphinstone et al., 1998b) supplemented with 50 mg/L rifampicin to suppress the growth of background bacteria. *D. solani* and *P. carotovorum* sp. *carotovorum* were incubated on the selective double layer-crystal violet pectate (DL-CVP) medium (Hélias et al., 2012) supplemented with 100 mg/L streptomycin, where the bacteria form distinct cavities in the upper layer of the medium. The selective media have been chosen as they showed a higher recovery rate from the environmental water samples in comparison to TSA supplemented with the respective antibiotic (data not shown). Moreover, they better suppress the growth of non-specific bacteria. The initial concentration of indigenous culturable bacteria in the natural oxic and anoxic water samples was assessed by plating 0.1 mL on non-selective TSA and low-nutrient Reasoner's 2A (R2A) agar (Oxoid; Thermo Fisher Scientific) and incubated at 25 °C. Anaerobic water samples were incubated anaerobically at 25 °C using Oxoid AnaeroGen sachets (Oxoid; Thermo Fisher Scientific) to create an anaerobic atmosphere in a closed container. Additionally, total numbers of culturable bacteria were enumerated in all oxic TDW microcosms from both temperatures at the end of the dieoff period of the SRP. For sampling, microcosms were mixed by vigorous shaking, then 0.1 - 0.3 mL of water sample was taken for detection of the bacteria by dilution plating on the respective medium in duplicates. Plates were incubated three to four days at 28 °C before counting. Water samples from anoxic microcosms were sampled through the rubber septum with a syringe and needle to maintain anaerobic conditions in the microcosm; afterwards the plates were incubated aerobically. We define dieoff as the process that renders bacterial cells not culturable anymore.

2.2.5 The die-off model

As a first step, the bacterial concentration defined as culturable cells per mL was plotted against time. The resulting graph gave insights into the course of the die-off. Three types of die-off models were considered, as shown in Figure 2.



▲ **Figure 2** Different die-off models were simulated with given parameter estimates (see values in the graph). The population concentration in CFU/mL is plotted against time in days. W+t stands for Weibull + tail, W for Weibull, and L for log-linear model.

Our results showed that in most cases, the bacteria did not follow a linear die-off behavior. Moreover, the initial bacterial population stayed stable over a period of time resulting in a curve with a shoulder (see Fig 2, model W+t and W). Another feature of the curve can be a tail-shape which represents a persistent population at the end of the experiment (see Fig 2, model W+t). To account for these curve characteristics, a model based on the work of Albert and Mafart (2005) to describe microbial dieoff was chosen. In the further text we refer to it as the Weibull + tail (W+t) model:

$$C_t = (C_0 - C_{res})\, e^{-(\alpha t)^{\beta}} + C_{res} \quad \text{(1: W+t)}$$

where C_t is the bacterial concentration in CFU/mL at time t, C_0 the initial bacterial concentration in CFU/mL (at time $t = 0$), C_{res} [CFU/mL] is the residual

bacterial population at the end of the observation period, and $t, \alpha, \beta > 0$. α [1/day] is a scale parameter and β [-] a shaping parameter to display convexity of a curve if $0 < \beta < 1$, or simulates a shoulder effect when $\beta > 1$. C_0, C_{res}, α and β are unknown parameters that have to be estimated.

The Weibull model (Peleg and Cole, 1998) describes microbial inactivation as a statistical distribution of die-off times. Thereby, it accounts for the heterogeneity of a bacterial population where different subpopulations exist with different resistances to the exposed stress. Consequently, α is not a single die-off rate constant that can be applied to the whole bacterial population, but the mean of the distribution describing the death times. Van Boekel (2002) concludes that the Weibull is an empirical model that can still be linked to physiological effects, where α relates to microbial inactivation, while the dependency of β can be linked to abiotic and biotic conditions and the adaptation of the bacteria to the external stress. Furthermore, the authors state that if $\beta < 1$, the remaining cells are better adapted to stress and have less probability of dying. In contrast, $\beta > 1$ implies that the cells are increasingly damaged and susceptible to stress. The Weibull + tail model can be reduced to the non-linear Weibull (W) model if there is no tailing and the parameter $C_{res} = 0$:

$$C_t = C_0 \, e^{-(\alpha t)^{\beta}} \qquad \text{(2: W)}$$

A further reduction of the Weibull model results in a log-linear model (L) if $\beta = 1$, corresponding to first order die-off. As consequence, the die-off probabilities are not time dependent anymore, and α is a constant rate parameter. Lastly, this implies that the bacterial population is homogenous and the cells are equally susceptible to environmental stresses.

$$C_t = C_0 \, e^{-(\alpha t)} \qquad \text{(3: L)}$$

A visual selection of the model just by looking at the curve shape is useful, but fails to exactly determine the best model fit. All three models were applied to fit the experimental data and the best model was chosen using the dimensionless Akaike Information Criterion (AIC) (Akaike, 1974). The AIC considers the goodness of fit and parsimony of the models. For our experiments, the model with the smallest AIC value was therefore chosen as best model.

Statistical analysis

The data was analyzed using the *R* software (R Core Team, 2022) (version (v.) 1.2.1335) with different statistical packages: (i) 'dplyr' to organize the data (*v. 0.8.3,* (Wickham et al., 2019)); (ii) 'nlstools' for the statistical analysis and non-linear modelling (*v. 1.0-2*, (Baty and Delignette-Muller, 2015)); (iii) 'investr' to plot the non-linear model including prediction intervals (*v. 1.4.0*, (Greenwell and Schubert Kabban, 2014)) and (iv) 'nlshelper' to use F-tests or ANOVA as analysis if the estimated coefficients were different between groups (*v. 0.2*, (Duursma, 2017a)). Non-linear modelling using *R* is well described by Duursma (2017b), as well as the utilization of the 'nlstools' package by Baty et. al. (2015). The package also allows to examine the model fit by analyzing the distribution of the residuals. The R code used in this study is described in the Supplementary information. The data frame consisted of a total of 986 observations with 4 to 22 data points per experiment. The various treatments of the microcosms, as well as the temperature and redox conditions were evaluated as categorical variables or factors.

2.3 Results

2.3.1 Description of field site/initial conditions

In natural TDW at 10 and 25 °C, the background culturable bacterial levels in the control microcosms were 10^2 CFU/mL grown on TSA and R2A, and reached 9.6 – 9.8 x10^3 CFU/mL after 16 days, grown on TSA or R2A respectively. In the control microcosms of anoxic AW with or without nitrate, the concentration of culturable indigenous bacteria was about 2.3 x10^2 – 4 x10^3 CFU/mL, grown on TSA or R2A respectively. Table S1 shows an overview of the enumeration of culturable microorganism on TSA and R2A present in the native TDW and AW. No background bacteria were culturable on SMSA supplemented with rifampicin. Only a few unspecific bacteria were growing on the upper layer of DLCVP supplemented with streptomycin that were clearly distinguishable from the cavity forming soft rot bacteria. Initially, no bacteria were detected when plating the filtrated TDW on TSA. However, at the end of the experiment with SRP at 25 °C, some colonies appeared (10 – 10^2 CFU/mL) when plating the filtered TDW, as maybe a few bacteria passed the filter and recovered during the experiment. Long-term persistence of the pathogens was demonstrated in the autoclaved microcosms where optimal conditions (available nutrients, no competition with other microorganism) resulted in growth of the pathogens in the beginning of the experiments. In the autoclaved microcosms

the physicochemical composition of the water is changed during the heating process. In contrast, the filtration only removes the biotic fraction from the water but leaves the abiotic conditions unchanged. This allows to study the influence of the biotic factors in oxic microcosms when comparing natural and 0.22 µm filtered TDW. Table 2 gives an overview of the water quality at the MAR pilot site.

The measured values of the chemical oxygen demand (COD) represent the total organic compounds in the water and they are representative for the range found in unpolluted surface waters (around 20 mg/L). Overall, the TDW is oxic and contains nitrate levels in the order of 50 mg NO_3/L. The AW is anoxic and almost free from nitrate (see also Acacia Water (2019)). The temperature of the TDW varies with the season, while the AW remains at a constant temperature of around 10 °C. Total phosphate and nitrate concentrations did not change after 0.22 µm filtration or autoclaving of the TDW.

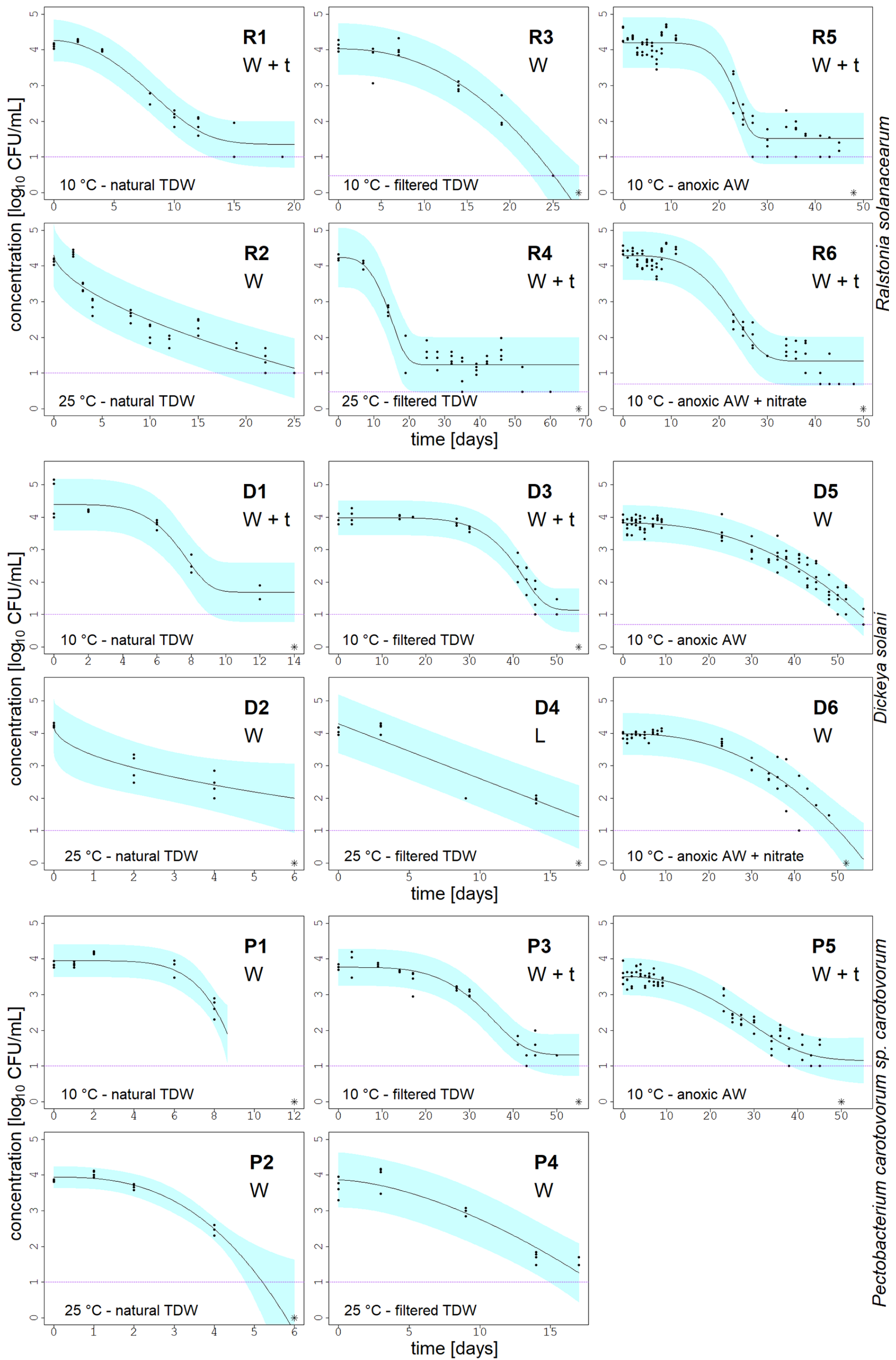
▼ **Table 2** Water quality of natural waters at a managed aquifer recharge site

Water quality parameters		**Tile drainage water**		**Aquifer water**
		2018/04/17	2018/12/10	2018/12/10
measured in the field	Electrical Conductivity [µS/cm]	1940	1490	1150
	pH	6.7	6.9	7.3
	Temperature [°C]	9.5	7.4	8.3
	O_2 [mg/L]	3.35	8.42	0.33
measured in the laboratory	Chemical Oxygen Demand (COD) [mg O_2/L]	21.2	29.5	20.6
	Nitrate (NO_3) [mg/L]	24.6	70.3	0.4
	Total phosphate (PO_4) [mg/L]	0	0.02	0.28
	Ammonium (NH_4) -N [mg/L]	0	0.17	0.94

2.3.2 Bacterial die-off in water microcosms

The die-off of three bacterial plant pathogens was studied in microcosms filled with natural or treated water at different temperatures. A total of 17 datasets were evaluated and the fitted die-off curves are shown in Figure 3. The experiments were concluded when the limit of detection of 3-10 CFU/mL was reached (visualized as horizontal dotted line in Figure 3) or the bacteria were no more detectable by plating (below the detection limit, as marked with an asterisk in Figure 3). To select the best model fit, the Akaike information criterion (AIC) values (Table S2) of the three models were compared after fitting the data, and the model with the smallest AIC is chosen. The Weibull + tail model, as well as the Weibull model were both selected eight times as the best model. Only in one case, the die-off of *D. solani* in filtered TDW at 25 °C, the log-linear model was selected as the best model, although the AIC values for the Weibull and log-linear model were very similar (see Table S2). Nevertheless, the Weibull + tail model could only be fitted to the data if the bacterial concentration remained constant at the end of the die-off period, resulting in a tail shape in the plotted curve. The results of experiments in autoclaved microcosms are shown in Fig S1. There, the bacteria started to grow at 10 and 25 °C reaching a concentration of 10^6 CFU/mL followed by a slow decline until they reached a concentration of at least 10^3 CFU/mL which lasted until the end of the observation period (150 days). The only exception from this trend was the dieoff of *R. solanacearum* in the autoclaved TDW at 10 °C which rendered nonculturable after a period of approximately 30 days. In the further subsections, the die-off of each bacterium is described in detail and Table 3 shows an overview of the estimated model parameter values. The ANOVA analysis revealed that our tested treatments resulted in significantly different bacterial dieoffs ($P < 0.05$). In anoxic AW, the addition of nitrate did not have a significant effect ($P = 0.23$) on the die-off of *R. solanacearum.* In contrast, the addition of nitrate had a significant effect on the die-off of *D. solani* under the same conditions ($P = 0.003$). There, the bacterial die-off was slower (56 days) if no nitrate was added, as nitrate addition accelerated the die-off period (48 days).

▼ **Figure 3** Die-off of *Ralstonia solanacearum*, *Dickeya solani*, and *Pectobacterium carotovorum* sp. *carotovorum* in microcosms under varying conditions, shown as $\log_{10}$ [CFU/mL] vs. time [days]. The experimental conditions are displayed in the lower left corner of each graph. Points represent the plate counts in duplicate of two microcosms per treatment. The solid black line displays the model fitted to the data. The blue area depicts the 95% prediction interval of the model and the purple dotted line is the detection limit. The model applied used to fit the data is shown in the right corner of each graph: W+t stands for Weibull + tail, W for Weibull and L for log-linear model. The asterisk sign accounts for the last measurement point, where no more viable colonies were detectable.



R1
W + t
10 °C - natural TDW
R3
W
10 °C - filtered TDW
R5
W + t
10 °C - anoxic AW
R2
W
25 °C - natural TDW
R4
W + t
25 °C - filtered TDW
R6
W + t
10 °C - anoxic AW + nitrate
Ralstonia solanacearum
concentration [log10 CFU/mL]
time [days]
D1
W + t
10 °C - natural TDW
D3
W + t
10 °C - filtered TDW
D5
W
10 °C - anoxic AW
D2
W
25 °C - natural TDW
D4
L
25 °C - filtered TDW
D6
W
10 °C - anoxic AW + nitrate
Dickeya solani
P1
W
10 °C - natural TDW
P3
W + t
10 °C - filtered TDW
P5
W + t
10 °C - anoxic AW
P2
W
25 °C - natural TDW
P4
W
25 °C - filtered TDW
Pectobacterium carotovorum sp. carotovorum

2.3.3 *Ralstonia solanacearum*

The population of *R. solanacearum* in natural TDW declined by 3-log_{10} within 19 days at 10 °C, and within 25 days at 25 °C (Fig 3, R1 & R2). The die-off in 0.22 µm filtered TDW took longer in comparison to the untreated conditions because most of the indigenous microbiota was removed during the filtration. Therefore, the faster die-off in natural water microcosms can be attributed to the presence of the microbiota because the abiotic factors stayed the same. Furthermore, the die-off in filtered TDW underlined the temperature sensitivity of *R. solanacearum.* While the detection limit in filtered TDW at 10 °C was reached after 25 days, the die-off in the same water type at 25 °C was more than two times longer. A similar observation was made in autoclaved TDW at 10 °C, where *R. solanacearum* is no longer culturable after a period of 30 days, even though the bacteria were culturable for a long period of 150 days at 25 °C (see S1 Fig). In filtered TDW at 25 °C, the bacteria persisted at a low concentration of ca. 10 CFU/mL for up to 60 days creating a long tailshape in the plotted curve (see Fig 3, R4), which can also be observed under anaerobic conditions for a period of ca. 25 days (Fig 3, R5 & R6). Surprisingly, the die-off of *R. solanacearum* under anoxic conditions took about 45 days and was two times slower than in natural TDW despite the low temperature of 10 °C and absence of oxygen. To summarize, regardless of the water microcosm or treatment, and a starting concentration of 10^4 CFU/mL, the concentrations of *R. solanacearum* decreased by 3-log_{10} within the first 25 days of the experiments but resulted in many cases in a persisting population (Fig 3, R2, R4-R6). In comparison to *D. solani* and *P. carotovorum* sp. *carotovorum*, the plotted curves of *R. solanacearum* have the most prominent shoulder and tailing phenomena. Four out of the six datasets are fitted best with the Weibull + tail model.

2.3.4 Soft rot *Pectobacteriaceae*

The inactivation behavior of the soft rot *Pectobacteriaceae* (SRP) *P. carotovorum* sp. *carotovorum* and *D. solani* were very similar as seen in Fig 3, D & P(1-6). We observed the quickest die-off of the tested SRP in natural oxic TDW at 25 °C, where the bacteria were culturable for four days (Fig 3, D2 & P2); and for a maximum of 12 days at 10 °C (Fig 3, D1 & P1). In contrast, the die-off in filtered TDW was decelerated at least three times compared with untreated TDW. In filtered TDW at 10 °C, the bacteria showed a comparably slow die-off: the populations were stable (shoulder phenomenon, Fig 3, D3 & P3) for about 30 days, after which the concentrations decreased continuously, reaching the detection limit after 50 days. The die-off in the same water type at 25 °C was much faster; within 17 days (Fig 3, D4 & P4). The faster die-off at 25 °C compared to 10 °C was noticeable in untreated and in filtered TDW,

implying lower resistance of the SRP at a higher temperature. Still, the die-off in autoclaved TDW was similar at both temperatures. Interestingly, the die-off of *D. solani* in filtered TDW at 25 °C is the only dataset that follows firstorder inactivation. The datasets of *D. solani* in anoxic AW at 10 °C could both be fitted with the Weibull model (Fig 3, D5 & D6), while the die-off of *P. carotovorum* sp. *carotovorum* in anoxic AW was fitted with the Weibull + tail. The tailing phenomenon that was clearly visible in the inactivation curves of *R. solanacearum* (e.g., Fig 3, R4), is shorter in the curves of the SRP (Fig 3, D1 & D3, P3 & P5). As with *R. solanacearum*, the die-off of the SRP can be better explained by using the non-linear regression models.

2.3.5 Parameter estimation

The parameter estimates of the best fitting model per bacterial inactivation curve are summarized in . The C_{res} values were similar in all models, indicating this concentration may be a critical density for the bacterial population to persist. Nonetheless, these values were slightly above the limit of detection. Note, that in all our experiments the bacteria were no more detectable by plating at the end of the observation periods. A persisting population was only observed in the autoclaved, artificial microcosms (see S1 Fig). The α parameter ranges between 0.03 and 4.61 1/day, where a higher α reflects a faster decline in concentration over time. Moreover, α and β both affect the bacterial decline. The shaping parameter β ranges between 0.49 and 5.63 [-]. The fitting plotted in R5 and R6 (Figure 3) have similar values for α and C_{res}, but differ in β. In fact, R5 has the longest shoulder where the initial population stays constant for nearly 20 days (β = 5.63). In contrast, the shoulder in R6 is smaller (β = 3.1) and observed during the first 15 days. There is also an influence of temperature on α and β in our experiments, as seen for example in the die-off of *P. carotovorum* sp. *carotovorum* in natural oxic TDW (see , P1 & P2). There, α is 0.15 at 10 °C while β is 5.61 (P1). At 25 °C the die-off is two times faster resulting in a higher α of 0.39, and β is 2.68 producing a two times shorter shoulder phase. We observed this relation of the shoulder phase duration in days and the parameter estimate also with the other two bacteria.

▼ **Table 3** Model parameter estimates and their 95% Gaussian confidence intervals shown in brackets

	dataset	[°C]	treatment	redox	$Log_{10}(C_0)$ [CFU/mL]	C_0 [CFU/mL]	$Log_{10}(C_{res})$ [CFU/mL]	C_{res} [CFU/mL]	α [1/day]	β [-]
Ralstonia solanacearum	**Weibull + tail**									
	R1	10	natural	oxic	4.3 [4.0; 4.5]	1.8 x10^4	1.4 [1.1; 1.7]	22	0.25 [0.14; 0.36]	1.7 [1.0; 2.4]
	R4	25	filtered	oxic	4.3 [3.9; 4.6]	1.7 x10^4	1.2 [1.1; 1.4]	17	0.12 [0.06; 0.16]	2.5 [1.0; 4.0]
	R5	10	natural	anoxic	4.2 [4.1; 4.3]	1.6 x10^4	1.5 [1.4; 1.7]	33	0.05 [0.04; 0.06]	5.6 [2.2; 9.1]
	R6	10	natural + NO_3	anoxic	4.3 [4.2; 4.4]	1.9 x10^4	1.3 [1.2; 1.5]	21	0.07 [0.04; 0.08]	3.1 [1.6; 4.5]
	Weibull									
	R2	25	natural	oxic	4.3 [3.9; 4.7]	2.0 x10^4			1.10 [-0.09; 2.28]	0.6 [0.4; 0.8]
	R3	10	filtered	oxic	4.0 [3.8; 4.3]	1.1 x10^4			0.10 [0.07; 0.13]	2.2 [1.5; 3.0]
Dickeya solani	**Weibull + tail**									
	D1	10	natural	oxic	4.4 [4.1; 4.7]	2.4 x10^4	1.7 [1.2; 2.2]	49	0.18 [0.13; 0.22]	3.9 [1.4; 6.4]
	D3	10	filtered	oxic	4.0 [3.9; 4.1]	9.6 x10^3	1.2 [0.8; 1.6]	14	0.03 [0.02; 0.03]	5.5 [3.5; 7.4]
	Weibull									
	D2	25	natural	oxic	4.2 [3.9; 4.6]	1.7 x10^4			4.61 [-8.63; 17.85]	0.5 [0; 1.0]
	D5	10	natural	anoxic	3.8 [3.7; 3.9]	6.6 x10^3			0.04 [0.03; 0.04]	2.2 [1.8; 2.7]
	D6	10	natural + NO_3	anoxic	4.0 [3.9;4.1]	9.6 x10^3			0.04 [0.03; 0.05]	2.4 [1.7; 3.1]
	First-order									
	D4	25	filtered	oxic	4.3 [4.0; 4.6]	2.0 x10^4			0.39 [0.29; 0.47]	
Pectobacterium carotovorum sp. carotovorum	**Weibull + tail**									
	P3	10	filtered	oxic	3.8 [3.7; 3.9]	5.8 x10^3	1.3 [1.1; 1.6]	20	0.04 [0.03; 0.04]	3.3 [2.3; 4.3]
	P5	10	natural	anoxic	3.5 [3.4; 3.6]	3.2 x10^3	1.2 [0.9; 1.5]	14	0.06 [0.04; 0.07]	1.9 [1.3; 4.3]
	Weibull									
	P1	10	natural	oxic	4.0 [3.8; 4.1]	8.9 x10^3			0.15 [0.13; 0.17]	5.6 [2.4; 8.9]
	P2	25	natural	oxic	3.9 [3.8; 4.1]	8.7 x10^3			0.39 [0.3; 0.47]	2.7 [1.5; 3.8]
	P4	25	filtered	oxic	3.9 [3.6; 4.2]	7.3 x10^3			0.18 [0.08; 0.26]	1.6 [0.9; 2.4]

2.4 Discussion

During MAR for agriculture, infiltrated TDW may contain plant pathogens that can still be present in the abstracted water after aquifer storage. Therefore, the die-off of bacterial plant pathogens in the water phase as one of the crucial removal processes during MAR was evaluated.

2.4.1 Influence of temperature

Overall, *R. solanacearum* was more persistent in the natural water microcosms than the soft rot *Pectobacteriaceae* (SRP), and the pathogens were differently susceptible to the tested temperatures. Whereas *R. solanacearum* copes better with the warmer conditions in the natural oxic TDW, the SRP survive better in the 10 °C microcosms, also supported by the results of the filtered TDW. The temperature dependency on the culturability of *R. solanacearum* has been shown earlier where the bacterium was monitored in a Spanish river over a two year period. The bacterium was not detectable with cultivation based techniques during the colder winter months, but reappeared when the temperature rose above 14 °C (Caruso et al., 2005). The absence of *R. solanacearum* after the exposure to lower temperatures can be linked to die-off, but also to the viable but non-culturable (VBNC) state the bacterium can enter (Elphinstone et al., 1998a), which was not evaluated in this study. To escape from hostile and low water temperatures during winter time, the bacteria survive in host plants growing along water streams. In particular, *R. solanacearum* was isolated from the bittersweet plant *Solanum dulcarama* (Elphinstone et al., 1998b), or from the stinging nettle *Urtica dioica* (Wenneker et al., 1999), from where it can get released when the water temperature starts to increase.

2.4.2 Influence of oxygen

It becomes clear that other factors than temperature influenced the die-off of the bacteria by comparing the die-off periods in natural oxic TDW and anoxic AW, both performed at 10 °C. Surprisingly, the die-off was not faster in anoxic microcosms but was decelerated at least two times. Whereas the SRP are described as facultative anaerobic bacteria that can switch their metabolism to nitrate respiration (Smid et al., 1993), little is known about the anaerobic metabolism of *R. solanacearum* as it is generally described as aerobic organism. Wakimoto et al. (1982) mentioned that *R. solanacearum* stayed viable in sterile anoxic water without stating further details. More recently, Dalsing et al. (2015) explored the nitrogen metabolism of

R. solanacearum phylotype I demonstrating its ability to grow better under anoxic conditions if nitrate (> 62 mg/L) was supplemented in a liquid broth medium compared to growth in the same broth without nitrate. To add a higher concentration of nitrate (e.g., 1.5 g/L, as tested in Dalsing et al. (2015) would be not representative for the concentrations found in our water types. In our study, the addition of 50 mg/L nitrate did not have a significant effect on the die-off of *R. solanacearum,* even though the AW has a very low nitrate concentration (0.4 mg/L). Nitrate could have increased the survival serving as terminal electron acceptor under the anoxic conditions. Therefore, our study is the first to analyze the die-off of *R. solanacearum* phylotype II under anoxic conditions in natural water. Further investigation should elucidate the metabolic activity of *R. solanacearum* under these conditions. *R. solanacearum* not only persisted under anoxic conditions, but was culturable for a prolonged period at 10 °C in the anoxic AW. For plant pathogens, the adaptation to low oxygen is important. They can encounter low oxygen conditions in the environment, or during the invasion of the plant host while thriving in the plant xylem; a requisite for a successful infection (Babujee et al., 2012; Lowe-Power et al., 2018). For example, the absence of oxygen induces virulence related genes in *D. solani* enhancing the chance of a successful plant invasion by the pathogen (Lisicka et al., 2018). Additionally, anaerobic nitrate respiration by the formerly named *Erwinia carotovora* was activated in the absence of oxygen; conditions that the bacterium experiences during potato tuber invasion (Smid et al., 1993).

2.4.3 Influence of microbiota

Temperature not only influences the pathogens themselves, but increases biotic interactions of the native microbiota. As reported by Álvarez et al. (2007), the die-off of *R. solanacearum* in natural river microcosms was much faster at 24 °C (4 days) than at 14 °C (28 days), which the authors explained with increased microbial activity at the higher temperature, that negatively affected the pathogen. In contrast to this study, we found in our experiments that *R. solanacearum* was longer culturable in natural TDW (about 20 days) at 25 than at 10 °C. Whereas for the SRP, we observed a faster decline within 4 days at a higher temperature (25 °C) and a better persistence at the lower temperature in natural TDW. Nevertheless, the temperature effect on the die-off of the SRP was negligible in autoclaved TDW microcosms, when comparing the inactivation curves at 10 and 25 °C (see S1 Fig). The long-term persistence in sterile aquatic microcosms, where the indigenous microbiota was absent, has been shown earlier for the SRP (Cother and Gilbert, 1990; van Doorn et al., 2008), as well as for *R.*

solanacearum (Elsas et al., 2001; Álvarez et al., 2007; Stevens et al., 2017). In addition, our results demonstrated that *D. solani* can persist in sterile autoclaved water for a prolonged time which seem to contrast with the findings of van Doorn et al. (2008) where *Dickeya* spp. rendered unculturable after a few days of incubation in sterile basin or ditchwater. *R. solanacearum* persisted two to three times longer in natural TDW at 10 and 25 °C than the SRP, which indicates that the former is better adapted to the conditions in the natural TDW and against increased biotic interactions at 25 °C. In the anoxic AW, the addition of nitrate to the natural AW microcosms might have also increased biotic interactions by stimulating the growth of denitrifies as part of the aquifer microbial community. This could explain the faster die-off of *D. solani* in AW supplemented with 50 mg/L nitrate. On the contrary, the die-off of *R. solanacearum* was not influenced by nitrate addition and did not shorten its survival, supporting its better adaptation against the existing microbiota. In this study, the indigenous microbiota was only represented by the culturable community (growing in TSA and R2A, respectively). Further research is required to elucidate any specific interactions between the examined pathogens and their environment including other microorganisms in the microbial community. For example, Lowe-Power et al. (2018) outlined that low cell densities of *R. solanacearum* sensed via quorum sensing, induce a metabolic strategy that allows the pathogen to exploit a broader variety of nutrients. This increases the pathogen's fitness and competition against other microbes, justifying the long tail in the die-off curves of *R. solanacearum* where the bacteria persist over an extended period at low concentrations. Interestingly, we observed similar and longer die-off periods in anoxic AW than in TDW for all the tested pathogens. Although TDW and AW presented comparable concentrations of culturable bacteria the composition of the microbiota might differ between both, as previously reported (Griebler and Lueders, 2009). These authors stated that the total diversity of the microbiota in shallow aquifers is lower than in the overlying surface, which could support the different die-off periods of the pathogens in natural TDW and AW, observed in the present study. In fact, the TDW used in our microcosms is rain water that passes through the upper nutrient rich soil rhizosphere, where microorganisms such as protozoa, bacteria and viruses can detach from the soil surfaces and end up in the TDW used for infiltration. This can result in a heterogeneous microbial community and its composition is dynamic depending on the seasonal variation. Although, the effects of the different fractions of the native microbiota on the pathogens were not accessed in this study, previous studies reported that the native microorganisms (e.g., protozoa, bacteria and bacteriophages) increased the inactivation of *R. solanacearum* significantly (Álvarez et al., 2007). Protozoa

play an important role in the interaction with the pathogens as they can reduce bacterial populations by grazing (Feichtmayer et al., 2017). They are aerobic organisms, thus, they will not be found as part of the active indigenous microbial community in the anoxic AW environment. In our study, the absence of protozoa in anoxic AW could be one factor influencing the prolonged persistence of the pathogens, under this condition. Furthermore, the pathogens themselves actively can increase their survival chance by producing enzymes and virulence factors, as reported for *D. solani*. These metabolic changes are relevant for the adaptation to unfavorable environments and to improve the competition against other microorganisms (Pédron et al., 2014). The obtained data suggests that the inoculated pathogens competed better against the AW microbiota than to the TDW, where fewer predators (e.g., protozoa) are present, or where the absence of oxygen induces metabolic responses, increasing the pathogen fitness in the AW. This study focused on the die-off kinetics of the pathogens and only analyzed the culturable part of the indigenous microbiota. Interactions between the pathogens and specific microorganisms or bacteriophages may affect the die-off kinetics and needs to be further addressed.

2.4.4 Model

The obtained inactivation curves of *R. solanacearum* in natural TDW are comparable with die-off periods from an earlier study in natural drainage water, where *R. solanacearum* was not culturable after 16 days at 12 °C, but still detectable (4 CFU/mL) at 20 °C after 32 days; both experiments by Elsas et al. used an inoculation concentration of 5 x10^3 CFU/mL (Elsas et al., 2001). The comparison of the *R. solanacearum* die-off curves with ours reveal similar features regarding the curve shape: initially, the die-off of *R. solanacearum* in natural oxic drainage water is fast and without a distinct shoulder, followed by a slower die-off until the bacterial population remains stable at a low concentration, and until the bacteria are no more culturable (Fig 3, R1+2). Although the die-off curves in both studies depict clearly a nonlinear trend, the authors (Elsas et al., 2001) only reported first order die-off rates. As a rough estimation of die-off times this might be sufficient, but it could underestimate the pathogen's survival in the environment, if applied in risk modelling and if potentially contaminated water is recycled as irrigation water. Therefore, we propose the use of the non-linear Weibull + tail model which includes parameters to better describe the die-off pattern of the bacterial plant pathogens, as it accounts for convex or concave (β parameter), as well as tail-shaped curve shape (C_{res}). The model has the advantage that it incorporates the simpler Weibull model and a log-linear model. As a result, the flexibility of the model allowed to fit 17 different datasets. Nevertheless, it is not always possible to compare the resulting parameter estimates of

two different bacteria. For example, β was similar (~ 5.5) in two cases: the die-off of *D. solani* in filtered TDW at 10 °C, representing a shoulder duration of around 35 days; and the die-off of *R. solanacearum* in anoxic AW, where the shoulder phase was observed for at least 15 days. Consequently, some general assumptions can be made evaluating the parameter estimates, but a direct comparison of the parameters between the bacterial species is not possible. On the one hand, this can be explained by the Weibull model equation itself (see model 1 or 2), where both α and β influence the decay time as the two parameters are inversely related: if a large β is needed to describe the shoulder, it means that α must become lower as otherwise the effective rate constant gets too high. On the other hand, it is not possible to compare the parameter estimates of different bacteria as each species has different metabolic stress responses, and thereby different lethal times. The Weibull model takes into account this heterogeneity within a bacterial population, assuming that the cells are not equally resistant to the environmental stresses they are confronted with (van Boekel, 2002). The existence of subpopulations has been described earlier. Elsas et al. (2001) characterized morphological differences of a *R. solanacearum* population. Along with these results, Álvarez et al. (2008) investigated the different survival strategies of *R. solanacearum,* that included the formation of viable but not culturable (VBNC) state, persister cells, or the change of their cell shape from bacilli to coccoid form that also alters their cell metabolic activities.

The C_{res} parameter which accounts for a persisting population is another important criterion for the model selection. The Weibull model considers the distribution of different stress tolerances within the population, but does not describe the mechanisms of the resistances as described in the previous paragraph (Brouwer et al., 2017). Even though the C_{res} parameter improves the prediction of the bacteria's die-off, if a persistent population is present, it also has a limitation. According to the Weibull + tail model, the bacterial concentration might never reach zero because it includes C_{res}. Therefore, the C_{res} parameter should not be handled as an absolute value, but as a fraction of the initial C_0 concentration in risk modelling. Otherwise, the final concentration after treatment of even low (< 10 CFU/mL) contaminated water would mathematically always result in C_{res} regardless of the initial C_0. However, under natural conditions, over much longer time frames, the populations get extinct and are no more present, which in our study was reached with the limit of culturability in all natural microcosm conditions. In our case, the prediction beyond the data cannot be executed with sufficient certainty and therefore, the situation where the bacteria get extinct cannot be reproduced with the Weibull + tail model (Albert and Mafart, 2005), but would require the addition of another parameter to the

formula. The use of different detection methods that are able to monitor lower concentrations could circumvent this problem or just postpone it to a later time point. When comparing with other studies that studied the die-off of *R. solanacearum* in natural waters (Elsas et al., 2001; Álvarez et al., 2007), a higher inoculation concentration (10^6 CFU/mL) did not increase the final concentration of the resistant population but increased the length of the die-off period. The awareness that such a persistent population exists, is especially important in the risk assessment as these bacteria may still cause disease. Die-off studies with human pathogens in water microcosms also illustrate that first-order inactivation does not sufficiently describe the pathogen's die-off because after an initially rapid inactivation, the pathogens persist at low concentrations for a prolonged period (Easton et al., 2005; Casanovas-Massana et al., 2018). Starved *R. solanacearum* populations from oligotrophic and sterile microcosms, were still able to cause disease after a prolonged period (Álvarez et al., 2008). Nevertheless, as these experiments have been conducted under rather artificial conditions and used the highly susceptible tomato plant as host, it makes it difficult to adopt them to natural environments. The C_{res} parameter can serve as a worst case scenario where a low bacterial populations survives in the water.

However, the die-off in the water phase is only one removal process during MAR and other die-off processes, such as soil attachment, need to be taken into account within the risk assessment. If bacteria are still present after the MAR treatment, their concentrations will be low and future research needs to address the pathogenicity of low-inoculum concentrations. In our study, we have shown that the three economically relevant bacterial plant pathogens *R. solanacearum*, *D. solani* and *P. carotovorum* sp. *carotovorum* can persist in natural waters days to weeks, thereby posing a risk if recycling water systems are applied for irrigation without further treatment or insufficient residence times. Our non-linear models describe well the progression of the bacteria's die-off in natural water environments and could be applied to other human or plant pathogens. The results will contribute to define the operation of a MAR system in order to provide safe irrigation water, where bacterial concentrations are so low that there is no risk inducing plant diseases.

2.5 Conclusions

In this research we determined the die-off kinetics of three bacterial plant pathogens (*Ralstonia solanacearum*, *Pectobacterium carotovorum* sp. *carotovorum*, and *Dickeya solani*) in natural oxic TDW and in natural anoxic groundwater below an agricultural

field. The decline in bacterial concentration by 3-log_{10} occurred between 6 to 50 days and was faster in oxic tile drainage water than in anoxic water from the aquifer. As a result, a variety of die-off curves with different shapes was obtained and we developed a flexible non-linear Weibull model that allowed to model the different bacterial die-off curves. These models are needed to reliably predict the die-off of relevant pathogens in aqueous environments and specifically in MAR systems. In the future, water reuse schemes will gain more importance as fresh water scarcity increases which will affect especially agricultural production that accounts for about 70% of fresh water use. MAR is an option to store fresh TDW in the subsurface for later use as irrigation water but water quality, particularly, related to the presence of plant pathogens needs to be considered. Our results will be implemented in microbial risk assessments setting guidelines for a safe application of water reuse schemes such as MAR. This study contributes to the knowledge about the survival of plant pathogens in the agro-ecosystem which is crucial for plant disease management.

2.6 Appendix A

The supplementary data contains Figure S1 showing the bacterial die-off in autoclaved TDW. Table S1 shows the enumeration of culturable bacteria in natural waters from the MAR site. Table S2 shows the results of the Akaike information criterion (AIC) to select the best fitting model.

The R script for non-linear modelling is described in File S1 and File S2 includes all bacterial enumerations to model the die-off, which can be found online at https://doi.org/10.1371/journal.pone.0250338.

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Chapter 3
Removal of bacterial plant pathogens in columns filled with quartz and natural sediments under anoxic and oxygenated conditions

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Highlights

- First study to analyse the transport of plant pathogenic bacteria in natural sands
- Bacterial removal was several $\log_{10}$ higher in natural sediments (18-40 $\log_{10}$ m^{-1}) compared to quartz sand (max. 6.8 $\log_{10}$ m^{-1})
- Column studies give insights on bacterial fate during simulated aquifer passage
- Results will serve as basis for the design of MAR systems

Abstract

Irrigation with surface water carrying plant pathogens poses a risk for agriculture. Managed aquifer recharge enhances fresh water availability while simultaneously it may reduce the risk of plant diseases by removal of pathogens during aquifer passage. We compared the transport of three plant pathogenic bacteria (*Ralstonia solanacearum, Dickeya solani, Pectobacterium carotovorum*) with *Escherichia coli* WR1 as reference strain in saturated laboratory column experiments filled with quartz sand, or sandy aquifer sediments. Bacterial and non-reactive tracer breakthrough curves were fitted with Hydrus-1D and compared with colloid filtration theory (CFT). Bacterial attachment to fine and medium aquifer sand was highest with attachment rates of max. k_{att1} = 765 day^{-1} and 355 day^{-1}, respectively. Attachment was the least to quartz sand (k_{att1} = 61 day^{-1}). In CFT, sticking efficiencies were higher in aquifer than in quartz sand but there was no differentiation between fine and medium aquifer sand. Overall removal ranged between <6.8 log_{10} m^{-1} in quartz and up to 40 log_{10} m^{-1} in fine aquifer sand. Oxygenation of the anoxic aquifer sediments for two weeks with oxic influent water decreased the removal. The results highlight the potential of natural sand filtration to sufficiently remove plant pathogenic bacteria during aquifer storage.

3.1 Introduction

Fresh water availability is decreasing as consequence of overuse and extreme weather events. With agriculture being the biggest water consumer claiming about 70% of the total fresh water, it relies heavily on the advancement of water management practices (UN-Water, 2021). One strategy to tackle fresh water availability in agricultural settings is managed aquifer recharge (MAR) which includes riverbank filtration, infiltration ponds, or aquifer storage and recovery (Dillon, 2005). The goal is to replenish aquifers and improve water quality as microbial pathogens are removed during soil filtration. Removal is crucial because surface waters used for MAR may contain pathogenic organisms. Their number should decline to safe levels prior to irrigation to avoid the spread of (plant) diseases (Hong and Moorman, 2005). Chemical, biological, and physical processes occurring at the soil-water interphase during MAR can remove pathogens (Ginn et al., 2002). This includes physical straining, dieoff in the water phase, (ir)reversible attachment to sediment grains, dieoff at the grain surface, and die-off through predation and competition with other microorganisms (Bradford et al., 2013). Several studies analysed the removal efficiency of MAR systems focusing on human pathogens and related human health risks after irrigation of crops or recreational areas (e.g., Toze (2004); Page et al. (2010); Ayuso-Gabella et al. (2011)). In contrast, no study investigated the removal of plant pathogens during MAR, although MAR treated water is often intended for irrigation and related consequences for plant health have high ecological and economic value (Hong and Moorman, 2005; Savary et al., 2019). Moreover, the implementation of legislations (Council, 2009; Commission, 2020) set requirements for water reclamation technologies which necessitates studies on the fate of plant pathogens. To our knowledge, only two studies investigated the fate of plant pathogens in saturated porous media. Liu et al. (2008) showed that biofilm coated glass beads increased the removal of the bacterium *Erwinia chrysanthemi*. Jeon et al. (2016) demonstrated a higher retention of zoospores of the oomycete *Phytophthora capsici* in iron-oxide coated saturated quartz sand in comparison to uncoated sand. Both studies used "fabricated", homogenous sediment to study principal transport processes. Natural sediments are heterogeneous in their lithological and chemical composition. They usually contain positively charged metal-oxide coatings that increase the number of favourable attachment sites and enhance the removal of the negatively charged microorganisms (Johnson et al., 1996; Bradford et al., 2013). Moreover, MAR usually results in oxygenation of aquifers and may lead to formation of additional iron-oxides. Studies on transport of microbial plant pathogens in natural sediments are only available within the context of slow sand filtration (SSF) (e.g., Ferreira et al. (2012); Lee

and Oki (2013)) which show a high efficiency to remove pathogens from greenhouse irrigation waters. For example, a horizontal slow sand filter with a length of 27.6 m removed 99.5% of *Fusarium* propagules (Prenafeta-Boldú et al., 2017). However, SSF and MAR differ in hydrological and geochemical conditions in terms of lithology, water saturation, redox conditions, and biological activity which makes a comparison of both systems difficult. The aim of our study was to analyse the removal of the following three economically and ecologically important plant pathogenic bacteria during transport in laboratory columns filled with aquifer sediments (or quartz) for the first time: *Ralstonia solanacearum* (brown rot), and the soft rot *Pectobacteriaceae* (SRP) *Dickeya solani* and *Pectobacterium carotovorum.* These bacteria all cause high crop losses to a broad variety of crops including potato and tomato but also can affect ornamental flower production (Hayward, 1991; Ma et al., 2007; van der Wolf and De Boer, 2007; Tjou-Tam-Sin et al., 2016). We compare their transport with *Escherichia coli* WR1 which has been used in previous field (Schijven et al., 2000) and column studies with natural sand (Hijnen et al., 2004; Hijnen et al., 2005), but not in quartz sand. The aquifer sediments were obtained from a MAR site used to produce and store irrigation water. We hypothesize that a higher removal is achieved in natural sediment as these contain more favourable attachment sites in comparison to clean quartz sand. Furthermore, the effect of oxygenation of the naturally anoxic sediments on removal rates was tested as this could lead to the formation of additional positively charged iron oxides. The resulting breakthrough curves (BTC) of the column experiments were modelled in Hydrus-1D using the advection-dispersion equation extended with a 1- or 2-site kinetic attachment/detachment model (Schijven et al., 2002; Schijven and Šimůnek, 2002). To extrapolate the results, log removals per meter were calculated for an indication of log removal under field conditions and for comparison with other studies (Pang, 2009).

3.2 Material and Methods

3.2.1 Porous media and aqueous solutions

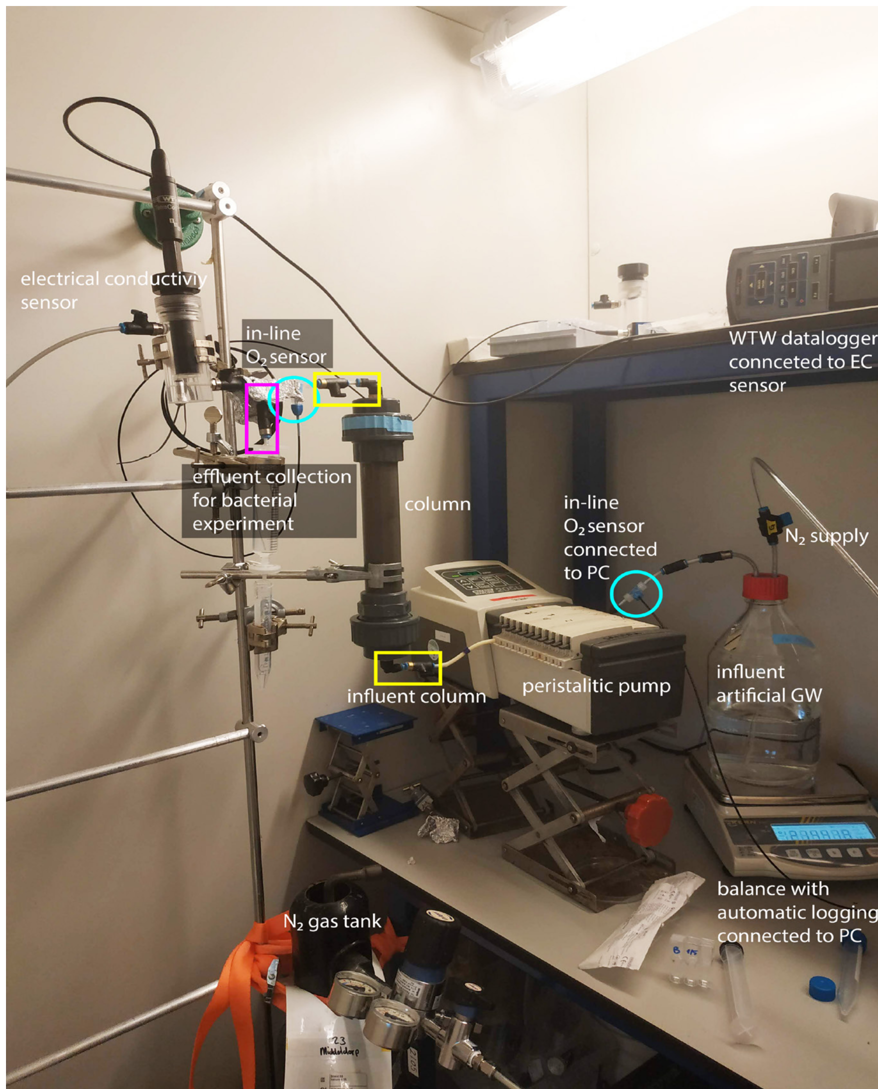
Fine to medium grained silica quartz sand (d_{50} = 260 μm) with a 99.5% SiO_2 content (M32, Sibelco, Belgium) was used in the quartz sand experiments. To remove any metal oxides the sand was acidwashed (Chu et al., 2001). The quartz sand was treated with 0.2 M citrate buffer containing 44.1 g L^{-1} sodium citrate and 10.5 g L^{-1} citric acid at 80 °C and mixed occasionally followed by the addition of 0.05 M sodium dithionite. Finally, the sand was washed with demineralized water until the electrical conductivity (EC) reached about 10 μS cm^{-1}. The water-sand mixture was autoclaved for 20 min at 121 °C

and stored until further use. For experiments with aquifer sand, sediment was obtained during the drilling operation of a MAR site in Breezand, the Netherlands (coordinates: 52.8883, 4.8221). The semiconfined sandy aquifer (11.5-33.0 m below surface level) of late Holocene and Pleistocene age lies below a confining Holocene clay/peat layer. Sediment samples were obtained from the boreholes using a 2 m sonic drill aqualock system with a core catcher. The sand cores were confined on both ends and stored in closed PVC tubes at 4 °C for one month before they were opened for sand sample collection. After opening, the samples were exposed to air but handled rapidly to avoid oxidation. Two soil samples from different depths were collected with a disinfected (70% EtOH) spoon for the column experiments: (i) fine aquifer sand from 12-14 m depth with d_{50}= 192 μm, and (ii) medium aquifer sand of d_{50}= 305 μm from 24 m depth. The coefficient of uniformity ($C_u = d_{60}/d_{10}$) is 1.6 for quartz sand, 2.0 for fine and 2.3 for medium MAR sand. A C_u<2 refers to uniform soils. A grain size distribution chart is shown in Figure S1 and Table S1 together with a chemical soil analysis in Table S2. Both soil samples are representative for the aquifer which consists to over 80% of fine to medium coarse sand (125-500 μm). The soil samples were placed in a zip-locked plastic bag of which excess air was removed before closure, and stored in a gas tight container initially flushed with N_2 gas together with two anaerobic gas generation sachets (Oxoid™ AnaeroGen™ 2.5 L, Thermo Fisher Scientific). The container was stored at 10 °C in the dark until further use. All three sand types (quartz, fine and medium aquifer) were extracted for total iron using the citrate-dithionite extraction method after Claff et al. (2010) and analysed using Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES).

An artificial groundwater (aGW) solution was prepared in autoclaved containers after Bolster et al. (2001) by adding the following salts to 1 L deionized water: 60 mg $MgSO_4x7H_2O$, 20 mg KNO_3, 36 mg $NaHCO_3$, 36 mg $CaCl_2$, 35 mg $Ca(NO_3)_2$, 25 mg $CaSO_4x2H_2O$. aGW was used during all experimental procedures including filling of the column and in all experiments. aGW had a pH of about 7.5 (± 0.2) and was prepared freshly, not autoclaved to avoid changes in water chemistry, and was kept at 10 °C. The aGW was selected to exclude variations in bacteria attachment resulting from variations in solution chemistry. It is not identical to the MAR groundwater but contains the major ions and has low ionic strength of about 3 mM which does not favour attachment (Foppen and Schijven, 2006). For the non-reactive salt tracer solution 1 g L^{-1} sodium chloride was added to the aGW. For all anoxic experiments, the aGW or tracer solution were made anoxic by flushing about 2 L with nitrogen gas for 1 h.

3.2.2 Column setup

Recommendations by Gilbert et al. (2014) were followed for the dimensioning of the column and selection of materials in the experimental setup (Figure 1).



▲ **Figure 1** Experimental column setup in the laboratory

We used a PVC column with a length of 23 cm and an inner diameter of 3.6 cm. Therefore, the representative elementary volume (REV) calculated by dividing the column diameter by the grain diameter results in a REV of 128 for quartz, 168 for fine MAR and 105 for medium MAR sand. These values are all higher than the minimum REV of 40 for microbiological experiments. The column was wet-packed by pouring the sand material into the column and applying a slow flow rate (<0.5 mL min^{-1}) of anoxic aGW with bottom-up flux to avoid air entrapment. Additionally, the column was continuously tapped while filling to remove air bubbles which required specific attention with natural sand. A metal mesh (100 μm pore size) was placed at the top and bottom of the column to retain the sand and was reinforced by a perforated metal plate (1 mm thick, 2 mm wide holes). Column packing was executed under aerobic conditions while flushing with N_2 at the column headspace and pumping anoxic aGW from bottom-up. The tubing (polyurethane) was used with appropriate connectors (Festo B.V., The Netherlands) and all materials were autoclaved prior to use. The column was rinsed carefully with 70% EtOH prior to use. A peristaltic pump (Watson Marlow 205U, UK) maintained a constant pump rate of 2.5 mL min^{-1} (± 0.1 mL min^{-1}) which was confirmed by logging the effluent gravitationally (Kern & Sohn GmbH, Germany). The velocity of 3.6 m day^{-1} was chosen according to an average velocity of 1.4-5.3 m day^{-1} within the MAR system between infiltration and abstraction well, considering only radial horizontal water flow in the most permeable layer of the aquifer. The velocity is higher next to the infiltration point and decreases with distance towards the extraction well located at 6 m distance. An oxygen flow-through cell (PreSens, Germany) at the column in- and outlet monitored the oxygen concentrations automatically. Additionally, a flow-through EC sensor at the outlet measured the EC in the effluent with a datalogger (WTW, Xylem Analytics Germany) to monitor the breakthrough curves of the non-reactive salt tracer. We conducted three experiments with different saturated porous media which are summarized in Table 1: (E1) clean quartz sand under oxic conditions, serving as worst-case scenario and reference experiment, conducted with all bacteria; (E2) fine aquifer sand under both anoxic and oxygenated conditions with *P. carotovorum*; (E3a) medium aquifer sand under anoxic conditions done with *R. solanacearum*, *E. coli*, and *P. carotovorum*, and (E3b) oxygenated medium aquifer sand with *P. carotovorum*. Experiments with quartz sand were conducted in duplicates and with freshly packed columns, while the experiments with fine aquifer sand were only done once per condition, but using the same column to subsequently test oxygenation. The experiments with medium aquifer sand were performed in triplicates using the same column, but were freshly packed for each bacterium. The transport of *P. carotovorum* in medium MAR

sand was first studied under anoxic conditions, followed by oxygenation of the same column to study the transport under oxic conditions. Thereby, only one parameter, namely the influence of oxygen, was changed. In between the replicate experiments, the column was flushed (1.5 mL min^{-1}) with anoxic deionized water over night to remove attached bacteria before the column was equilibrated again with aGW. At the beginning of the bacterial breakthrough experiments, an effluent sample was taken shortly after the start of the pathogen loading when bacterial breakthrough was not yet expected. The effluent sample was analyzed for the presence of the selected pathogen by dilution plating. Oxygenation of the column with fine aquifer sand (E2) was executed by flushing (1.5 mL min^{-1}) the column for two days with oxic aGW until the effluent O_2 concentration remained stable. This flushing was extended to two weeks in experiments with medium aquifer sand (E3b) to achieve more extensive oxidation of the sediments and potentially more formation of reactive surface metal oxide coatings. All experiments were performed in a cooling chamber at 10 °C (± 1.5) except for experiments with *R. solanacearum* which had to be performed within a laminar flow cabinet at room temperature due to the bacteria's quarantine status. The column was operated in upflow direction at a pump rate of 2.5 mL min^{-1} to ensure saturated conditions. Each experiment was setup as following: (1) Filling of the column with sand material and equilibration with about 13 pore volumes (PVs) aGW. (2) Breakthrough experiments with the non-reactive salt tracer solutions. There, the sodium chloride solution was pumped for 85 min (ca. 2.4 PVs) followed by switching back to the aGW solution until the initial EC was measured. (3) Bacterial breakthrough experiments where the bacterial suspension containing ca. 10^6 CFU mL^{-1} was also pumped for 85 min followed by switching back to the aGW solution. Effluent samples of ca. 0.5 mL were collected manually in reaction tubes at adequate time points and evaluated by dilution plating.

▼ **Table 1** Experimental conditions for column experiments in different sand types

		Saturated porous media	Bacterium	Temp °C	Redox
Silica quartz sand, Acid-washed	E1	fine d_{50} = 260 µm	*D. solani*	10 (± 1.5)	Oxic
			P. carotovorum		
			E. coli		
			R. solanacearum	[a]RT (23 ± 2)	
Natural sand from MAR site	E2	Fine d_{50} = 200 µm	*P. carotovorum*	10 (± 1.5)	Anoxic
					[b]Oxic
	E3a	Medium d_{50} = 300 µm	*E. coli* WR1	10 (± 1.5)	Anoxic
			R. solanacearum	[a]RT (23 ± 2)	
			P. carotovorum	10 (± 1.5)	Anoxic
	E3b				[c]Oxic

[a]RT = room temperature; [c]Continuous column flushing with oxic artificial ground water (aGW) for 2 days; [c]Continuous column flushing with oxic aGW for 2 weeks

3.2.3 Inoculation solution

R. solanacearum race 3 biovar 2 (phylotype II) strain IPO-1828, *D. solani* IPO-2266, and *P. carotovorum* IPO-1990 are plant pathogenic bacteria present in surface waters and were used in this study. Their transport was compared with *Escherichia coli* WR1 (NCTC 13167). The selected bacteria are all gram-negative, rod-shaped, flagellated and have a similar size of 1.2-2.5 μm in length and 0.4-1 μm in width. Zeta potentials have been obtained from literature and they show that all pathogens are negatively charged: -9.85 mV for *P. carotovorum* (Gutierrez-Pacheco et al., 2018), -22 mV for *E. coli* WR1 (Schijven et al., 2008), -21.3 mV for *R. solanacearum* (Yan et al., 2004) and -25.03 mV for *Dickeya* spp., formerly named *Erwinia chrysanthemi* (Liu et al., 2008). Bacterial suspensions were prepared by incubating the respective bacteria in oxic liquid medium overnight for 15 h at 28 °C (*E. coli* 12 h at 37 °C) on a rotary shaker at 100 rpm. Therefore, the bacteria were harvested during the late log and early stationary growth phase. Details on bacteria and culture media preparation can be found in the supplement (S2). The grown cultures were harvested by centrifugation (3500 x *g*, 20 min at room temperature), followed by washing and resuspending the pellet in a quarter strength Ringer's solution (Sigma-Aldrich; St. Louis, USA). This pelleting and washing step was repeated twice to remove any excess broth. The bacterial suspension was then diluted to reach an optical density of 0.1 at 600 nm representing a concentration of 10^8 CFU mL^{-1}, and was used to inoculate 250 mL of (anoxic) aGW to reach a final concentration of 10^6 CFU mL^{-1}. The concentration of the inoculation suspension (C_0) and the inoculated aGW before and at the end of the breakthrough experiment, and enumeration of the bacterial effluent concentrations were confirmed by dilution-plating using selective media (S2) (Elphinstone et al., 1998; Hélias et al., 2012). Furthermore, bacterial hydrophobicity was analysed using the microbial adhesion to hydrocarbon (MATH) test as described by Gargiulo et al. (2008).

3.2.4 Data analysis and modelling

Breakthrough curves of the non-reactive salt tracer and bacteria were analyzed with Hydrus-1D (Simunek et al., 2005). Solute transport of the non-reactive tracer is described by the advection-dispersion equation under steady state flow conditions and a constant head:

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial x^2} - \nu\frac{\partial C}{\partial x} \quad (1)$$

where C is the solute concentration in the liquid phase [M L^{-3}], t is time [T], D is the hydrodynamic dispersion coefficient [L^2 T], x is distance [L] and ν the average interstitial water velocity [L T^{-1}]. Bacterial transport is modelled

by an extended form of the advection-dispersion equation accounting for two kinetic sites with reversible bacterial attachment and detachment rate coefficients (Schijven et al., 2002):

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial x^2} - v\frac{\partial C}{\partial x} - (k_{att1} + k_{att2})C + k_{det1}S_1\frac{\rho_B}{\theta} + k_{det2}S_2\frac{\rho_B}{\theta} \quad (2)$$

$$\frac{\rho_B}{\theta}\frac{\partial S_1}{\partial t} = k_{att1}C - k_{det1}S_1\frac{\rho_B}{\theta} \quad (3)$$

$$\frac{\rho_B}{\theta}\frac{\partial S_2}{\partial t} = k_{att2}C - k_{det2}S_2\frac{\rho_B}{\theta} \quad (4)$$

where ρ_B is dry bulk density [M L^{-3}]; θ is porosity [-]; S_1 and S_2 are the concentrations of attached bacteria at two kinetic sites [M M^{-1}]. k_{att} and k_{det} [T^{-1}] are the attachment and detachment rate coefficients, respectively, of free and attached microorganisms, while subscripts 1 and 2 refer to the two different kinetic sites. In Hydrus-1D, normalized C/C_0 values were used as input values and to model the tailing of the BTC. Log transformation of C/C_0 was applied to overcome the great difference between maximum and minimum bacterial effluent concentrations (Schijven and Šimůnek, 2002). Porosity θ and longitudinal dispersivity α_L [L] ($\alpha_L = D/v$) were both determined from salt tracer experiments and used as input values to modael bacterial BTC. Growth or dieoff of the bacteria was not considered within the time span of the experiments (about 9 h) as we have shown in batch experiments that dieoff in the water phase took only place after more than 24 h (Eisfeld et al., 2021). Hydrus-1D allows to compare the fit of a 1- or 2-site kinetic model by evaluating the coefficient of determination R^2 and the Akaike Information Criterion (AIC) as output data from Hydrus-1D. The AIC considers both the goodness of fit and parsimony of the models. For our experiments, the model with the smallest AIC value was therefore chosen as best model.

The advection-dispersion equation (2) accounts for reversibility of the bacterial attachment. We compare these results with the CFT that assumes a single grain size with perfect sphericity and smoothness where bacteria attach irreversibly. We used the equations described by Tufenkji and Elimelech (2004) to calculate the single-collector contact efficiency (η_0) and sticking efficiency (α). To derive α, the C/C_0 input values were calculated using the equation described by Schijven et al. (2000):

$$\log\left(\frac{C}{C_0}\right) = \frac{x}{2.3}\frac{\left(1-\sqrt{1+4\alpha_L\frac{k_{att1}+\mu_l}{v}}\right)}{2\alpha_L} \quad (5)$$

which employs the Hydrus-1D output values α_L and k_{att1} from the salt and bacterial breakthrough experiments, respectively. Finally, the C/C_0 is used to estimate microbial log-removal per meter expressed by λ [log_{10} L^{-1}] (Pang, 2009).

3.3 Results and Discussion

3.3.1 Chloride tracer BTC

The non-reactive salt tracer allowed to determine the physical water flow characteristics of the column. The normalized BTCs of the chloride EC effluent concentrations (C/C_0) were fitted in Hydrus-1D to obtain longitudinal dispersivity α_L and porosity θ as shown in Table 2. In quartz sand, these parameters were evaluated for each experiment individually and the porosity was 0.37 - 0.40 with α_L of 0.038 - 0.045 cm. The same parameters were obtained in the aquifer sand experiments by combining the data of the chloride BTCs of the replicate experiments per bacterium. For *R. solanacearum* in medium aquifer sand, only two salt tracer tests were executed. The porosity in fine and medium aquifer sand was lower (0.31 - 0.34) than in the quartz sand experiments, whereas α_L was considerably higher ranging between 0.28 – 1.5 cm. α_L of medium aquifer sand used for experiments with *E. coli* was 1.51 cm, thereby similar to the columns filled with fine sand (1.46 cm). Differences in porosity and longitudinal dispersivity may arise from combining data of the tracer BTCs of the aquifer sand, but also from column packing or variation in grain size distribution of the natural aquifer sand (Figure S1). These variations in dispersivity have also been reported by other authors (Schijven et al., 2002; Oudega et al., 2021) but have little influence on the fitting and adsorption parameters.

▼ **Table 2** Fitting parameters of the tracer BTC in different sand media. Standard error of the parameters is presented in parentheses.

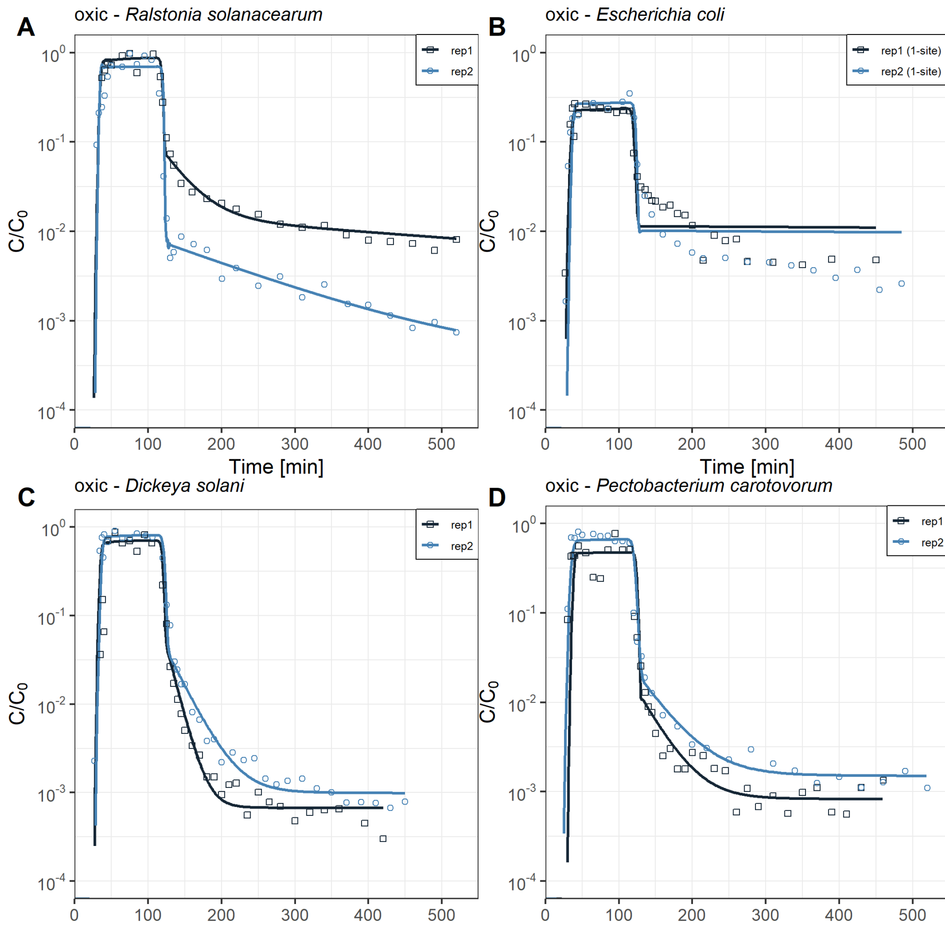
		Non-reactive salt tracer		
		Θ [-]	α_L [cm]	R^2
Quartz sand				
Pc	rep 1	**0.40** (8.41 x10^{-4})	**0.039** (5.32 x10^{-3})	1.0
	rep 2	**0.39** (1.41 x10^{-3})	**0.103** (1.25 x10^{-2})	0.999
Ds	rep 1	**0.38** (1.12 x10^{-3})	**0.045** (0.006)	0.998
	rep 2	**0.38** (1.38 x10^{-3})	**0.056** (0.007)	0.999
Rs	rep 1	**0.37** (1.26 x10^{-3})	**0.038** (8.31 x10^{-3})	0.998
	rep 2	**0.37** (9.67 x10^{-3})	**0.012** (5.76 x10^{-2})	0.790
Ec	rep 1	**0.38** (9.05 x10^{-4})	**0.061** (6.07 x10^{-2})	0.999
	rep 2	**0.39** (1.93 x10^{-3})	**0.039** (1.09 x10^{-2})	0.999
Anoxic medium aquifer sand				
Ec	rep 1-3	**0.33** (2.85 x10^{-3})	**1.5** (0.08)	0.997
Rs	rep 1-3	**0.31** (2.64 x10^{-2})	**0.28** (0.41)	0.508
Pc	rep 1-3	**0.33** (2.28 x10^{-3})	**0.37** (0.04)	0.994
Oxygenated medium aquifer sand				
Pc	rep 1-3	**0.32** (1.63 x10^{-3})	**0.346** (2.74 x10^{-2})	0.997
Fine aquifer sand				
Pc	anoxic + oxygenated	**0.34** (5.10 x10^{-3})	**1.46** (1.51 x10^{-1})	0.992

Pc = P. carotovorum, Ds = D. solani, Ec = E. coli WR1,
Rs = R. solanacearum; Θ = porosity, α_L = longitudinal dispersivity,
R^2 = coefficient of determination to evaluate the model fit to the data

3.3.2 Bacterial transport in quartz sand

The bacterial BTCs are shown in Figures 2+3. The modified advection-dispersion equation (2) for bacterial transport implemented in Hydrus-1D was applied to fit the bacterial BTCs. Table 3 shows the attachment and detachment parameters for a 1- or 2-site kinetic model. In most cases, the 2-site kinetic model showed a better fit when comparing the R^2 and AIC (Table S4). All BTCs of quartz and natural MAR sand were analysed for blocking by fitting the blocking parameter S_{max1} (e.g., Hornstra et al. (2018)) in Hydrus-1D. In blocking, available grain surfaces get occluded by for example attached bacteria which decreases the retention of other bacteria as less favourable sites are available (Ryan and Elimelech, 1996). Adding the blocking parameter did not improve model fit considering the AIC. In all experiments, the bacterial BTCs coincided with the tracer BTCs, but the bacterial BTCs

showed a lower C/C_0 as consequence of bacterial attachment which we describe as removal. Figure 2 presents the results of the duplicate bacterial BTCs in quartz sand. Additionally, Figure S2A compares the BTCs of the four bacteria where C/C_0 is presented linearly, while Figure S2B displays the same results using log-transformed C/C_0 values which allowed to identify the tails of the BTCs caused by detachment. *E. coli* had the highest removal in quartz sand, followed by *P. carotovorum* and *D. solani*. *R. solanacearum* was least removed reflected by a high C/C_0 of 0.85 in replicate 1 and a low k_{att1} of 6 day^{-1}. In comparison, k_{att1} was ten times higher for *E. coli* (61 day^{-1}, replicate 1) and the C/C_0 peak was 0.18. The bacterial BTC of *E. coli* was best fitted with the 1-site kinetic model and therefore, removal is solely guided by kinetic site 1. The BTC of the plant pathogenic bacteria were best fitted with the 2-site kinetic model but a comparison of the AICs often showed little difference indicating that the 1-site model would also be an adequate fit. At kinetic site 2, attachment occurred to a lesser extent than at site 1 (k_{att2} = 1 - 4 day^{-1}). While kinetic site 1 showed little detachment (k_{det1} = 0.03 – 2.2 day^{-1}) detachment at site 2 was very high ranging from 11–112 day^{-1}. Note that k_{att1} of *D. solani* and *R. solanacearum* and all k_{det2} rate coefficients show a very high standard deviation. Its implication will be discussed in the section of parameter evaluation. Overall, the plant pathogenic bacteria were removed to a lesser extent than *E. coli* and their BTC peak had a higher C/C_0 value. Reported bacterial characteristics such as cell size, surface charge or growth stage are similar for the selected bacteria. Moreover, the results of the hydrophobicity test (Table S3) indicate that all bacteria are hydrophilic with little difference (2.1–8.9%) in partitioning into the hydrophobic phase. Therefore, other bacterial characteristics like the specific surface structure (e.g., lipopolysaccharides) may cause the different transport behaviours (Gilbert et al., 1991; Ginn et al., 2002). Nevertheless, these were not further analysed and should be addressed in future studies.



▲ **Figure 2** Duplicate experiments of bacterial breakthrough experiments in quartz sand under oxic conditions (rep = replicate). The normalized effluent concentrations (C/C_0) are plotted as a function of time (min) on a semi-log scale. Solid lines are the fitted models obtained from Hydrus-1D and the symbols are the corresponding experimental data in the same color. All breakthrough curves (BTCs) have been modelled with the 2-site kinetic model, expect for the BTCs of Escherichia coli WR1 which was modelled with the 1-site kinetic model.

▼ **Table 3** Fitting parameters of the bacterial breakthrough curves in different sand media. Standard deviation of the parameters is presented in parentheses.

Bacterial breakthrough experiments

		Hydrus-1D results					Calculated results			
		k_{att1}	k_{det1}	k_{att2}	k_{det2}	$^{a}R^2$	C/C_0 peak	η_0	α	λ
Quartz sand		[day^{-1}]	[day^{-1}]	[day^{-1}]	[day^{-1}]		[-]	[-]	[-]	[$\log_{10}$ m^{-1}]
Pc	rep 1	**28** (3)	**0.04** (0.02)	**1** (1)	**49** (39)	0.92	0.45	0.009	0.117	2.7 (0.5)
	rep 2	**17** (16)	**0.09** (0.08)	**1.4** (2)	**39** (36)	0.77	0.63	0.009	0.067	
Ds	rep 1	**15** (59)	**0.05** (0.01)	**4** (2)	**112** (28)	0.95	0.65	0.011	0.047	1.4 (0.3)
	rep 2	**8** (9)	**0.10** (0.02)	**2** (1)	**57** (14)	0.95	0.79	0.011	0.027	
Rs	rep 1	**6** (21)	**2.2** (1.39)	**4** (3)	**45** (47)	0.99	0.85	0.011	0.018	0.42 (0.2)
	rep 2	**1** (1)	**0.03** (0.15)	**1** (1)	**11** (18)	0.73	0.97	0.011	0.003	
Ec	rep 1 (1-site)	**61** (2)	**0.58** (0.30)			0.92	0.18	0.012	0.176	6.8 (0.4)
	rep 2 (1-site)	**51** (2)	**0.49** (0.44)			0.9	0.24	0.012	0.149	
Medium aquifer sand (anoxic)										
Pc	rep 1	**184** (10)	**0.38** (0.13)	**25** (6)	**30** (9)	0.86	1.21×10^{-2}	0.009	0.617	19 (1.3)
	rep 2 (1-site)	**129** (3)	**0.55** (0.25)			0.88	3.9×10^{-2}	0.009	0.451	
	rep 3 (1-site)	**202** (3)	**0.55** (0.16)			0.85	7.9×10^{-3}	0.009	0.670	
Rs	rep 1	**161** (8)	**0.03** (0.04)	**24** (25)	**33** (27)	0.9	1.7×10^{-2}	0.012	0.424	18 (1.7)
	rep 2	**208** (4)	**0.25** (0.19)	**31** (26)	**25** (35)	0.94	6.0×10^{-3}	0.012	0.534	
	rep 3	**155** (16)	**0.07** (0.11)	**25** (18)	**23** (22)	0.93	2.0×10^{-2}	0.012	0.410	
Ec	rep 1	**355** (8)	**0.4** (0.06)	**75** (8)	**0.4** (7)	0.91	4.1×10^{-3}	0.013	0.540	28 (2)
	rep 2	**551** (28)	**0.13** (0.07)	**107** (25)	**19** (3)	0.63	6.3×10^{-4}	0.013	0.725	
	rep 3	**419** (8)	**0.26** (0.08)	**132** (26)	**79** (21)	0.84	2.2×10^{-3}	0.013	0.604	
Medium aquifer sand (oxygenated)										
Pc	rep 1 (1-site)	**53** (2)	**0.58** (0.17)			0.96	2.41×10^{-1}	0.01	0.182	13 (4)
	rep 2 (1-site)	**215** (3)	**1.48** (0.16)			0.8	5.78×10^{-3}	0.01	0.657	
	rep 3 (1-site)	**103** (5)	**0.75** (1)			0.69	7.05×10^{-2}	0.01	0.338	
Fine aquifer sand										
Pc	anoxic (1-site)	**765** (91)	**0.91** (0.79)			0.81	1.0×10^{-4}	0.014	0.569	40
	oxygenated	**515** (30)	**0.86** (0.38)	**317** (415)	**155** (258)	0.21	8.1×10^{-4}	0.014	0.440	31

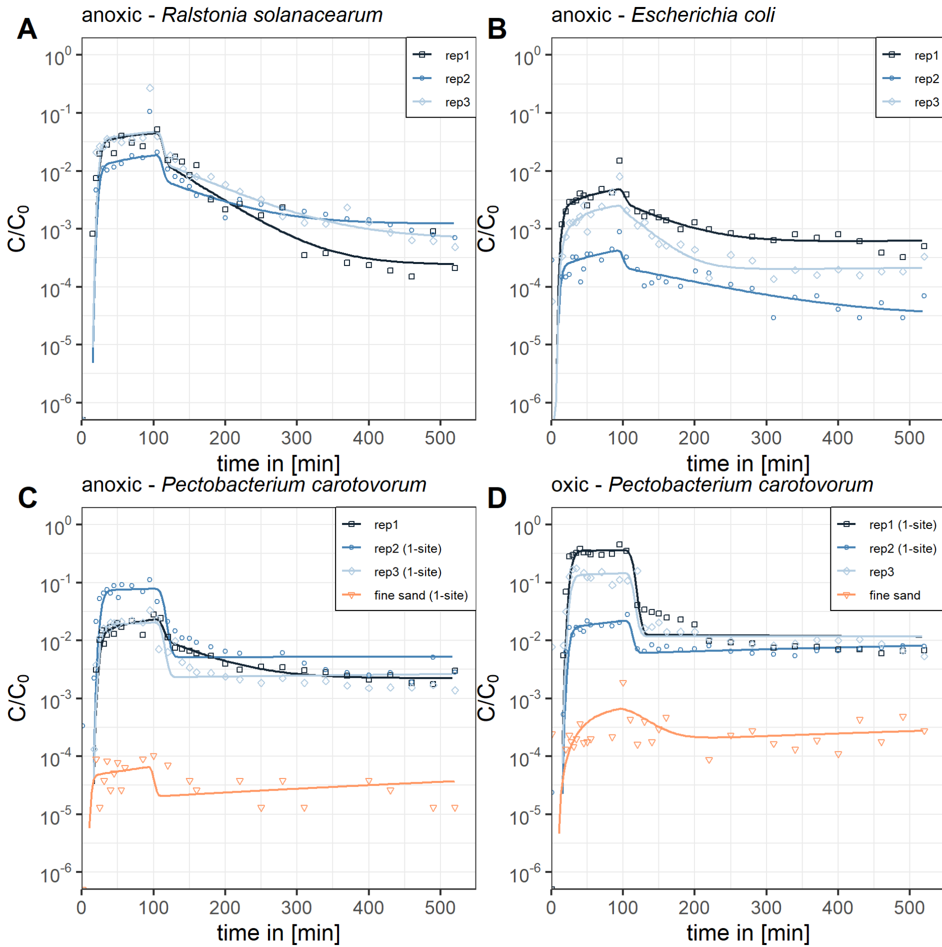
Pc = *Pectobacterium carotovorum*, *Ds* = *Dickeya solani*, *Rs* = *Ralstonia solanacearum*, *Ec* = *Escherichia coli* WR1; Θ = porosity, α_L = longitudinal dispersivity, R^2 = coefficient of determination to evaluate the model fit to the data; η_0 = single collector contact efficiency; α = sticking efficiency; λ = removal per meter distance, average removal from replicate experiments is shown.

3.3.3 Bacterial transport in aquifer sand

Bacterial BTCs in medium aquifer sand are shown in Figure 3A-D. The replicate experiments for each bacterium show a higher variability in aquifer than in quartz sand as a result of the greater heterogeneity of the sand material represented by a higher C_u. In some experiments, retained bacteria from a previous replicate experiment detached and were measured in the effluent before the actual bacterial breakthrough was expected. Yet, these concentrations were low (<10^2 and once 10^3 CFU mL^{-1}) and were in the range or lower than the tail concentrations caused by detachment. The peak breakthrough of the bacteria and the overall removal was not influenced. For example, during the transport of *E. coli* in anoxic medium MAR sand (replicate 2 and 3), about 10^2 CFU mL^{-1} were detected in the first sample taken after one minute. However, k_{att1} as well as the overall removal were higher in replicate 2 and 3 in comparison to replicate 1 which did not yet contain attached bacteria.

In anoxic medium aquifer sand, the C/C_0 values were at least three orders of magnitude lower than in the quartz sand. Differences in attachment to quartz and natural MAR sand can also be explained by the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory (Verwey et al., 1948; Derjaguin and Landau, 1993). It describes the repulsive and attractive forces between the bacterial cell and grain surface. Clean quartz sand has a negative surface charge (negative zeta potential) that repulses negatively charged bacteria (Elimelech et al., 2000). Unfavorable attachment can occur when the distance between grain surface and bacterial cells gets small enough and the attractive van der Waals forces dominate over the repulsive double layer. Favorable attachment occurs in natural sediments where metal oxides depict favorable attachment sites that attract negatively charged bacteria. Fine and medium MAR sand had the same amount of Fe- and Al-metal oxides (Table S2) although the total extractable iron was higher in medium aquifer (21 ± 0.6 mg L^{-1}) than in fine aquifer sand (6.8 ± 0.7 mg L^{-1}, Table S5). Grain size played an important role in the retention of the bacteria as the MAR sand has a larger fraction of fine sands compared to quartz providing a greater total surface area for attachment. Likewise, the retention in fine MAR sand has been greater than in medium MAR sand. They varied in the fraction of fine sands smaller than 250 μm which was about 77% in fine and 30% in medium MAR sand. These fractions of very fine sands, silt, and clay (<32 μm; 4.7% in fine and 3.2% in medium MAR sand) reduce the pore space which can result in a retention of the bacteria by size-exclusion or a longer travel distance through the column. This increases contact time and the chance of attachment of bacteria to the sand material (Murphy and Ginn, 2000).

As observed in the quartz sand experiments, *E. coli* showed higher removal than the plant pathogenic bacteria comparing the BTCs where the peak C/C_0 is on average 2.3×10^3 for *E. coli* and 1.9×10^2 for *P. carotovorum*, and 1.4×10^2 for *R. solanacearum*. Moreover, k_{att1} coefficients were about ten times higher in medium aquifer sand ranging from 129 – 551 day^{-1}. Detachment at site 1 is negligible as values are small (<1 day^{-1}), similar to the quartz sand experiments. The highest removal was achieved in fine aquifer sand under anoxic and oxygenated conditions, where *P. carotovorum* had a k_{att1} of 765 day^{-1} and 515 day^{-1}, respectively. The peak C/C_0 values in fine aquifer sand were very low (1.0×10^{-4} under anoxic and 8.1×10^{-4} under oxygenated conditions) and effluent concentrations were at the limit of detection (Figure 3C+D). As a result of the high removal, the breakthrough peak in fine sand columns was less pronounced in comparison to the shape of the BTCs in quartz and medium aquifer sand experiments. The effluent concentrations in the experiment with oxygenated fine sand were scattered and resulted in a poor model fit (R^2 = 0.21). Although the data of the anoxic fine sand experiments were also scattered, a reasonable fit using the 1-site kinetic model was achieved (R^2 = 0.81). Here, the initial breakthrough of the bacteria was noticeable which has a greater influence on R^2. Overall, all BTCs of *P. carotovorum* in aquifer sand were better fitted with the 1-site kinetic model, while the transport of *E. coli* and *R. solanacearum* in medium aquifer sand showed a better fit applying the 2-site kinetic model. The k_{att2} and k_{det2} values were in a similar range for *R. solanacearum* (about 25 day^{-1}), while for *E. coli*, k_{att2} was higher (75-132 day^{-1}) than k_{det2} (0.4-79 day^{-1}). The results agree with the observations by Schijven et al. (2002) that site 1 is characterized by relatively fast attachment and slow detachment, while site 2 is characterized by relatively fast attachment and fast detachment, implying that attachment to site 1 mostly determines removal.



▲ **Figure 3** Bacterial breakthrough curves in medium (shades of blue) and fine (orange) aquifer sand (rep = replicate). Normalized effluent concentrations (C/C_0) are plotted as a function of time (min). Solid lines represent the fitted models obtained from Hydrus-1D and the symbols represent the corresponding dataset in the same colour. Two-site kinetic models are shown if not stated other in the legend. Triplicate experiments (rep. 1-3) in anoxic medium aquifer sand of *Ralstonia solanacearum* (A), *Escherichia coli* (B), and *Pectobacterium carotovorum* (C) are shown. Triplicate experiments in oxygenated medium aquifer sand of *P. carotovorum* are shown (D). BTC of *P. carotovorum* in fine aquifer sand is shown in orange under anoxic (C) and oxygenated (D) conditions.

3.3.4 Parameter evaluation

The 1-and 2-site kinetic models and associated parameter estimates resulted in a good fit of the bacterial breakthrough data as confirmed by AIC and R^2. Yet, we often found little differences between the 1- and 2-site kinetic models in terms of goodness of fit (AIC and R^2, Table S4), indicating both models would be a reasonable choice and that kinetic site 2 is less relevant (e.g., *E. coli* in quartz sand). The rate coefficients of kinetic site 2 influenced the shape of the BTC and improved model fit, as for example the BTC of *P. carotovorum* replicate 2 in anoxic medium aquifer sand (Figure 3C). There, the 2-site kinetic model allowed a smooth transition from the declining limb towards the tail of the BTC, governed by k_{att2} and k_{det2} (Schijven et al., 2000). In contrast, the 1-site kinetic model (replicate 1 and 3) failed to model a smooth transition from the peak to the decline of the BTC and the curve shape of the tail. Nevertheless, Figure 3C shows that the 1-site model represents sufficiently the curve shape of the BTCs. Consequently, site 2 was less important for the removal of the pathogens which was also represented by the high standard deviations of the parameters at site 2 (e.g., k_{det2} = 49 ± 39 day^{-1}, *P. carotovorum* in quartz sand). The peak C/C_0 was mainly determined by k_{att1} which was confirmed by calculating C/C_0 using only k_{att1} as input value using equation (5). The calculated C/C_0 and modeled C/C_0 by Hydrus-1D were in the same range. k_{det1} determined the height of the tail and the values of C/C_0 at the tail are magnitudes lower than at the peak breakthrough in all experiments. In the aquifer sand experiments, the tail was about three orders of magnitude lower than the peak C/C_0 and in all experiment, the tail could only be visualized using the semi-log scale (see Figure S2). Therefore, the removal mechanism can be simplified and only described by irreversible attachment that neglects detachment.

We compared results of the reversible attachment-detachment model with CFT to evaluate k_{att1}. In CFT, the removal of colloids (bacteria) is described with the single collector contact efficiency (η_0) and the sticking efficiency (α) from which an irreversible deposition rate can be calculated (Pang et al., 2021). The results of η_0 and α are shown in Table 3. η_0 was similar in quartz and aquifer sand (0.009-0.014). α values were low in quartz sand (0.018 – 0.176) reflecting little attachment, while higher collision frequencies were represented by high α in aquifer sand (0.182 – 0.725). Previously reported (Hijnen et al., 2005) sticking efficiencies for *E. coli* WR1 in column experiments with finer natural sandy soil (d_{50} = 180 μm) were 0.341 at 0.5 m day^{-1} and 0.424 at 0.9 m day^{-1}, therefore lower than our results for *E. coli* in medium aquifer sand (α = 0.540 – 0.725, at 3.6 m day^{-1}). Moreover, CFT predicts that finer sand results in higher sticking efficiencies. Yet, in our results, sticking

efficiencies of *P. carotovorum* in medium aquifer sand were similar or higher than in fine sand. CFT does not consider heterogeneities of the porous medium such as metal oxides that pose favourable attachment sites, nor physiological bacterial characteristics like hydrophobicity or surface charge. The attachment-detachment model accounts for these heterogeneities which is reflected in higher k_{att1} comparing transport of *P. carotovorum* in anoxic fine (765 day^{-1}) and medium aquifer sand (e.g., rep. 1: 184 day^{-1}). Finally, although k_{att1} mostly governs removal, detachment has to be taken into account as detached microorganisms re-contaminate the water source and might pose a risk to the end-user. Results of Knappett et al. (2014) pointed out detachment should not be ignored at the field scale.

3.3.5 Microbial removal rates

To evaluate our results on a larger scale we analysed $\log_{10}$ removal per meter that only takes into account irreversible attachment to site 1. These ranged between 0.42 for *R. solanacearum* and 6.8 $\log_{10}$ m^{-1} for *E. coli* in quartz sand. A similar removal (4.69 log m^{-1}) in quartz sand columns (d_{50} = 250 μm) were obtained for *E. coli* J6-2 by Weaver et al. (2013). Our quartz sand experiments allowed to investigate bacterial transport under very controlled conditions as the sand has a defined grain size and no favourable attachment sites. There were no physicochemical differences between the quartz experiments, except that the experiment with *R. solanacearum* was done at room temperature. Previous research showed that temperature had little effect on bacterial deposition (Kim et al., 2009) but it did influence the retention of viruses (Sasidharan et al., 2017). Consequently, differences in transport are due to the nature of the bacterial species and their metabolic state which influences cell characteristics like hydrophobicity and surface charge (Murphy and Ginn, 2000; Bradford et al., 2013).

Compared to quartz sand, the removal in anoxic medium aquifer sand was much higher for *R. solanacearum* (18 $\log_{10}$ m^{-1}) and *P. carotovorum* (19 $\log_{10}$ m^{-1}) and even 28 $\log_{10}$ m^{-1} for *E. coli*. Similar removal of about 20 log m^{-1} were presented by Oudega et al. (2021) using column experiments with natural heterogeneous gravel material and the bacterium *Bacillus subtilis* and a bacteriophage. Yet, the authors found a much lower removal in the field (0.2 log m^{-1}) which they explained by preferential flow paths that may occur in their studied aquifer and enhance the bacterial transport. These large discrepancies between column and field studies have also been summarized by Pang (2009) and need to be considered in risk assessments. The highest

removal (40 log_{10} m^{-1}) was observed in oxic fine aquifer sand for *P. carotovorum*. This highlights the importance to know about the geological structure of a site. Although the aquifer sands used in our study had a similar d_{50} of 192 µm (fine) and 305 µm (medium), their removal capacity differed greatly. This may be caused, amongst others, by the very fine fraction (<125 µm) which was 10% in fine sand and only 3% in medium aquifer sand (Table S1). Therefore, a detailed geological description of a MAR site is very useful to predict removal of pathogens and field tests should be included if feasible.

3.3.6 Influence of oxygenation

In this research, the influence of oxygenation of the aquifer sand material and effects on the transport of *P. carotovorum* was investigated. The column filled with medium aquifer sand was first operated under anoxic conditions followed by flushing the column with oxic aGW to simulate infiltration of oxic water into an anoxic aquifer. Oxygen levels in the column influent and effluent remained below 0.1 mg L^{-1} during anoxic experiments. During the flushing, the influent O_2 concentration was about 12 mg L^{-1}, while the effluent O_2-levels were on average 1.4 mg L^{-1} lower than the influent indicating O_2 consumption by chemical and/or biological reactions within the column. Reactive transport modelling has shown that the injection of oxic water into an anoxic aquifer will modify the biological and geochemical properties of the aquifer and thereby, could affect the microbial transport over time (Kruisdijk and van Breukelen, 2021). Ferrous iron (e.g., from minerals like pyrite) is oxidized and results in formation of positively charged iron oxide coatings which can increase bacterial attachment due to their negative surface charge (Johnson et al., 1996). For example, virus attachment was reduced under anoxic conditions in field experiments (Schijven and Hassanizadeh, 2000), while sub(oxic) conditions increased virus removal due to positively charged metal oxides (Hornstra et al., 2018). In contrast, our results unexpectedly show that removal of *P. carotovorum* decreased under oxygenated conditions compared to prior anoxic conditions. The average removal was 19 (± 1.3) log_{10} m^{-1} under anoxic and 13 (± 4) log_{10} m^{-1} under oxic conditions. The transport in oxygenated fine aquifer sand also resulted in lower removal, although the flushing with oxygen-rich water was done for a shorter time (2 days). Here, the removal was 40 log_{10} m^{-1} under anoxic and 31 log_{10} m^{-1} under oxic conditions. On the one hand, lower retention during oxic experiments may have been a result of fewer available attachment sites due to retained cells of *P. carotovorum* during the anoxic medium MAR experiments. On the other hand, we expect that most the bacteria died-off during the two weeks flushing with oxic aGW (Eisfeld et al., 2021) or detached during flushing with deionized water which changed the ionic strength (Foppen et al., 2007). Moreover, the retention

of *P. carotovorum* in oxic medium MAR sand in replicate 2 was just as high (k_{att1} = 215 day^{-1}) as during the anoxic experiments (k_{att1}= 129-202 day^{-1}) which indicates that there were still sufficient attachment sites available. The two other replicate experiments (1+3) under oxic conditions showed less removal under oxic conditions calculated sticking efficiencies that were lower in oxic compared to the anoxic experiments in medium and fine aquifer sand.

As stated earlier, oxygen-rich water modifies the geochemistry of an anoxic aquifer, but also acts as an environmental signal for microorganisms that may trigger metabolic changes which consequently modifies attachment. As response to anoxic conditions, microorganism can modify their gene expression which may lead to the production of different surface structures (e.g., lipopolysaccharides) and alterations in surface charge as shown for *E. coli* (Landini and Zehnder, 2002). Castro and Tufenkji (2008) analysed the transport of *E. coli* O157:H7 and *Yersinia enterocolitica* under low and high oxygen concentrations. While *E. coli* had a higher sticking efficiency under oxic conditions, attachment of *Y. enterocolitica* was increased under anoxic conditions. The authors assumed that growing the bacteria either aerobically or anaerobically resulted in differences in the cell surface structure and surface charge. Similarly, the plant pathogenic bacteria have adaptation strategies that changes their transcriptional response and surface properties in response to environmental changes (Lisicka et al., 2018). To conclude, expected improvement in removal due to oxygenation was not observed as the duration of the oxygenation may have been insufficient to increase the presence of iron-oxides. Future research is needed to further investigate the effects of oxygenation on the porous media and its effects on the pathogens and natural microbiota to evaluate it overall effects on pathogen transport.

3.3.7 Applicability to MAR systems

Column experiments are an important tool in the assessment of bacterial transport because environmental conditions can be better controlled than in the field. Nevertheless, upscaling of these results to field scale needs to be done with caution as results may overestimate the actual removal in the field, particularly when the variation in grain size distribution with depth is poorly characterized (Pang, 2009; Oudega et al., 2021). The quality of the water used for injection should also be considered in translating column results to the field scale. In our study, high inoculation concentrations (10^6 CFU mL^{-1}) were used to enable measuring the breakthrough over a longer period, but this does not represent concentrations of plant pathogenic bacteria found in surface

waters which are about 0.1 - 10^3 CFU mL^{-1} (Wenneker et al., 1999). Moreover, the applied flow velocity of 3.6 m day^{-1} represents high groundwater flow during constant injection. Under realistic conditions, water will be injected during rain events but recovered at a different time point and the velocity in the aquifer will be lower especially during periods without infiltration. Lower velocity results in higher contact time of the pathogens with the grain surface which favours attachment, and residence time is an essential factor in MAR operation (Hendry et al., 1999; Massmann et al., 2008). Field experiments with pathogenic organisms are often prohibited. Therefore, we included *E. coli* WR1 as reference organism for future field experiments. Yet, *E. coli* WR1 had the highest removal in quartz and aquifer sand in comparison to the plant pathogenic bacteria which makes it unsuitable for direct comparison to these pathogens. In a future study, an alternative surrogate for field experiments should be tested in column experiments beforehand to evaluate the comparability with the plant pathogens. Nevertheless, the results showed that the aquifer sands were an effective filter to remove bacterial pathogens. Future work will combine the results of this study on filtration processes with those of die-off experiments with the same plant pathogens (Eisfeld et al., 2021) in a quantitative microbial risk assessment model to aid the design (e.g., minimal travel distances and residence times) of MAR systems.

3.4 Conclusions

Plant pathogenic bacteria are a threat to global agricultural production and dissemination of plant diseases via contaminated irrigation water has often been observed. Irrigation water availability is under pressure due to water scarcity and the deterioration of (surface) water quality. We studied if aquifer storage and recovery as solution for water scarcity could deliver safe irrigation water by means of plant pathogen removal during transport in natural sediments. In laboratory column experiments, we showed the effective removal of three plant pathogenic bacteria in natural sediments from a MAR site and compared the transport with the human pathogen *E. coli* WR1. Removal parameters were obtained by modelling the results in Hydrus-1D. The heterogeneous natural aquifer sands had a higher (up to several log_{10}) pathogen removal capacity than quartz sand. Oxygenation of the anoxic aquifer sands did not increase removal, contrary to our expectations. Tailing caused by detachment has been observed in all BTCs but was several magnitudes lower than the peak C/C_0 suggesting that it may be neglected. However, detachment has to be considered as trace levels of highly infectious pathogens may still be present after soil treatment. The quartz sand experiments

represent non-favourable conditions for attachment and results may be used as worst-case scenario in risk assessments. In conclusion, our study confirms the potential of MAR as a natural water treatment technology in agricultural settings to store and supply both fresh, and bacterially safe irrigation water.

3.5 Appendix B

The supporting information consists of S1 that describes in detail the bacteria and culture media used in this study. Figure S1 shows the grain size distribution chart of the used sands and Figure S2 shows the BTCs in quartz sand on a linear an semi-logarithmic scale. Table S1 shows the grain size analysis and Table S2 the chemical analysis of the aquifer sands used in the column experiments. Table S3 the results of the hydrophobicity (MATH) test and Table S4 lists the R^2 and AIC output parameters from Hydrus-1D to compare the 1-and 2-site kinetic model. Table S5 shows results of the iron content of the sands used in the experiments. The supplementary data to this article can also be found online at https://doi.org/10.1016/j.watres.2022.118724

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Chapter 4
Dose-response relationship of *Ralstonia solanacearum* and potato in greenhouse and *in vitro* experiments

This chapter is based on:
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Highlights

- First study to analyse the dose-response relationship of potato and *R. solanacearum* under low inoculum concentrations
- Dose of $5x10^2$ cells/plant of *R. solanacearum* can cause latent infections using soil-soak inoculation
- Dose of $5x10^2$ cells/plant of *R. solanacearum* required to cause visual bacterial wilt symptoms
- *In vitro* study allowed to study the effect of very low inoculation concentration (0.5 CFU/plant)
- Dose-response results are an integral element of quantitative microbial risk assessments

Abstract

Ralstonia solanacearum is the causative agent of bacterial wilt of potato and other vegetable crops. Contaminated irrigation water contributes to the dissemination of this pathogen but the exact concentration or biological threshold to cause an infection is unknown. In two greenhouse experiments, potted potato plants (*Solanum tuberosum*) were exposed to a single irrigation with 50 mL water (non-invasive soil-soak inoculation) containing no or 10^2 – 10^8 CFU/mL *R. solanacearum*. The disease response of two cultivars, Kondor and HB, were compared. Disease development was monitored over a three-month period after which stems, roots and tubers of asymptomatic plants were analyzed for latent infections. First wilting symptoms were observed 15 days post inoculation in a plant inoculated with $5x10^9$ CFU and a mean disease index was used to monitor disease development over time. An inoculum of $5x10^5$ CFU per pot ($1.3x10^2$ CFU/g soil) was the minimum dose required to cause wilting symptoms, while one latent infection was detected at the lowest dose of $5x10^2$ CFU per pot (0.13 CFU/g). In a second set of experiments, stem-inoculated potato plants grown *in vitro* were used to investigate the dose-response relationship under optimal conditions for pathogen growth and disease development. Plants were inoculated with doses between 0.5 and $5x10^5$ CFU/plant which resulted in visible symptoms at all doses. The results led to a dose-response model describing the relationship between *R. solanacearum* exposure and probability of infection or illness of potato plants. Cultivar Kondor was more susceptible to brown-rot infections than HB in greenhouse experiments while there was no significant difference between the dose-response models of both cultivars in *in vitro* experiments. The ED_{50} for infection of cv Kondor was $1.1x10^7$ CFU. Results can be used in management strategies aimed to reduce or eliminate the risk of bacterial wilt infection when using treated water in irrigation.

4.1 Introduction

The *Ralstonia solanacearum* species complex causes bacterial wilt and comprises three different species, namely *R. solanacearum*, *R. seudosolanacearum* and *R. syzigii* (Safni et al., 2014). Together they affect more than 200 plant species present in tropical to temperate climates. Hosts include *Solanaceous* crops, groundnut, banana and plantain, weeds, some tree species and ornamental plants (Hayward, 1994). In temperate climates, potato (*Solanum tuberosum*) production is affected by brown-rot, caused by *R. solanacearum* phylotype II (race 3 biovar 2) which is a strain adapted to cooler regions (Janse, 1996). As a result of a high number of potato brown rot outbreaks in Europe during the 1990's, the pathogen complex had been put under quarantine status in the European Union with the aim to prevent its spread and eradicate the pathogen after outbreaks (Directive, 1998; 2006).

The emergence of brown rot has predominantly been associated with the use of (latently) infected seed tubers. Trading of seed and ware potatoes forms the highest risk of disease spread internationally (EFSA et al., 2019). For example, the first occurrence of brown rot in the Netherlands was detected in a ware potato field in 1992, presumably introduced through infected non-certified seed tubers (Janse et al., 1998). Natural dispersal of brown rot is mainly caused by contaminated irrigation water as surface water used in irrigation can become contaminated with run-off water from *R. solanacearum* infected agricultural fields or through contact with effluent water coming from potato processing industry (EFSA et al., 2019). *R. solanacearum* has been repeatedly detected in surface waters in the Netherlands and other European countries and disease outbreaks were directly linked to the use of contaminated surface water in irrigation (Janse, 1996; Wenneker et al., 1999). Moreover, *R. solanacearum* survives in plants such as bittersweet nightshade (*Solanum dulcamara*) and to a lesser extent in stinging nettle (*Urtica dioica*). They often grow along ditches next to agricultural fields from where the bacterium is released into the environment in high numbers (Olsson, 1976; Elphinstone et al., 1998). Eventually, this led to an irrigation ban on the use of surface water for (seed) potato production in the Netherlands and other European countries. Although this measure has effectively reduced the number of brown rot incidences (Janse, 2012) farmers were confronted with increased water scarcity. Rainfall can be insufficient to meet crop water requirements during the cropping season. In potato production, irrigation is essential to ensure a high tuber yield and potatoes are specifically sensitive to water stress during tuber initiation (Alva, 2008). Water scarcity in agriculture will

further increase since surface water is not available for irrigation anymore and groundwater extraction is often restricted or prohibited as a result of climate change (Eyring et al., 2021). Consequently, farmers are looking for different fresh water sources which often include the recycling of irrigation water as done in greenhouse cultivation or the treatment of water with lower quality (e.g., treated wastewater) (Hong and Moorman, 2005). Additionally, more efficient irrigation systems like drip irrigation can be installed to reduce water use (Shock et al., 2002). Although these water management strategies help to overcome water shortages, (plant) pathogenic micro-organisms and other (agro)pollutants may accumulate in water recycling systems (Ristvey et al., 2019). Therefore, treated water needs to meet quality requirements for irrigation water. Populations of pathogenic organisms need to be lower than the biological threshold which is the minimum inoculum level or dose which is required to cause a response (infection) in the host (Lamichhane and Bartoli, 2015).

In nature, a minimum pathogen concentration is required to establish an infection which had to withstand detrimental environmental conditions and the host defense system. Under optimal conditions for the pathogen, however, the concentration to infect a host may be as low as a single cell. Information on the biological threshold for plant pathogens in irrigation water is scarce despite its relevance (Stewart-Wade, 2011). For *R. solanacearum*, most infection studies analyzed the resistance of potato cultivars or the virulence of different *R. solanacearum* strains using high concentrations of about 10^6-10^8 CFU/mL (e.g., Lebeau et al. (2010); Aliye et al. (2015); Mori et al. (2015)). Bowman and Sequeira (1982) tested wilt-resistant potato clones against a highly virulent strain of *Pseudomonas solanacearum* from Mexico using stem-inoculation with 5.6×10^{-1} to 1.2×10^8 CFU/plant which resulted in 50% wilted plants at an inoculum of 3-100 CFU/plant and 2.1×10^6 CFU/plant in a more resistant clone of *S. phureja.* However, invasive infection via stem-inoculation may overestimate the disease response and cannot simulate natural exposure of the pathogen through contaminated irrigation water. Montanelli et al. (1995) inoculated potato plants by non-invasive root inoculation to differentiate between resistant and susceptible cultivars. The authors concluded that a dose of 5×10^6 CFU/plant was necessary to identify resistant plants. However, infections were still observed in all three cultivars at the lowest tested concentration of 5×10^5 CFU/plant. To our knowledge, only one study by Singh et al. (2014) investigated the effect of low concentrations (10^1-10^{10} CFU/mL) of *R. solanacearum* on potted tomato plants in a greenhouse setting. In their experiments, a single irrigation event containing a dose of 5×10^3 CFU of *R. solanacearum* resulted in bacterial wilt incidence. At an inoculum of 5×10^2 CFU, infections were only observed when roots had been injured before irrigation.

As part of a quantitative microbial risk assessment, we investigated the dose-response relationship between *R. solanacearum* and potted potato in greenhouse experiments. In addition, the minimum concentration required under optimal conditions for disease development was assessed in experiments with *in vitro* plants using cultivars with different pathogen susceptibility. Dose-response models are essential in risk assessments and have already been applied in studies related to human health and pathogen exposure after the consumption of treated drinking water (Schijven et al., 2019). Dose-response models in the context of plant health are scarce (Boelema, 1985) or used to evaluate the efficacy of biological controls for plant pathogens (Montesinos and Bonaterra, 1996; Smith et al., 1997). The aim was to develop a dose-response model that eventually can be applied to value the effectivity of (irrigation) water treatment systems that remove pathogens and provide safe irrigation water.

4.2 Material and Methods

4.2.1 Bacterial strain and growth conditions

R. solanacearum phylotype II (race 3 biovar 2) strain IPO-4187 was received from the working collection at Wageningen UR and used in this study. Strain IPO-4187 was derived by making *R. solanacearum* strain PD2762, isolated from potatoes in the Netherlands in 2016, rifampicin resistant following the protocol described by van der Wolf et al. (2022). The strain belongs to sequevar 1 (*R. solanacearum* phylotype IIB-1) (EPPO, 2018). The strain was kept at 80 °C using the multi-purpose protect cryobeads system (Technical Services Ltd, Lancashire, GB) and revived on casamino-acid-peptone-glucose (CPG) agar before the experiment (Hendrick and Sequeira, 1984). CPG is composed of 1 g/L casamino acids, 10 g/L peptone, 5 g/L glucose and 15 g/L agar. Inoculation suspensions were prepared from cultures grown overnight in CPG broth at 28 °C while shaking (150 rpm). Cultures were harvested by centrifugation (3500 x *g*, 20 min at room temperature) followed by washing and resuspending the pellet in a quarter strength Ringer's solution (in the following referred to as "Ringer's solution"; Sigma-Aldrich; St. Louis, USA). This pelleting and washing step was repeated twice to remove any excess broth. Lastly, Ringer's solution was added to resuspend the pellet and the bacterial suspension was then diluted to reach an optical density of 0.1 at 600 nm representing a concentration of approximately 10^8 CFU/mL which was confirmed by dilution-plating. *Ralstonia solanacearum* was detected on the semi-selective medium South Africa (SMSA) (Elphinstone et al., 1998) supplemented with 50 mg/L rifampicin to suppress the growth of background

bacteria and 200 mg/L cycloheximide to suppress fungal growth. Duchefa Biochemie (Haarlem, NL), Sigma-Aldrich (St. Louis, MO, USA), and Fisher Scientific (Hanover Park, IL, USA) were our chemical suppliers.

4.2.2. Greenhouse experiments

4.2.2.1 Plants and growth conditions

During the summers of 2019 and 2020, the dose-response relationship of *R. solanacearum* and potato (*Solanum tuberosum*) was assessed in a climate controlled compartment of the Unifarm greenhouse facilities of Wageningen University & Research, Wageningen (NL). Certified pest-free minitubers of cultivar (cv) Kondor (2019) and HB (2020) were obtained from Agrico (Emmeloord, NL) and stored at 4 °C. Both cultivars are susceptible for *R. solanacearum* but there is no further information available how resistant these strains are towards the pathogen. For the experiment, 100 mini-tubers were presprouted in the light for about two weeks at 15 °C until they were planted in 5 L plant pots containing 4 kg clay loam obtained in spring 2019 from an agricultural field of Unifarm which was prepared for potato cultivation. The soil consisted of about 27% clay, 33% sand and 32% silt. Half of the soil was used for the experiment in 2019 while the rest was stored at 4 °C for the experiment in 2020. The air dried soil was roughly sieved (1 x 1 cm mesh size) before filling the pots. In each pot, an about 10 cm deep hole was dug in the soil where one mini-tuber was placed inside with the sprouts put up and covered with a few centimeters of soil. Pots were placed on saucers and the soil was watered from above. Temperature of the greenhouse was set to 23 °C and 70% humidity during the experiment. After emergence of the plants, a 16-h light period was achieved using supplemental lighting (high-pressure sodium lamps, 150 W/m^2) when needed. Till inoculation of the potato plants, the soil was daily watered from above. Once a week fluid nutrient solution (Yara Netherlands, Vlaardingen, NL) for potted tomato plants was applied following the manufacturer's guidelines. Five days before inoculation, plants received restricted watering. After inoculation, the plants were watered via the saucers to avoid crosscontamination caused by splashing or formation of aerosols. To avoid water saturation of the soil of bacterial wilt diseased plants, the irrigation volume was adapted to the plant's needs. The plants were inspected weekly for symptom expression till the end of the experiments.

In addition to the potato plants, 6 (2019) or 10 (2020) tomato plants cv Moneymaker were grown in the same greenhouse compartment to check the virulence of the *R. solanacearum* inoculum used in the potato dose-response experiments. Watering was done the same way as with the potato plants. The tomato plants were inspected weekly for symptom expression during four weeks.

Simultaneous with the 2020 dose-response experiment, the die-off of a *R. solanacearum* population was studied in soil without the presence of a host plant. The experiment was done in two sets, each consisting of a control and three soil contaminated pots, respectively. One set (disturbed) was used for sampling of the soil 7, 14, 21, 28, 35 and 54 days post inoculation (dpi). The other set was sampled only at the end of the experiment (undisturbed) to exclude influence of the soil sampling on the pathogen's persistence. The control pot of the undisturbed set was used to monitor the soil moisture, temperature and humidity during the experiment (Hobo Soil Moisture Smart Sensor connected to a data logger, Onset Computer Corporation, MA, USA). Pots (5 L) filled with 4 kg of the same soil as used in the dose-response experiment were used. The soil of the *R. solanacearum* population die-off was watered from above during the length of the experiment as no plant roots were present to suck the water from the saucer.

4.2.2.2 Selection of inoculation doses

The goal was to simulate natural infection of potato plants by *R. solanacearum* using a single irrigation event with contaminated water. Several factors were considered in the selection of doses. First, we used irrigation data from a potato growing field in the North of the Netherlands (53.2945693 N, 7.0045595 E). There, the drip irrigation doses for potato cropping were monitored for three consecutive years during the cropping season (2016-2018) and ranged between 53 and 176 mm (Acacia Water, 2019b; a). The highest irrigation dose was supplied during the dry summer in 2018. Secondly, we considered *R. solanacearum* concentrations in contaminated surface water from different regions in the Netherlands which were up to 10^2 CFU/mL during the summer months (Wenneker et al., 1999). In a conservative scenario, the maximum dose applied to a potato plant during the cropping season would result in about $7x10^5$ CFU; calculated from 176 mm irrigation, a soil surface in the pot of 415 cm^2, and the concentration of R. solanacearum in contaminated surface water (10^2 CFU/mL). Based on this dose, we selected a range of doses from $5x10^2$ to $5x10^9$ CFU per pot as shown in Table 1A.

▼ **Table 1** Setup for dose-response experiments in greenhouse (A) and *in vitro* (B) for potato cultivars Kondor and HB, where potato plants were inoculated with selected concentrations of *R. solanacearum*.

A. Greenhouse experiments										
Number of plants per cultivar		5	15	15	15	15	10	10	10	5
inoculation concentration[1]	CFU/mL	control	10^1	10^2	10^3	10^4	10^5	10^6	10^7	10^8
estimated dose	CFU	control	$5x10^2$	$5x10^3$	$5x10^4$	$5x10^5$	$5x10^6$	$5x10^7$	$5x10^8$	$5x10^9$
Dose per g soil (4 kg soil)	CFU/g	control	0.13	1.3	13	$1.3x10^2$	$1.3x10^3$	$1.3x10^4$	$1.3x10^5$	$1.3x10^6$
Dose per soil surface area[2]	CFU/cm^2	control	1.2	12	$1.2x10^2$	$1.2x10^3$	$1.2x10^4$	$1.2x10^5$	$1.2x10^6$	$1.2x10^7$
B. *In vitro* experiments										
Nr. of plants per repetition and cultivar (2 repetitions)		5	15	15	15	15	10	10	10	
inoculation concentration[3]	CFU/mL	control	$5x10^2$	$5x10^3$	$5x10^4$	$5x10^5$	$5x10^6$	$5x10^7$	$5x10^8$	
estimated dose	CFU	control	0.5	5	50	500	$5x10^3$	$5x10^4$	$5x10^5$	

[1] 50 mL applied for non-invasive soil-soak inoculation; [2] soil pot surface area = 415 cm^2;
[3]1 µL applied on stem wounding.

4.2.2.3 Inoculation procedure

Five days before inoculation, plants received restricted watering. In the dose-response experiments, unwounded potato plants (36 days old in 2019, 30 days old in 2020) were inoculated via a soil-soak inoculation assay (Tans-Kersten et al., 2001) by pouring 50 mL of *R. solanacearum* suspension with the respective concentration onto the soil around the stem, followed by watering with 200 mL water to let the inoculum penetrate into the soil. The pot surface area was 415 cm^2, therefore, the plants were irrigated with a total of 6 mm of which 1.2 mm (50 mL) were the inoculation suspension. Of the 100 plants grown for each experiment 95 plants were inoculated with bacterial suspension. Five plants treated with 50 mL Ringer's solution served as negative controls (Table 1A). As we expected few infections in the lower inoculum concentrations (10^1 – 10^4 CFU/mL), 15 plants were inoculated per concentration while 10 plants per concentration were inoculated with the higher concentrations (10^5 – 10^7 CFU/mL), and 5 plants with the highest concentration of 10^8 CFU/mL. After inoculation, the plants were sorted in 5 replicate blocks of 20 plants in which each dose was

represented proportionally (e.g., 1 control plant per block or 3 plants of the dose $5x10^2$ CFU).

In the experiment in which the die-off of *R. solanacearum* in soil was studied without plants, soil was contaminated with the pathogen following the same procedure as was used in the dose-response experiment. Per pot, 50 mL of a 10^8 CFU/mL bacterial suspension was applied onto the soil corresponding to $1.3x10^6$ CFU/g. The controls were treated with Ringer's solution.

Three (2019) or five (2020) tomato plants were inoculated by injecting ca. 25 µL of a suspension of 10^8 CFU/mL of *R. solanacearum* via the leaf axil of the 2nd or 3rd true leaf into the stem. The same number of plants were used as control, respectively, and injected with Ringer's solution.

4.2.2.4 Disease scoring

The potato plants were monitored weekly after inoculation by visual inspection for typical bacterial wilt symptoms. The monitoring period was 38 dpi for cv Kondor and 54 dpi for cv HB. Per plant, the total amount of stems was counted and each stem was scored individually using the following categories: 0 – no wilting; 1 – 0-25% of stem wilted; 2 – 26-50% of stem wilted; 3 – 51-75% of stem wilted; 4 – 76-100% of stem wilted. Per plant and time point a disease index (DI) was calculated:

$$DI = \frac{\sum wilting\ scores\ per\ stem}{total\ number\ of\ stems}$$

Finally, a mean DI value was calculated considering the total number of plants for each treatment and their respective DIs. Healthy plants were also included in the calculation of total number of stems.

4.2.2.5 Plant and soil sampling

At the end of the dose-response experiments, plant material (stems, tubers and roots) and a mixed soil sample consisting of bulk and rhizosphere soil were collected per plant to test for the presence of *R. solanacearum*.

Segments of all stems per asymptomatic plant were collected by cutting 4-5 cm long pieces ca. 5 cm above the soil and placed without preceding surface sterilization in an universal extraction bag (Bioreba Ag, Reinach, CH). For symptomatic plants, only the stems showing symptoms were collected. Stem samples were further processed on the day of sampling as described below. Stem segments of tomato plants which served as pathogenicity

check for *R. solanacearum* were collected in the same way and processed without preceding surface sterilization. After collecting the stem samples, the plant pot was turned upside down in a clean plastic tub of which the inside had been disinfected with 70% EtOH and covered with a clean paper towel. Then, the different subterranean plant plants and soil were carefully separated to take samples.

Tubers were removed by hand from the stolons and placed into a paper bag. Adhering soil on the tubers was not removed to avoid damaging of the peel. The paper bags were stored max. 12 weeks inside a storage box at room temperature together with silica gel to remove moisture and avoid rotting during storage. Before further processing, tubers were gently wiped with paper tissue to remove dried adhering soil. The peel at the stolon end was removed with a disinfected (70% EtOH) knife and the vascular tissue lying below spooned out, placed in a zip-lock extraction bag (4x6 cm, 0.09 mm thick LDPE, unbranded) and processed as described below.

Roots were subsequently cut off from the stem base with disinfected (70% EtOH) scissors, separated from the bulk soil and vigorously shaken in two subsequent batches of 250 mL tap water to remove attached soil. In cv Kondor, only the roots of non-symptomatic plants were analyzed and as positive control, two roots samples of symptomatic plants inoculated with $5x10^8$ CFU. The washed roots were dabbed dry on tissue paper and stored overnight in a plastic bag at room temperature till surface sterilization. Before surface sterilization, the root sample was transferred to a 180 mL screw-cap plastic container (Corning, Gosselin, NY, USA). Then, 0.01% Tween 80 solution was poured over the roots and gently agitated by hand for 5 min to soak off attached soil particles and fatty components. After draining off the Tween 80 solution, the roots were covered with 1 : 4 diluted household bleach (active reagent sodium hypochlorite ca. 5%) and gently agitated for another 5 min. Finally, the surface sterilized roots were washed four times with tap water, before being transferred into a zip-lock extraction bag (8x6 cm, 0.09 mm thick LDPE, unbranded) and processed as described below. After removal of the plant parts, the remaining soil was thoroughly mixed by hand. Randomly selected soil samples were transferred with a disinfected (70% EtOH) spoon to a zip-lock plastic bag till a total of ca. 30 g soil was collected. Excess air was pushed out before closing the bag. Soil was stored at room temperature till being further processed as described below.

The top of the soil in the pots of the disturbed set of the *R. solanacearum* population development experiment was sampled weekly and the monitoring period was 53 days after soil contamination. Per pot, soil was collected from three randomly selected locations in the upper soil layer by digging a 5 cm deep hole with a disinfected (70% EtOH) spoon which was carefully closed again after sampling. Then, the collected soil was mixed and about 6 g composite soil sample was transferred to a 15 mL falcon tube and directly processed as described below.

4.2.2.6 Sample processing

Samples of plant material in extraction bags were weighed, and then macerated by crushing at 2 bar pressure for about 20-30 sec in a Sample crusher (AAA Lab Equipment, Roelofarendsveen, NL). Then Ringer's solution (buffer volume equivalent to twice the weight of the plant material) was mixed through the crushed material for about 5 minutes to allow bacteria to diffuse out of the tissues into the fluidal phase. To soil samples, a volume of Ringer's solution equivalent to twice the weight of the soil material was added, followed by vigorous vortexing for 12 min. Plant macerates were spread-plated undiluted on SMSA supplemented with 200 mg/L cycloheximide and 50 mg/L rifampicin. Additionally, serial dilutions of soil suspensions were prepared (undiluted, 10x and 100x diluted in Ringer's solution) and also spread-plated in duplicates. After incubation for 3-5 days at 25 °C, plates were inspected for the presence of *Ralstonia*-like colonies. Colonies from the soil samples of the *R. solanacearum* soil population development experiment were enumerated. Although the SMSA supplemented with rifampicin suppressed successfully the growth of non-target bacteria after plating suspensions from plant material, some non-target bacteria grew when plating the soil samples. To identify *R. solanacearum*, all *Ralstonia*-like colonies re-isolated from plant or soil material were checked with colony-PCR. To do so, a *Ralstonia*-like colony was selected from the plate and suspended in 50 μL de-ionized water in a PCR reaction tube where it was boiled for 10 min at 95 °C using a PCR cycler, after which it could be stored at -20 °C. To identify the *R. solanacearum* by PCR, we followed the protocol of Schönfeld et al. (2003) which amplifies a *fliC* fragment (5′- GAA CGC CAA CGG TGC GAA CT-3′; 3′-GGCGGCCTTCAGGGAGGTC-5′) and results in a 400 bp PCR product that was visualized on a 1% agarose gel. To the PCR reaction mix, 100 nm of the forward and reverse primer were given together with 5 μL of the boiled colony.

4.2.3 *In vitro* experiments

Using stem-inoculated potato *in vitro* plants, we investigated the effect of very low doses of *R. solanacearum* on its host under conditions considered optimal for multiplication of the pathogen and disease development. *In vitro* plants of *S. tuberosum* cv Kondor and HB were obtained from Agrico (Emmeloord, NL). Plants were propagated in clear polypropylene tissue culture vessels (Duchefa Biochemie, Haarlem, NL) filled with Murashige & Skoog (MS) agar. This medium consisted of 4.4 g/L MS salts, 30 g/L sucrose and 7 g/L plant agar, with a final pH of 5.8. *In vitro* plants were maintained in a light incubator at 25 °C and 16 h photo period. For the experiment, only the top part of the shoot of each plant was propagated to obtain a uniform sample. One shoot was placed in a 'De Wit' culture tube filled with 7 mL MS (Duchefa Biochemie, Haarlem, NL). The experiment was executed in two independent replicates using both potato cultivars and 80 plants per cultivar and repetition. In total, 320 plants were tested and Table 1B shows the overview of the experimental setup. Before inoculation, the cut shoots were grown for about 50 days. Plants were inoculated by firstly wounding the stem of the plants with a sterile needle (0.8 mm diameter, B. Braun Melsungen AG, Germany) ca. 2 cm above the agar. Secondly, 1 µL of bacterial suspension with the respective concentration was pipetted on the wound. The droplet was absorbed within about 15 minutes by the plant, after which the tubes were closed and wrapped with cling film. Disease symptoms were monitored after 10 and 17 dpi using the same disease scoring scale as in the greenhouse experiments. To detect latent infections, plants were analysed for the presence of *R. solanacearum* by re-isolating the bacterium from stem tissue in the same way as the greenhouse experiments. In short, plants were cut with a sterile knife 1-2 cm above the inoculation point, placed in a plastic zip-lock extraction bag (4x6 cm, 0.09 mm thick LDPE, unbranded), macerated and suspended in 0.5 mL of Ringer's solution. Suspensions were enumerated in Tryptone Soya Agar (TSA; Oxoid, Basingstoke, UK) supplemented with 50 mg/L rifampicin using the pour plating method in 24-well plates. For the pour plating, autoclaved TSA was maintained liquid at 50 °C and supplemented with rifampicin. 100 µL of diluted plant extract (undiluted, 10x, 100x, 1000x) was added in duplicates into 24-well plates, after which 300 µL of the liquefied TSA+rifampicin medium were added to each well while shaking steadily to allow mixing before the agar solidified. As control, 100 µL of Ringer's solution was added to the medium. Plates were incubated at 25 °C for 3-4 days until bacterial colonies within the medium were counted using a binocular.

4.2.4 Dose-response model

The response of a host after being challenged with a pathogen can be described in two steps (Teunis and Havelaar, 2000; Teunis et al., 2018). At first, the pathogen has to successfully enter the host, overcoming its natural defense barriers and multiply to infect the plant. Assuming that every pathogen has the same fixed probability p_m to enter the host, overcome its barriers (p_1 , ..., p_m) and cause a disease, the dose-response relationship for infection is described as an exponential dose response model:

$$P_{inf}(cV|p_m) = 1 - e^{-p_m cV} \quad (1)$$

where p_m quantifies the host-pathogen interaction, c is the pathogen concentration in a certain volume V, where cV represents the exposure dose, the number of pathogens. However, it is more realistic that the interaction between host and pathogen is heterogeneous and not constant. Instead of using a fixed probability of infection described by $p_{m,}$ the infection is described with the infection parameters α and β. In this case, the host-pathogen interaction follows a Beta probability distribution, which can be described by the Beta-Poisson model for microbial infection α and β are the infection parameters and ${}_1F_1$ is the confluent hypergeometric function (Teunis and Havelaar, 2000):

$$P_{inf}(cV|\alpha,\beta) = 1 - {}_1F_1\,(\alpha, \alpha+\beta; -cV) \quad (2)$$

α and β are Monte Carlo sample pairs (joint distribution) reflecting uncertainty and variability of infectivity. They were transformed to improve parameter estimation:

$$u_1 = \frac{\alpha}{\alpha+\beta};\; w_1 = log\left(\frac{u_1}{1-u_1}\right)$$
$$v_1 = \alpha+\beta;\; z_1 = log(v_1) \quad (3)$$

w_1 is thereby a measure of infectivity (location) and z_1 is a measure of variation in infectivity (spread). In a second step, the conditional probability of illness (symptomatic plants) within the group of infected plants is described with the hazard model of illness dose response.

$$P_{ill|inf}\,(cV) = 1 - \left(1 + \frac{cV}{\eta}\right)^{-r} \quad (4)$$

Similarly to infection, illness parameters r and η were transformed into location parameters w_2 and z_2:

$$u_2 = \frac{r}{r+\eta};\; w_2 = log\left(\frac{u_2}{1-u_2}\right) \qquad (5)$$

$$v_2 = r + \eta;\; z_2 = log(v_2)$$

Log-likelihood ratio (LR) testing was applied to select the best dose-response model for infection, exponential (Eq. 1) or Beta-Poisson (Eq. 2) (Teunis et al., 1996). LR tests were also used to compare individual dose-response datasets (e.g., stem infection) with pooled datasets (all observations of infections of different plant parts are combined: stem, root or tuber infection). Also, we compared the dose-response models of both cultivars to test whether a pooling of these datasets was admissible. Therefore, values for parameters p_m or α and β that maximize the log-likelihood function were estimated. The log-likelihood function is described as:

$$\ell\,(\alpha,\beta) = -2\,\sum_{i=1}^{k}\{I_i \log\, P_{inf}(D_i) + \,(T_i - I_i)\,\log(1 - P_{inf}\,(D_i)\} \qquad (6)$$

where ℓ is the likelihood, k the number of doses, T_i the number of plants exposed a certain dose of which I_i were infected. P_{inf} represents the values obtained from Eq. (1) or Eq. (2) with $D_i = c * V_i$. The parameter values that optimize this function ($\hat{\ell}$) are $\widehat{p_m}$, $\hat{\alpha}$ and $\hat{\beta}$.

The best fit of the dose-response relation given a specific dataset is achieved with the maximum likelihood estimates $\hat{\alpha}$ and $\hat{\beta}$. The likelihood supremum without any constraints is calculated as followed:

$$\ell_{sup} = \sum_{i=1}^{i=n}\left[I_i \log\left(\frac{I_i}{T_i}\right) + \,(T_i - I_i)\,\log\left(\frac{T_i - I_i}{T_i}\right)\right] \qquad (7)$$

The model was written and run in JAGS (Just Another Gibbs Sampler, v4.3.0) (Plummer, 2015) from R (v4.1.2) (R Core Team, 2022) to assess uncertainty. JAGS is a system for Markov chain Monte Carlo (MCMC) sampling for Bayesian hierarchical models. The source code of the JAGS model and LR testing is provided in S1. For each Monte Carlo simulations, 3 chains were run in parallel; after a burn-in of 1000 iterations, the model was run for 10^5 iterations. Wide priors were used for the parameters, and their influence on the posterior predictive samples was checked by using different means and variances for *w* and *z*. Prior values together with the source code can be found in S1.

4.2.5 Die-off model for soil population development

The die-off of *R. solanacearum* populations in non-cultivated soil was modelled with a non-linear Weibull + tail model as described in Eisfeld et al. (2021).

$$C_t = (C_0 - C_{res})\, e^{-(\alpha t)^{\beta}} + C_{res} \tag{8}$$

where C_t is the bacterial concentration [M/L^3] at time t [T], C_0 the initial bacterial concentration [M/L^3] (at time $t = 0$), C_{res} [M/L^3] is the residual bacterial population at the end of the observation period. α [1/T] is a scale parameter and β [-] a shaping parameter to display convexity of a curve if $0 < \beta < 1$, or simulates a shoulder effect when $\beta > 1$. If $C_{res} = 0$, a non-linear Weibull model is obtained. Plus, the model can be reduced to a log-linear die-off model if β = 1. The Weibull + tail, the Weibull and the log-linear model can be compared using the AIC (Akaike Information Criterion). The model with the best fit has the lowest AIC. Note that the inoculation concentration at t = 0 was 1.3×10^6 CFU/g but the first sample was taken 7 dpi to allow an adaptation of the bacterium to the soil environment and not disturb by sampling.

4.3 Results

4.3.1 Dose-response relationship in the greenhouse

In two greenhouse experiments, the disease response of potted potato plants to *R. solanacearum* was monitored for 38 and 54 days using cultivars Kondor and HB, respectively. Plants were inoculated by irrigation with contaminated water containing different concentrations of the bacterium. Tomato plants cv Moneymaker were stem inoculated (5×10^8 CFU) during both experiments. The tomato plants showed wilting mild symptoms after 7 dpi with a mean disease index of 2. At 14 dpi, all plants showed heavy wilting symptoms (DI = 4) which confirmed the virulence of the strain (see Figure 1A). The potato cultivars differed in their growth characteristics as cv HB developed very long stems and grew to about two times the height as cv Kondor (Figure S1). The tuber yield was lower in cv HB than in cv Kondor. While a potato plant of cv Kondor had on average nine progeny tubers with an average weight of 170 g, cv HB had on average two progeny tubers with an average weight of 57 g (Table S1).

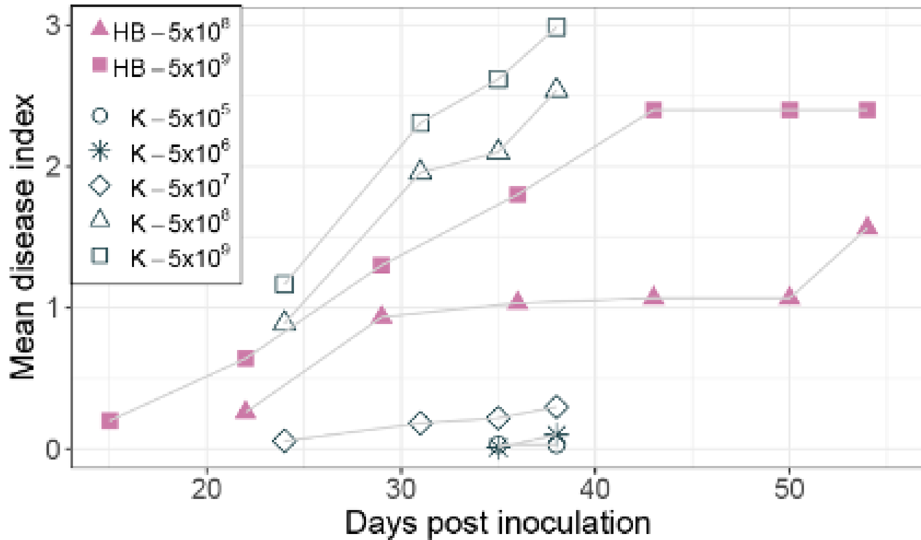
4.3.1.1 Disease development

Inoculation of potatoes with high bacterial numbers (5×10^8 and 5×10^9 CFU) resulted in fast disease symptom development as shown in Figure 1B. Figure 2 shows the development of the disease progress by scoring wilting symptoms of the stems and calculating the mean DI for each treatment over time.

First wilting symptoms were observed in plants inoculated with $5x10^9$ CFU at 15 dpi in cv HB and at 24 dpi in cv Kondor exposed to the same dose. In the experiment with cv Kondor, some plants (also controls) were affected by yellowing and brown spots of unknown cause. This may have hampered the clear identification of early stages of *R. solanacearum* infections, but overall it was possible to distinguish between the browning and wilting symptoms. In the end, 19 plants of cv Kondor with wilting symptoms were observed after inoculation with $5x10^5$ – $5x10^9$ CFU (Table 2). At a lower dose of $5x10^5$ CFU or $5x10^6$ CFU, only 1 of the 15 or 10 plants showed symptoms, respectively. This resulted in a maximum mean DI of 3 at $5x10^9$ CFU and a minimum DI of 0.03 at $5x10^5$ CFU. Only seven plants of cv HB showed bacterial wilt symptoms at the two highest inoculation concentrations. For both cultivars, wilting increased steadily until about 40 dpi if the plants had been treated with a higher dose (Figure 2). Thereafter, the disease progress slowed down in cv HB which was monitored longer (about 20 days) than cv Kondor. This resulted in a plateau phase from 43 to 54 dpi. In this plateau phase, all symptomatic plants of cv HB inoculated with $5x10^8$ CFU or $5x10^9$ CFU became 100% wilted and the mean DI did not increase anymore. Only at the last observation point (54 dpi), a newly wilted plant that had been treated with $5x10^8$ CFU, was observed which resulted again in an increase of the mean DI. In some plants, very fast disease

▲ **Figure 1 A**: Tomato cv Moneymaker were stem-inoculated (10^8 CFU) to confirm the virulence of *Ralstonia solanacearum* 4187; two inoculated plants next to a non-inoculated control plant (right). **B**: Bacterial wilt in potato cv Kondor after soil-soak inoculation with $5x10^8$ CFU, left plant shows symptoms 24 dpi and right plant 35 dpi; **C**: Progeny tubers of a potato plant inoculated with $5x10^9$ CFU; stolon ends have been removed for analysis; tubers show typical infection symptoms of *R. solanacearum* in different stages of infection indicated by the arrows – the vascular ring discolors (a) and white bacterial ooze emerges (b) followed by browning of the vascular ring (c) and rotting of tuber tissue (d).

development was observed. For example, one plant of cv HB inoculated with $5x10^8$ CFU did not show any symptoms at 22 dpi but thereafter all four stems wilted 100% within one week (29 dpi). Overall, we observed more wilting and infections in cv Kondor than in cv HB and symptoms were only observed at a dose higher than $5x10^5$ CFU. At the highest dose, 100% of the plants of cv Kondor were wilted and 50% plants of cv HB.



▲ **Figure 2** Development of bacterial wilt disease of potato plants cv Kondor (K, open black symbols) and HB (filled purple symbols) in greenhouse experiments inoculated with different doses of *Ralstonia solanacearum* in CFU, as indicated in the legend. The mean disease index was assessed by scoring the wilting symptoms of stems on a scale from 0 (no wilt) – 4 (100% wilted). The score of no wilt (0) is not displayed in the graph.

4.3.1.2 Analysis of plant material

At the end of the experiment, different plant parts (stem, root or tuber) were analysed for *R. solanacearum*. Thereby, the presence of *R. solanacearum* in symptomatic plants was confirmed and latent infections (infected plants not showing wilting symptoms) were found (Table 2). In total, nine plants of cv Kondor were latently infected mostly in the stems and roots of plants exposed to a low dose ($5x10^2$ or $5x10^3$ CFU). In the experiment with cv HB, three plants with latent infections were observed of which two of them in the roots, inoculated with $5x10^3$ or $5x10^5$ CFU. In some of the tubers produced by

the plants, infections were already so advanced that disease symptoms were visible on the tuber skin (browning around the stolon) or inside when cutting the tubers in half (Figure 1C). Only at a high dose of at least $5x10^7$ CFU infected progeny tubers were found in both cultivars. Moreover, some symptomatic plants of cv Kondor treated with a high dose did not produce progeny tubers, or tubers were already rotten at the end of the experiment and could not be further analysed. At the end of the experiments, from each plant, a mixed sample of bulk of rhizosphere soil was analysed. For cv Kondor, soil remained contaminated with *R. solanacearum* at all tested concentrations except for $5x10^4$ CFU while for cv HB the soil samples were found positive only at the two highest inoculation concentrations.

▼ **Table 2** Symptomatic (wilting) and non-symptomatic infections of potted potato plants cv Kondor and HB after a single non-invasive soil soak inoculation with different doses of *Ralstonia solanacearum*

	Dose (CFU)		**Ctrl**	**$5x10^2$**	**$5x10^3$**	**$5x10^4$**	**$5x10^5$**	**$5x10^6$**	**$5x10^7$**	**$5x10^8$**	**$5x10^9$**
	Nr. of exposed plants		**5**	**15**	**15**	**15**	**15**	**10**	**10**	**10**	**5**
cv Kondor	Nr. of plant parts infected with *R. solanacearum*[1]	stem	0	1	2	0	2	3	5	10	5
		root	0	1	0	0	0	0/9	5/9	2/2	-
		tuber	0	0	0	0	0	0	4	8/8	4/4
		soil	0	3	1	0	5	1	4	10	5
	Nr. of infected plants[2,3]		0	1	2	0	2	3	5	10	5
	Nr. of plants showing wilting symptoms (illness)[3]		0	0	0	0	1	1	2	10	5
	Nr of latently infected plants[4]		0	1	2	0	1	2	3	-	-
cv HB	Nr. of plant parts infected with *R. solanacearum*[1]	stem	0	0	0	0	0	0	1	4	3
		root	0	0	1	0	1	0	0	3	2
		tuber	0	0	0	0	0	0	0	1/5	0/1
		soil	0	0	0	0	0	0	0	4	5
	Nr. of infected plants[2,3]		0	0	1	0	1	0	1	4	3
	Nr. of plants showing wilting symptoms (illness)[3]		0	0	0	0	0	0	0	4	3
	Nr of latently infected plants[4]		0	0	1	0	1	0	1	0	0

[1] Based on SMSA dilution plating and confirmation by colony-PCR (when two numbers are separated by a slash the first number represents the number of infected parts and the second number the number of samples tested); [2] any plant part infected (stem, root or tuber); [3] these numbers were used as input infection or illness data in the dose-response (DR) model; [4] plants infected with *R. solanacearum* but without wilting symptoms.

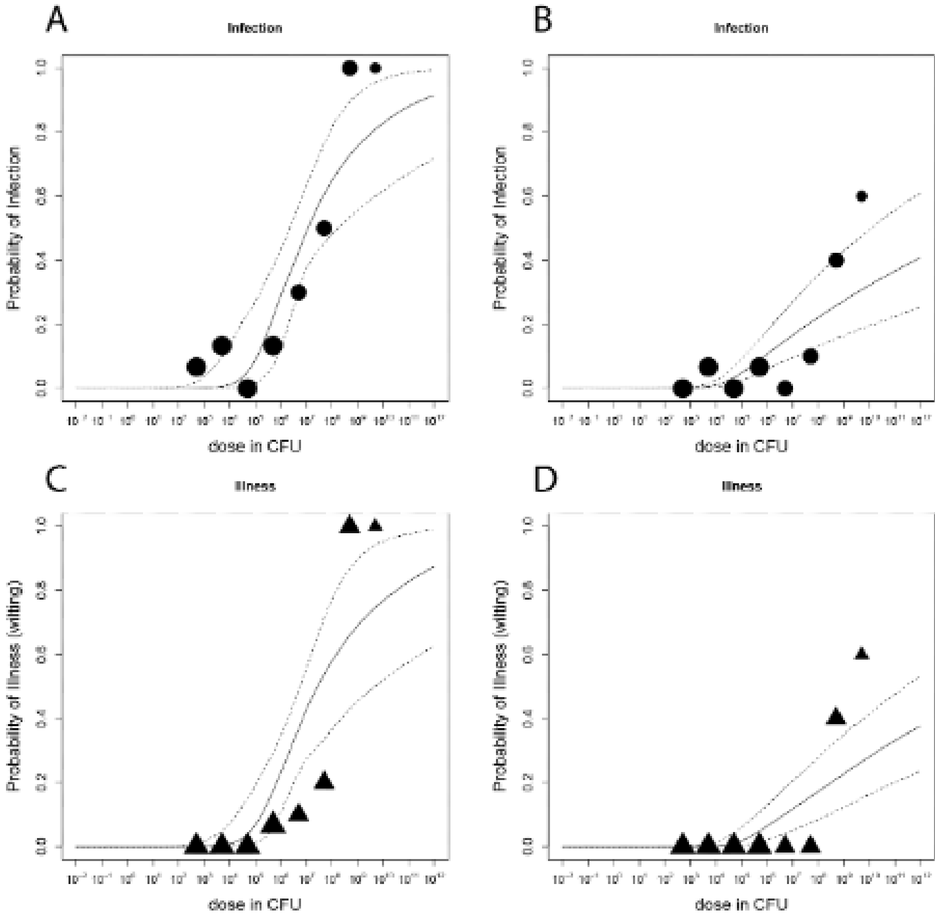
4.3.1.3 Dose-response model

Dose-response relations for *R. solanacearum* infections of potato plants are shown in Figure 3 and estimated infection and illness parameters are presented in Table 3. For the dose-response model infections of all plant parts (stem, root or tuber) at the end of the experiments were considered. A plant that was found positively infected in two different plant parts (e.g., stem and root) was only counted once (Table 2). The Beta-Poisson model was a better model than the exponential model according to the LR test. The dose-response model indicated a higher probability of infection or illness of cv Kondor by *R. solanacearum* than of cv HB. While the dose-response relation showed a similar trend for both cultivars at lower inoculation concentrations (e.g., 10% infection probability at a dose of $1.3x10^6$ CFU in cv Kondor and $7.2x10^6$ CFU in cv HB, the model is steeper for cv Kondor indicating higher probability of infection at a higher inoculation concentration. The ED_{50} (dose required to infect or wilt 50% of the plants) of infection was $1.1x10^7$ CFU for cv Kondor. Ninety percent of the plants of cv Kondor were predicted to be infected at a dose of $2.7x10^{12}$ CFU, whereas at the same dose for cv HB only 40% of the plants are predicted to get infected. The dose-response models for both cultivars predicted that there exists a 0.3% infection probability at a dose of about $2x10^4$ CFU. The illness dose-response model was similar to the infection dose-response model for both cultivars. Nevertheless, less symptomatic plants were observed at low doses in comparison to high inoculation doses.

▼ **Table 3** Estimated parameters of dose-response models for infection and illness and respective values of the 95% confidence interval.

	potato cv	DR model		2.5%	97.5%		2.5%	97.5%	ED_{50}
Greenhouse experiments	**Infection**		**α**			**β**			
	Kondor	bp	0.17	0.07	0.38	$3.7x10^5$	$1.6x10^3$	$2.2x10^6$	$1.1x10^7$
	HB	bp	0.03	0.02	0.05	$2.6x10^4$	$8.8x10^3$	$6.1x10^4$	$*1.2x10^{10}$
	Illness (symptomatic plants)		**r**	2.5%	97.5%	**η**	2.5%	97.5%	**ED_{50}**
	Kondor	bp	$1.6x10^5$	0.04	$6.2x10^3$	$4.5 x10^5$	$3.1x10^{-5}$	$8.4x10^4$	$2.7x10^7$
	HB	bp	$4.7x10^2$	0.05	$4.9x10^3$	$2.6 x10^4$	$7.6x10^3$	$6.1x10^4$	$*2.5x10^{11}$
***In vitro* experiments**	**Infection**		**α**	2.5%	97.5%	**β**	2.5%	97.5%	**ED_{50}**
	pooled Kondor + HB	bp	0.46	0.11	0.89	0.13	$6.0x10^{-3}$	0.41	0.90
	Illness (symptomatic plants)		**r**	2.5%	97.5%	**η**	2.5%	97.5%	**ED_{50}**
	pooled Kondor + HB	bp	$1.4x10^4$	0.19	$4.8x10^3$	$9.4 x10^2$	$1.1x10^{-5}$	$1.1x10^2$	1.13

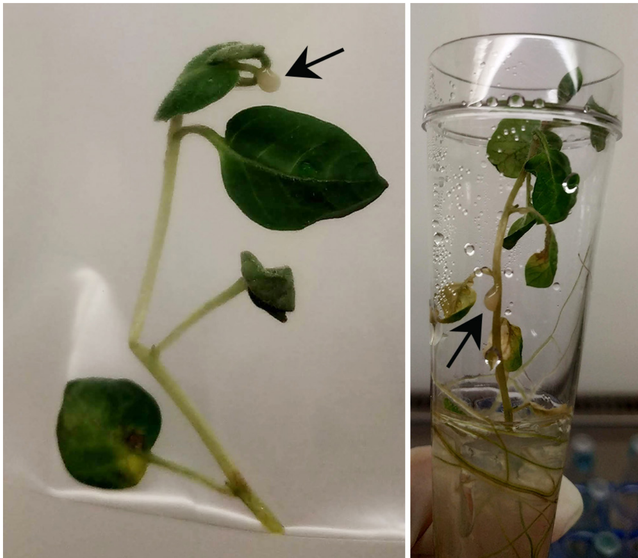
DR = dose-response; cv = cultivar; bp = Beta-Poisson; ED_{50} = dose required to infect or wilt 50% of the plants; * value taken from upper limit of 95% confidence interval



▲ **Figure 3** Dose-response relations of potato plants cv Kondor (A, C) or cv HB (B, D) and *Ralstonia solanacearum*; **A+B**: Probability of infection (stem, root, or tuber) with increasing dose in colony forming units (CFU); **C+D**: Probability of illness (wilting) with increasing dose. Each graph shows the median and 95% range of the probability of infection or illness, indicated by the dashed line, calculated by the dose-response model as a function of dose. Available data are shown as a bubble chart in which the symbol size is proportional to the number of plants challenged.

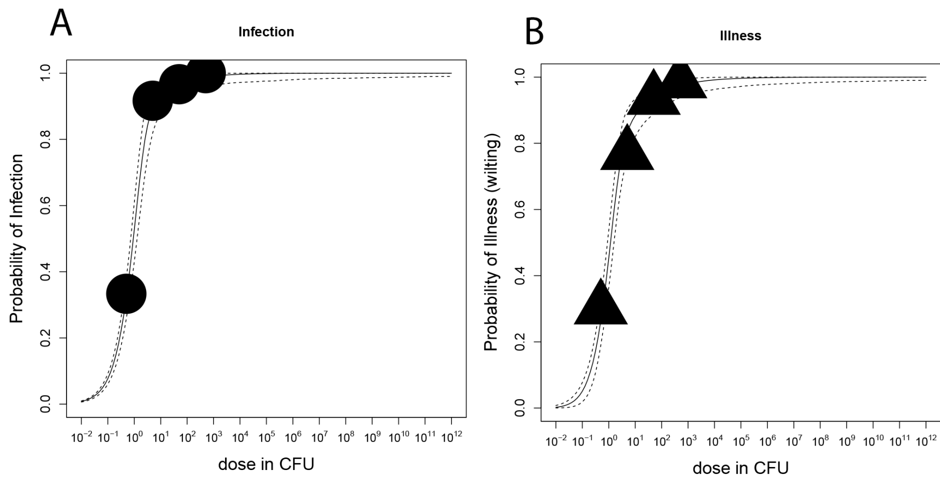
4.3.2 Dose-response relationship under *in vitro* conditions

Both potato cultivars were tested under sterile *in vitro* conditions to evaluate the dose response under conditions considered optimal for multiplication of *R. solanacearum* and disease expression. In contrast to the greenhouse experiments, the stems of the potato plants were wounded first, followed by placing 1 μL of bacterial suspension on the wound. This resulted in faster and more severe disease responses (Table 4). Some of the plants even showed oozing of bacterial slime from the stem containing a high concentration of *R. solanacearum* (Figure 4). First wilting was recorded at 10 dpi in all higher inoculation concentrations (>100 CFU). Six wilted plants of cv HB (replicate 1) were found after inoculation with 50 CFU and one wilted plant after inoculation with 5 CFU in cv Kondor. Another week later at 17 dpi, all plants of both cultivars in both replicates showed heavy wilting symptoms and even at an inoculation dose of 0.5 CFU about 20-30% of plants were wilted (Table 4). The ED_{50} for infection of *in vitro* plants was as low as 0.90 CFU. After the observation period, plants were harvested and analysed for the presence of *R. solanacearum* which led to the detection of a few (2-3) latently infected plants at lower inoculum of 0.5 and 5 CFU. Nearly all plants inoculated with higher inoculum were symptomatic after two weeks.



▲ **Figure 4** *In vitro* plants infected with *Ralstonia solanacearum* at 15 dpi; Arrows indicate oozing of bacterial slime, containing high concentrations of bacteria.

The dose-response relation of *in vitro* potato plants and *R. solanacearum* is shown in Figure 5 and corresponding parameter estimates are presented in Table 3. According to the LR test, all datasets of cultivars Kondor and HB could be pooled and the Beta-Poisson model was the better model than the exponential. In comparison to the greenhouse experiments the dose-response graph of *in vitro* experiments is shifted to the left as a much lower dose already caused infection or illness. As in the greenhouse experiments, the dose-response model for illness and infection were similar, indicating that the probability of an infection at a given dose has the same probability to result in illness.

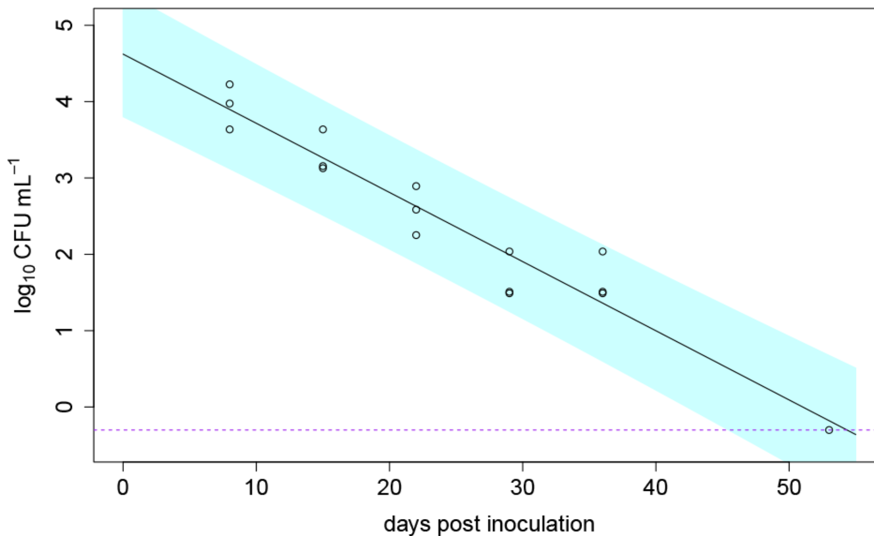


▲ **Figure 5** Dose-response relations of potato plants grown *in vitro* and *Ralstonia solanacearum*; **A**: Probability of stem-infection with increasing dose in colony forming units (CFU); **B**: Probability of illness (symptomatic plants) with increasing dose. Each graph shows the median and 95% range of the probability of infection or illness, indicated by the dashed line, calculated by the dose-response model as a function of dose. Available data are shown as a bubble chart (symbol size proportional to the number of plants challenged).

4.3.3 Die-off in infested soil

The die-off of a soil population of *R. solanacearum* without the presence of a plant was monitored simultaneously to the greenhouse experiments in 2020. At the end of the experiment (54 dpi), *R. solanacearum* was detectable in one of three inoculated pots which were sampled regularly (disturbed set of pots). The die-off of *R. solanacearum* in soil without the presence of a host plant was modelled using a non-linear Weibull model. Best model fit (comparing the AIC) was achieved using the log-linear die-off model $C_t = C_0\, e^{-\alpha t}$. The die-off

in soil is shown in Figure 6. The predicted model parameters resulted in a C_0 of 10^4 CFU/g (± 1.5) and a die-off rate of α = 0.09 (± 0.007) 1/day. At 54 dpi, the undisturbed set of pots (soil analysed only at 54 dpi) were analysed for the bacterium. There, *R. solanacearum* was only detected in one of the three inoculated pots which was similar to the results of the disturbed pots.



▲ **Figure 6** Die-off of *Ralstonia solanacearum* over time in in three pots filled with clay loam soil without the presence of a host plant; the data shown as open circle symbols were fitted with a log-linear die-off model (solid line); the dashed purple line indicates the detection limit and the blue band indicates the 95% prediction interval; at the last measurement point 54 days post inoculation, the bacterium was only detectable in one of the three pots.

4.4 Discussion

In potato production, water is crucial to support plant growth and tuber production. Due to decreasing availability of fresh water of adequate quality, water treatment schemes are required to provide irrigation water and secure food production. However, plant pathogens such as *R. solanacearum* may not be fully removed during the treatment and pose a risk spreading plant diseases. This is the first study to investigate the dose-response relationship between *R. solanacearum* and potato plants grown in pots in a greenhouse setting, simulating infection through irrigation with contaminated water

at low concentrations. The presented dose-response model can be used in quantitative microbial risk assessment (QMRA) to evaluate water treatment systems on their pathogen removal capacity.

At first, we found that the two tested potato cultivars showed different susceptibility to *R. solanacearum* under greenhouse conditions. Inoculation of cv Kondor with the two highest doses ($5x10^8$ and $5x10^9$ CFU) resulted in 100% infection of all plants, whereas only 50% of plants cv HB were infected when inoculated with the same doses. Susceptibility of a cultivar is determined by its genetic traits and breeding for resistance is an important tool to control bacterial wilt although so far no resistant potato cultivar exists (Huet, 2014). Although cv HB showed a higher resistance against *R. solanacearum*, it had less favourable agronomic traits because it produces very long stems and less progeny tubers than cv Kondor. As a result, cultivation of cv HB has been discontinued by the breeding company. Additionally, although partially resistant or tolerant cultivars can be desirable in countries with a high level of endemic infections, they may also contribute to an unnoticed multiplication of latently infected plant material and a spread of the pathogen. In this way, partially resistant or tolerant cultivars may even contribute to the build-up of *R. solanacearum* infections over time (Swanson et al., 2005).

In both cultivars, latent infections were discovered after maceration and analysis of the plant material. Studying the locations of infection within the plant allowed to retrace the infection pathway of *R. solanacearum*. According to literature, after the soil-soak inoculation, the bacterium first enters the plant through root openings followed by a systemic colonization of the xylem vessels of the stems (Siddiqui et al., 2014). All root-infected plants of cv Kondor were also stem infected indicating fast and successful colonization. Plants of cv HB inoculated with $5x10^3$ and $5x10^5$ CFU were only infected in their roots indicating an early infection stage. It is not clear if these root infections would have developed into a systemic colonization after the time span of the experiment. Note that not the whole root system of each plant was analysed. Therefore, it is possible that some root infections remained undetected. Consequently, sampling of roots can be useful for detecting early infections but may underestimate the actual infection incidence as processing of the complete root system is laborious. Our experiments demonstrated that latent infections also occurred at lower inoculation concentrations in stem and root although to a very low extent. The infected plants may develop symptoms when observed over a longer time period as the dose-response model predicts the same probability for infection or

illness after contact with a given dose. Latent infections of propagation material contribute to the spread of plant diseases. Nevertheless, infected progeny tubers were only found at high inoculum densities (>10^7 CFU) but not when irrigated with low doses. This is an important outcome as trading of latently infected seed tubers poses a high risk spreading brown rot disease internationally (EFSA et al., 2019).

While the greenhouse experiments simulated irrigation with contaminated water under natural conditions, *in vitro* experiments allowed to study the dose-response relationship under conditions (high temperature, availability of water and nutrients, absence of competition by other micro-organisms) expected to be optimal for the multiplication of *R. solanacearum* and for disease expression at very low inoculation concentrations. Moreover, stem-inoculation is more accurate in applying a certain dose where the bacterium is brought in direct contact with the plant. Soil-soak inoculation simulates natural contamination but not all bacteria may have reached and invaded the plant. As a result, even the lowest dose of 0.5 CFU caused infections under *in vitro* conditions using stem-inoculation. Note that we assumed the bacterial inoculation suspension to be Poisson distributed where on average 0.5 CFU should be present in 1 µL, but the actual doses might have been higher or lower than 0.5 CFU because of the random distribution of the bacterial cells in the inoculation solution (Haas et al., 2014b; Haas et al., 2014a). Eventually, the invasive inoculation method by stem injury promoted disease development even at the lowest dose. Singh et al. (2014) showed that injuring the roots of tomato plants (grown in a greenhouse in pots with autoclaved soil mixture) increased wilt disease. In their experiments, an inoculum level of 10^2 CFU/mL resulted in 63% wilting when roots were injured, while plants with uninjured roots inoculated with 10^3 CFU/mL resulted in only about 7% of diseased plants. In their experiments no wilted plants were observed when inoculated with 10 CFU/mL regardless of root wounding. In contrast, in our greenhouse experiments, latently infected plants (stem and root) were found after inoculation with minimally 10 CFU/mL (dose of 5×10^2 CFU). However, symptoms were only observed at an inoculum level of 10^4 CFU/mL (dose of 5×10^5 CFU) in our experiments. Consequently, the information gained from the *in vitro* experiments showed a precise dose-response relationship but it should be used with caution. It shows that under these conditions, fast and severe disease expression (wilting and oozing) may occur. But the *in vitro* results may not be useful to estimate the dose-response relationship under field conditions as most external influences on the host-pathogen system are excluded. Moreover, the *in vitro* experiments did not reproduce the different susceptibilities of the two tested potato cultivars as their disease progress

was similar. In the *in vitro* experiments, the bacteria were directly introduced into the vascular system of the plant by stem injection. The pathogen did not have to recognize, attach and penetrate the plant to overcome its external barriers when colonizing the plant during infection (Huet, 2014). In the field, bacterial infections are facilitated by wounds which are caused by feeding insects on the stem or root nematodes, or by natural openings where secondary roots will emerge (Champoiseau et al., 2009). In such a case, the dose-response model obtained from *in vitro* experiments may be adequate to estimate the high infection risk of potato plants by *R. solanacearum*. In the greenhouse experiments, however, root openings may have also been present in potato plants with growing roots. Plus, the natural soil from an agricultural field still contained organisms that may have damaged the root surface. Habe (2018) presented an *in vitro* assay that allowed to differentiate resistances between bacteria. In their study, *in vitro* plants were grown in sterile vermiculite with MS liquid medium and soil-inoculated with >10^2 CFU/mL, but lower doses as in our study have not been tested. The use of *in vitro* plants may be an effective way to study the pathogen virulence as symptom development occurred already at a very low dose of even a single cell. Currently, pathogenicity has to be tested on a host under greenhouse conditions (EPPO, 2018). The *in vitro* assay offers a less laborious and less expensive alternative to the standard pathogenicity test. Currently, a zero tolerance policy in Europe prohibits the use of surface water in which *R. solanacearum* has been detected for irrigation of potato crops (Directive, 1998). Our dose-response results indicate that this legislation is appropriate if high concentrations of *R. solanacearum* (e.g., released from wild host plants) are present in surface water. However, *R. solanacearum* will generally be found at lower concentrations in surface waters of maximum 10^3 CFU/mL (Wenneker et al., 1999; Álvarez et al., 2007) which only have a very low probability of infection. Moreover, water treatment like natural sand filtration can reduce bacterial concentrations by several $\log_{10}$ to improve water quality which decreases the risk spreading brown rot using treated irrigation water (Eisfeld et al., 2022). As with drinking water, irrigation water quality regarding microbiological safety can be analysed using QMRA (Drinkwaterbesluit, 2011). In this context, dose-response models are an important component of QMRA to evaluate risks related to water reuse in irrigation.

Finally, practical aspects of potato production should be considered when applying dose-response models. First, irrigation water quality is not only important during crop cultivation but also before planting. Farmers may need to irrigate their soil to guarantee sufficient soil moisture which promotes root development and seed germination (Letnes, 1958). Therefore, we also

analysed the persistence of *R. solanacearum* after soil irrigation without a potato plant present where the bacterium persisted for up to 54 dpi when inoculated with 1.3x10^6 CFU/g. In comparison, soils of the greenhouse experiments where plants were irrigated with high doses, similar to the soil die-off experiment, all soils remained contaminated with *R. solanacearum*. Therefore, our results indicate that the presence of a potato plant prolonged the pathogen's soil persistence even though the plant was not infected (e.g., cv Kondor treated with 10^2 CFU). Plants release root exudates and nutrients which attract the bacteria by chemotaxis towards the root surface (Yao and Allen, 2006). Consequently, persisting pathogen populations in the soil introduced via contaminated irrigation water can infect the planted seed tubers (Messiha et al., 2007). Second, the irrigation method may influence the dose-response results. This study analysed soil-soak inoculation to simulate drip irrigation where the bacteria can enter the plant via the roots. In overhead irrigation, also the leave surface gets in contact with the irrigation water which may result in a different dose-response model. Third, the irrigation frequency should be considered as continuous irrigation with low contaminated water may result in a build-up of the pathogen in the soil from where it can infect the plant. Lastly, research should explore the dose-response relationships with other hosts and plant pathogens. Our research showed that variability exists even between the same host-pathogen system if different host cultivars are used as potato cultivars depict different susceptibilities to brown rot (Bowman and Sequeira, 1982; Montanelli et al., 1995). In a risk assessment, we recommend applying the dose-response model of cv Kondor as it had a higher infection probability which will deliver more conservative risk estimates.

To conclude, due to increasing water scarcity in agriculture, water reuse schemes will gain more importance and regulations should consider water treatment as an effective way to reduce plant pathogens. Although a 100% removal can never be guaranteed, QMRA in combination with dose-response models can analyse the effectiveness of water treatment (Schijven et al., 2011; Verbyla et al., 2016). Dose-response models are an essential element of risk analysis as they allow to translate pathogen exposure into risk of infection which has a direct practical implications. If exposure after a water treatment remains too high, modifications or additional treatments can be included in order to minimize the pathogen related risks and provide sufficient water quality and quantity in agriculture.

4.5 Appendix C

Supplementary data contains Figure S1 that compares the growth characteristics of the two potato cultivars used in the study. Table S1 summarizes weights of the plant material collected at the end of the greenhouse experiments. The R source code together with the JAGS model can be accessed online at https://www.frontiersin.org/articles/10.3389/fpls.2022.1074192/full#supplementary-material.

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Chapter 5
QMRA of *Ralstonia solanacearum* in potato cultivation: risks associated with irrigation water recycled through managed aquifer recharge

This chapter has been submitted to the journal Science of the Total Environment and is accpted for publication with minor/moderate revision.

Highlights

- First QMRA focusing on plant health and risks of water reuse in agriculture
- Aquifer storage provides irrigation water and removes bacterial plant pathogens
- ASTR combines bacterial removal by water die-off and by attachment to aquifer sand
- A one meter soil passage predicts sufficient bacterial removal by attachment
- QMRA helps to support decision-making processes for water resource management

Abstract

Agricultural aquifer storage recovery and transfer (ASTR) stores excess fresh water for later reuse in irrigation. Moreover, water quality improves because chemical and biological pollutants will be removed by degradation and attachment to the aquifer material. The source water may contain the bacterial plant pathogen *Ralstonia solanacearum* which causes plant infections and high yield losses. We used quantitative microbial risk assessment (QMRA) to investigate the removal of *R. solanacearum* during ASTR to predict infection risks of potato plants after irrigation with the recovered water. Laboratory experiments analyzed the ASTR treatment by investigating the bacterial die-off in the water phase and the removal by attachment to the aquifer sediment. Die-off in the water phase depends on the residence time and ranged between 1.3 and 2.7 log_{10} after 10 or 60 days water storage, respectively. A subpopulation of the bacteria persisted for a prolonged time at low concentrations which may pose a risk if the water is recovered too early. However, the natural aquifer sand filtration proofed to be highly effective in removing *R. solanacearum* by attachment which depends on the distance between injection and abstraction well. The high removal by attachment alone (18 log_{10} after 1 m) would reduce bacterial concentrations to negligible numbers. Upscaling to longer soil passages is discussed in the paper. Infection risks of potato plants were calculated using a dose-response model and ASTR treatment resulted in negligible infection risks of a single plant, but also when simulating the irrigation of a 5 ha potato field. This is the first QMRA that analyzed an agricultural ASTR and the fate of a plant pathogen focusing on plant health. QMRA is a useful (water) management tool to evaluate the treatment steps of water reclamation technologies with the aim to provide safe irrigation water and reduce risks disseminating plant diseases.

5.1 Introduction

Freshwater is a critical resource for irrigated agriculture to obtain sufficient crop yields and securing food security of the growing world population. Nevertheless, freshwater scarcity increases as a result of climate change and ongoing groundwater exploitation (FAO, 2022). Additionally, surface water may contain plant pathogens and is therefore not suitable for irrigation as pathogens present in irrigation water pose a threat to global crop production and food security causing high economical losses (Hong and Moorman, 2005). This study focuses on the plant pathogenic bacterium *Ralstonia solanacearum* which has been found in surface waters and disease outbreaks have been linked to contaminated irrigation water (Janse, 1996). The pathogen originated from South America and was probably introduced in Europe through latently infected seed material. The cold-adapted strain of *R. solanacearum* (phylotype II, race 3 biovar 2) threatens potato and tomato production in Europe and is found in surface waters where it sheds in plants growing along waterways. Irrigation with contaminated (surface) water has been recognized as primary source of infection on a local scale (EFSA et al., 2019). After severe disease outbreaks in Europe in the 1990's, irrigation of (seed) potatoes has been prohibited which successfully reduced disease incidences and less positive findings of *R. solanacearum* in surface water (Directive, 2006; Janse, 2012).

Managed aquifer recharge (MAR) is a nature based solution to provide a fresh water reservoir in times of need while simultaneously improving water quality (Pyne, 1995). Aquifer storage and recovery (ASR) is a specific type of MAR where water is intentionally infiltrated during wet periods via injection wells and recovered from the subsurface using the same well (Dillon, 2005). Source waters of different origins and water qualities (e.g., wastewater, urban stormwater, rain water) can be infiltrated and improvement of the water quality will strongly depend on the aquifer material, its geochemical composition, and microbial community (Bekele et al., 2018). In an ASR, pathogens or chemical pollutants will die-off or degrade to a certain extent after a given storage time. However, it is difficult to exactly predict the travel distance of the recharged water as it depends on the infiltration volume and the porosity of the different aquifer sand layers. In contrast, an aquifer, storage, transfer and recovery (ASTR) system uses a spatially separated abstraction well. This forces the water to flow from infiltration towards the abstraction well through the porous aquifer medium before it is recovered. This offers additional pathogen removal during the soil passage in the

subsurface while the known distance between both wells adds a computable filtration step (Dillon et al., 2009). Pathogen removal occurs at the soil-water interphase and is governed by chemical, biological, and physical mechanisms (Ginn et al., 2002).

The treatment efficiency of AS(T)R and risks related to the reuse of water from different sources can be analyzed using quantitative microbial risk assessment (QMRA). QMRA in the context of MAR has already been used to quantify human health risks when MAR treated water is used for the irrigation of recreational areas, or the production of raw consumed vegetables (Ayuso-Gabella et al., 2011; Page et al., 2015; Masciopinto et al., 2020). These studies highlight the potential of MAR to enhance microbiological water quality and the potential of QMRA to reveal weak points of a MAR treatment scheme. For example, Masciopinto (2020) showed that MAR recharge with treated wastewater resulted in low health risks when using the recovered water. However, the authors also found increased pathogen concentrations in reclaimed water after extended drought when the dry and touristic season coincide in the coastal study area. Then, higher amounts of wastewater were produced and dilution by rainwater was lacking. This resulted in increased pathogens loads in the reclaimed water. The related human health risks may not exceed a certain target concentration. For example, the acceptable concentration of *Campylobacter* in drinking water is 10^4 cells/L (WHO, 2011). Overall, studies focused on human health risks but neglected the risks of plant pathogens potentially being present in source waters intended for MAR recharge. Therefore, currently no target concentration ('safe' concentration) for plant pathogens in irrigation water exists. Only the plant pathogen Pepper mild mottle virus (PMMoV) has been investigated in risk assessments where it served as an indicator for human viruses in wastewater treatment (Symonds et al., 2018). Verbyla et al. (2016) included PMMoV as surrogate to study its removal during riverbank filtration and applied QMRA to analyze human health risks related to the consumption of raw lettuce that had been irrigated with MAR treated water. To our knowledge, no study used QMRA to evaluate AS(T)R treatment for the production of irrigation water with the focus on plant health.

In this research, we used QMRA to analyze an agricultural ASTR system for the irrigation of potato plants with the aim to assess changes in microbiological water quality and quantify the infection risks of potato plants after irrigation with ASTR treated tile drainage water. The setup and operation of the ASTR system will influence its treatment efficiency. Therefore, the aim of our

study is to determine the critical parameters during ASTR operation that will enhance pathogen removal and to propose an operation scheme to reclaim water for irrigation without risking plant infections. The selected distance between injection and abstraction well may play a significant role as a greater distance will increase the natural filtration processes. Furthermore, bacterial die-off in the water phase and attachment to soil grains depend on velocity and residence time which will impact the fate of pathogens. The calculated risks of using ASTR-treated water for irrigation have to be compared with the current situation in which all safety measurements are followed (e.g., prohibition of surface water irrigation). At present, infections of potato plants are still found incidentally indicating that transmission pathways other than irrigation with surface water play a role in the epidemiology of the pathogen (Janse, 2012). Moreover, the risk of drought related yield losses due to insufficient irrigation water has to be balanced with the risk for disease outbreaks after using ASTR treated irrigation water (Breukers et al., 2008).

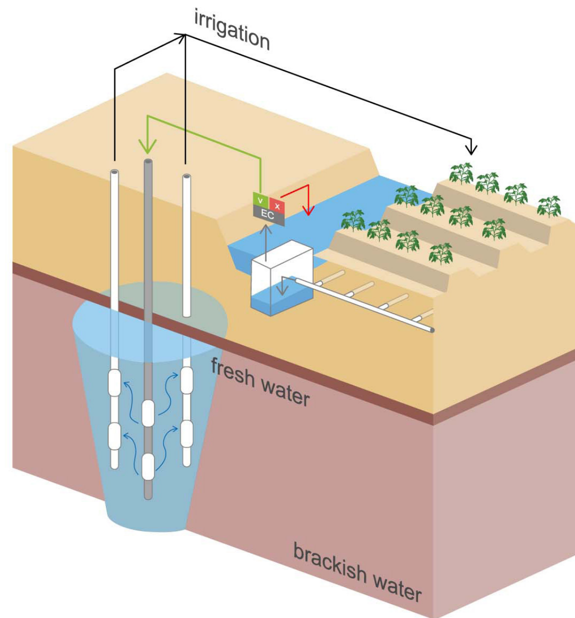
The studied ASTR site uses tile drainage water (TDW) collected after excess rain events from the agricultural field. Although low pathogen concentrations are expected in the TDW, it may mix with *R. solanacearum* contaminated surface water. We hypothesize that the concentration of pathogens and other (agro) pollutants like fertilizers will get reduced during aquifer storage due to die-off over time and removal by sorption. However, it is currently not allowed to use the recovered water from an ASTR for the irrigation of seed potatoes. The installation of water reclamation for agriculture is also hampered as there exist no target concentrations for plant pathogens in reclaimed water as for human pathogens, e.g., 10^{-4} cells/L of *Campylobacter* in drinking water (WHO, 2011). These reference values are missing to analyse the efficiency of a water treatment system. A recent policy used QMRA to establish minimum requirements for water reuse in irrigation regarding human health risks but neglected plant pathogens (Alcalde-Sanz and Gawlik, 2017; Commission, 2020). Therefore, the QMRA of agricultural MAR, and in specific ASTR, with focus on plant pathogens can serve as a tool in legislative decision processes to promote the implementation of ASTR to secure agricultural production by providing safe irrigation water.

5.2 Material and Methods

5.2.1 Agricultural ASTR – site description

The QMRA was applied to a pilot ASTR system (Figure 1) situated in an agricultural area in a polder in the North-Western part of the Netherlands (coordinates: 52.8883, 4.8221). The ASTR system stores water during wet periods in the underlying confined aquifer using wells from where it can be recovered in dry periods for irrigation. After rain events, tile drainage water (TDW) is collected from a 10 ha of agricultural land and injected via a vertical well in a sandy anoxic aquifer (11.5 – 33.0 m below surface level (b.s.l.)) of late Holocene and Pleistocene origin, below a confining Holocene clay/peat layer. The rain water reaches the tile drains (located about 0.7 m b.s.l.) as it percolates through the top soil from where chemical and biological agro(pollutants) may be released and carried along. The tile drains end up in a collection drain, from which TDW is discharged to a storage tank (ca. 1 m^3) when the phreatic groundwater level rises. As the water level exceeds a threshold, a pump within the storage tank is activated. First, disc filters (pore size: 40 μm) treat the pumped TDW to remove suspended solids to avoid clogging of the screens of the infiltration wells. However, these filters have no effect on pathogen removal due to their large pore size. The native aquifer is anoxic, brackish and has a constant temperature of about 10 °C. A freshwater storage is created through the infiltration of the oxic, fresh TDW. As consequence, the infiltrated water will undergo different biochemical reactions. For example, oxygen was reduced within two days and nitrate within 4-7 days using push-pull tests to assess aquifer reactivity (Kruisdijk and van Breukelen, 2021). Changes in water quality will depend on the aquifer composition, its hydrogeochemistry and the composition of the microbiota (Bekele et al., 2018). Moreover, changes will depend on the residence time of the water within the aquifer (time between infiltration and abstraction event) and the soil passage length which is determined by the distance between infiltration and abstraction well of an ASTR system. The outcomes of the QMRA will help in the design of an ASTR to determine the required soil passage to improve water quality sufficiently. The TDW may get contaminated with plant pathogens when contaminated surface water overflows adjacent fields after heavy rain events and enters the drainage system. Additionally, farmers may use level controlled drainage allowing surface water to enter the drainage system to increase water levels in the agricultural field. Pathogens and any other chemicals entering the tile drainage water need to be removed during the recharge process.

In the studied ASTR system, the soil passage is 7 m which cannot be changed anymore after drilling of the infiltration and abstraction wells. In contrast, the residence time is variable and can be controlled by the farmer. The water flow velocities within the aquifer are variable and depend on the pumping rate. For example, the studied system has two infiltration wells with a maximum pump rate of 10 m^3 h^{-1} per well which may result in flow velocities of up to 5.4 m day^{-1}, considering only radial horizontal water flow in the most permeable layer of the aquifer. Moreover, the four abstraction wells are installed with a pump rate of 20 m^3 h^{-1} per well. Although the pumping activities will result in different flow velocities, the background groundwater flow is about 0.01 m day^{-1} if no infiltration or abstraction is taking place.



▲ **Figure 1** Schematic representation of an agricultural field connected to a managed aquifer recharge site. The site is designed as an aquifer, storage, transfer, and recovery (ASTR) system. Excess rain water reaches the tile drainage system buried at about 70 cm depth. The collection drain terminates into a concrete reservoir where the electrical conductivity (EC) and turbidity of the tile drainage water is measured. If the EC or turbidity is below a set threshold value, the water is infiltrated via the injection well (depicted in gray). From there, the water travels through the sandy aquifer to the abstraction wells (depicted in white) and can be used for irrigation

5.2.2 Risk assessment

QMRA calculates risks probabilities associated to specific scenarios and comprises four steps: (i) hazard identification, (ii) exposure assessment, (iii) dose-response analysis and (iv) risk characterization as elaborated in the sections below (Haas et al., 2014). At first, the biological hazard causing harm for the crop health is identified. Then, the hazard's concentration in the source water and its removal during ASTR by different treatments are assessed to determine the exposure concentration. The dose-response analysis determines the infection risk of a potato plant given a certain exposure concentration. Finally, the results of hazard identification, exposure assessment and dose-response analysis are combined to formulate the risk characterization and analyse different scenarios including their variability and uncertainties.

Data analysis was performed using R (v.4.1.2, R Core Team (2022)) and the packages *gsl* (Hankin, 2021), *truncnorm* (Mersmann et al., 2018), and *fitdistrplus* (Delignette-Muller and Dutang, 2015). Graphics were prepared with the package *ggplot2* (Wickham, 2016). A random sample distribution was drawn using Monte Carlo sampling (n = 10'000) from all input parameters, using the parameter's mean value and standard deviation. Creating such a large random sample size allows to account for uncertainty and variability of all parameters which are used in the risk model. The distributions of the results are presented in box-whisker-plots where the 75% percentile represents the conservative and the 25% the optimistic estimate. It was assumed that the parameters follow a normal distribution, if not stated otherwise (Table 1).

5.2.3 Hazard identification

This study focused on *Ralstonia solanacearum* as the biological agent which is a hazard in agricultural production. *R. solanacearum,* together with *R. pseudosolanacearum* and *R. syzigii,* comprise the *R. solanacearum* species complex (RSSC) (Fegan and Prior, 2005). The three individual species can cause bacterial wilt in more than 200 plant species worldwide ranging from tomato to ornamental flowers (Hayward, 1991; Tjou-Tam-Sin et al., 2016). Here, we focus on *R. solanacearum* (phylotype II) which causes brown rot in potato in temperate climates. *R. solanacearum* originated from South America and was introduced into the EU through international seed trading which is the main route of pathogen distribution (EFSA et al., 2019). The pathogen complex has a quarantine status and is regulated in the European Union (Directive, 2000). As consequence of severe disease outbreaks in Europe in the 1990's, irrigation of (seed) potatoes has been prohibited.

Additionally, irrigation of starch and consumption potatoes is prohibited in areas with brown rot contaminated surface water (Directive, 2006). Member states of the EU are required to conduct yearly surveys to restrict the further spread of the pathogen with the aim to eradicate the disease. In the surveys, surface waters near potato producing areas and potato seed lots are investigated for the presence of *R. solanacearum* (Directive, 1998). Hosts like *Solanum dulcamara* (bittersweet nightshade) often grow along waterways where they can get infected with *R. solanacearum*. The pathogen multiplies within the host without showing disease symptoms and gets released into the water when the environmental conditions are favorable (EFSA et al., 2019). Furthermore, the pathogen can survive in topsoil for up to 200 days and its persistence may be prolonged if plant debris are present (Messiha et al., 2009; Tomlinson et al., 2011). In greenhouse experiments, the effect of different concentrations of *R. solanacearum* on two potato cultivars has been determined and a dose-response model was developed (Eisfeld et al., 2022a). It will be used in the QMRA to determine the infection risk of potato plants by *R. solanacearum* and is described in one of the sections below.

5.2.4 Exposure assessment

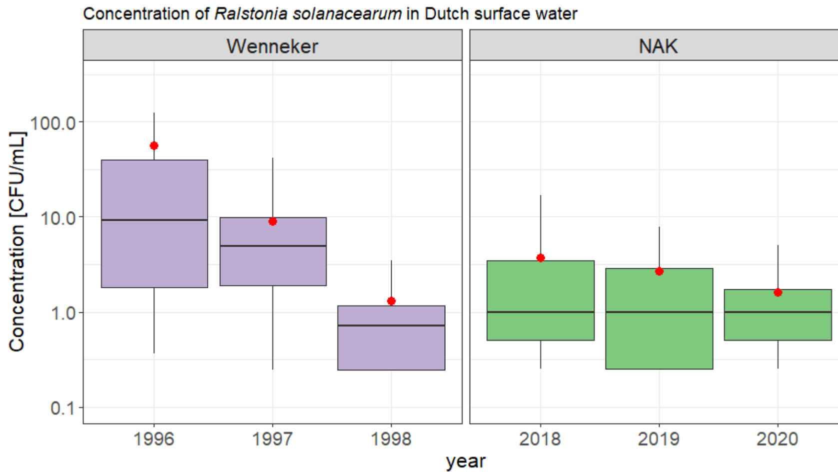
5.2.4.1 Source water quality

TDW collected after rain events is the source water used for storage in the ASTR system. However, the concentration of *R. solanacearum* in TDW has not been analyzed in this study. Tomlinson et al. (2009) surveyed *R. solanacearum* in the Nile delta of Egypt in canal waters along potato growing fields and found concentrations of 0.1-0.2 CFU mL^{-1} in canal waters. The authors also analyzed the drainage water in designated 'Pest-Free-areas' where the bacteria were not detectable. Consequently, the bacterial concentration in TDW is expected to be lower than in surface water or zero but there exists the risk that surface water containing *R. solanacearum* contaminates the drainage water. This may occur when agricultural fields are flooded during storm events by overflowing ditch water (Janse, 1996). Additionally, farmers use surface water in controlled drainage to raise water levels below the agricultural field (subirrigation) which is beneficial for plants during certain development stages (de Wit et al., 2022). However, if the surface water contains plant pathogens this increases the risks of plant infections. Instead, ASTR treated water can be used for such controlled drainage and reduce the risk of plant infections. Lastly, an ASTR system may use exclusively surface water for aquifer storage which poses a higher risk that plant pathogens enter the aquifer which have to be removed during storage.

As a conservative scenario, we assume that concentrations of *R. solanacearum* in drainage water are equal to surface water. *R. solanacearum* has been detected in surface waters in the Netherlands at maximum concentrations of 10^2 - 10^3 CFU mL^{-1} in the summer months. Figure 2 shows the distributions of *R. solanacearum* concentrations in surface water during six different years. There is a strong seasonal fluctuation influenced by water temperature (Wenneker et al., 1999; Caruso et al., 2005). During the winter months in the Netherlands, when water temperatures are below 10 °C, the pathogen was detected at very low concentrations (0.5 CFU mL^{-1}) or even below the detection limit (Wenneker et al., 1999). The seasonal variability of the bacteria in surface water is an important factor in temperate climates as the farmer can choose to infiltrate only during the winter months when lower pathogen concentrations are present and where most precipitation surplus can be expected. This may be different in other climate zones where temperature remain stable during the year as well as concentrations of *R. solanacearum* in surface waters.

The analysis of the distributions is done using *fitdistrplus* in R (Delignette-Muller and Dutang, 2015). The left panel ('Wenneker') is a dataset (n = 104) retrieved from the graphs of Wenneker et al. (1999) and bacterial concentrations were recorded during summer and winter months in 1996-1998. During the 1990's, higher concentrations of *R. solanacearum* were observed as a result of several brown rot outbreaks in Dutch potato cultivation. In September 1996, highest concentrations of max. 10^3 CFU mL^{-1} were observed at a water temperature of 25 °C. Strict hygiene measures, seed testing and a ban on the use of surface water for irrigation resulted in lower disease incidences. Consequently, the concentrations of *R. solanacearum* found in surface waters also decreased which are represented by the more recent dataset (n = 590) from 2018-2020, obtained from the 'Nederlandse Algemene Keuringsdienst (NAK)' van Duivenbode (2021). There, the maximum concentration of *R. solanacearum* was 65 CFU mL^{-1}. The recent surveys are only performed twice per year during the warmer summer months, for example in 2020 from 3-19 of June and 4-14 of August. As input data for the risk assessment, the surface water concentrations of *R. solanacearum* in the years 2018-2020 (Figure 2, 'NAK' data) were used which follow a log-normal distribution (Table 1). The concentrations in Dutch surface water are comparable with surveys of other countries. In the UK, high concentrations of max. 600 CFU mL^{-1} were found during July and August 1994 downstream of infected *Solanum dulcamara* plants while the bacterium remained undetectable from November until June (Elphinstone and Matthews-Berry, 2017). More recent river sampling in England and Wales only detected the

bacterium at a maximum concentration of 68 CFU mL^{-1} at 15 °C. Twenty-six rivers were tested at 54 locations in begin September 2014 and 2015 and only one river tested positive at both testing locations during both years (APHA, 2015). Moreover, Caruso et al. (2005) reported low concentrations (10-80 CFU mL^{-1}) of *R. solanacearum* in Spanish rivers and a dependency on water temperature.



▲ **Figure 2** Concentration of *Ralstonia solanacearum* in Dutch surface water shown as box-whisker plots with the 5-95% confidence interval. The bottom and top of the box represent the first and third quartiles (25th and 75th percentile values) and the red dot indicates the mean value. Data were obtained from yearly surveys reported by Wenneker et al. (1999) (left panel) and the 'Nederlandse Algemene Keuringsdienst (NAK)' (van Duivenbode, 2021) (right panel). The 'Wenneker' data was obtained during periodical sampling from 1996 to 1998 during winter and summer months. The 'NAK' data was obtained during sampling events in the summer months of June to August.

▼ **Table 1** Input parameters for the quantitative microbial risk assessment (QMRA) to calculate the infection risk of potato plants by *Ralstonia solanacearum* after irrigation with managed aquifer recharge (MAR) treated water.

Model parameter	unit	value; standard deviation	reference
Source water concentration			
C_s	CFU mL^{-1}	LOGN(0.08; 1.25)	van Duivenbode (2021), NAK dataset from years 2018-2020 (during summer months)
Recovery efficiency			
R	-	N(0.91; 0.024)	Pradhanang et al. (2000)
Removal by die-off in water phase (Equ. 1)			
Weibull + tail model			Eisfeld et al. (2021) cf. Figure 3(R5)/Table 3
a	day^{-1}	N(0.05; 0.002)	
b	-	N(6.7; 2.0), truncated at (0; Inf)	
C_0	CFU mL^{-1}	N(15900; 1)	C_0 relates to the experimental inoculation concentration used in the batch experiments
C_{res}	CFU mL^{-1}	N(33; 1.2)	
Removal by irreversible attachment (Equ. 3)			
Attachment to quartz sand			Eisfeld et al. (2022b)
k_{att}	min^{-1}	N(0.007; 0.009), truncated at (0; Inf)	
α_L	cm	N(0.038; 0.008)	
α_{L-2} (100 cm soil passage)	cm	N(0.17; 0.037)	Upscaled longitudinal dispersivity
α_{L-3} (200 cm soil passage)		N(0.33; 0.072)	
Attachment to aquifer sand			
k_{att}	min^{-1}	N(0.121; 0.018), truncated at (0; Inf)	
α_L	cm	N(0.45; 0.30)	
α_{L-2} (100 cm soil passage)	cm	N(2.0; 1.32)	Upscaled longitudinal dispersivity
α_{L-3} (200 cm soil passage)		3.9	
Velocity			
$v_{average}$	cm min^{-1}	0.246	Average velocity during infiltration or abstraction occur (3.5 m day^{-1})
$v_1 - v_2$	m day^{-1}	1 - 5.4	Upscaled velocity
Volume of irrigation			
176	mm	per growing season	Acacia Water (2019b)
35.2	L	total volume per plant per growing season	
Dose-response model (Equ. 7)			
α	-	bp(0.17; 0.08)	Eisfeld et al. (2022a)
β	-	bp(3.6x10^5; 9.7x10^5)	
ASTR operation			
a) soil passage			
x_1	cm	23	Column length from Eisfeld et al. (2022b)
x_2	cm	70	Required soil filtration distance needs to be known during ASTR design stage; the pilot ASTR site has a 7 m soil passage
x_3	cm	100	
b) residence time			
$t_1 - t_2 - t_3$	days	10 – 30 – 60	Residence time can be set by the farmer

LOGN = lognormal distribution; N = normal distribution, UNIF = uniform distribution; bp = Beta-Poisson distribution; α_L = longitudinal dispersivity; katt = irreversible attachment parameter

5.2.4.2 Die-off in the water phase

The first natural treatment during aquifer recharge relies on the bacterial die-off in the water phase. It depends on the residence time which is the storage period between injection and abstraction events, and can be determined by the farmer. For the QMRA, three residence times of 10, 30 and 60 days were compared. Subsurface travel time of 60 days combined with specific setback distances is considered safe in drinking water production in the Netherlands (CBW, 1980). A short residence time of 10 days will remove less pathogens but represents a greater flexibility for the farmer using recovered water for different purposes. However, infiltration will mostly occur during the wet winter months while water recovery is needed during the drier cropping periods. Therefore, residence times of one or several months might be more realistic.

A non-linear Weibull + tail model described the bacterial die-off in natural oxic TDW and anoxic aquifer water from a MAR site (Eisfeld et al., 2021). The removal is described by :

$$z_{die-off} = \frac{C}{C_0} = e^{-(at)^b} - \frac{C_{res}}{C_0}\, e^{-(at)^b} + \frac{C_{res}}{C_0} \quad (1)$$

where C_0 [M L^{-3}] is the inoculation concentration which has been used in the batch experiments (about 10^4 CFU mL^{-1}). C is the bacterial concentration after time t [T]. *a* [T^{-1}] and *b* [-] are the model parameter estimates which influence the curve shape. C_{res} [M L^{-3}] represents a more persisting residual concentration which depends on the inoculation concentration for which is accounted using the ratio of C_{res}/C_0. The improvement in water quality of the infiltrated water after a given residence time is calculated as:

$$C_t = C_s * z_{die-off} \quad (2)$$

where C_s [M L^{-3}] is the bacterial concentration in the source water which will decrease to C_t [M L^{-3}] by water die-off after a given residence time t. The maximum removal which can be predicted is about 3-log_{10} due to the experimental conditions which observed the bacterial die-off from an inoculation concentration of 10^4 CFU mL^{-1} to about 10^1 CFU mL^{-1}.

The die-off was monitored under oxic and anoxic conditions as the redox conditions will change during ASTR. The infiltrated TDW is oxic but due to biological and chemical interactions with the aquifer material (e.g., organic carbon), oxygen will be depleted within 1-2 days (Kruisdijk et al., 2022a). For the risk assessment, the experimental parameters of the die-off of *R. solanacearum* in anoxic aquifer water at 10 °C are used (Table 1). There, the die-off was longest and bacteria were no more detectable after 55 days. The die-off was described with the Weibull + tail model. The die-off curve under these conditions was characterized by an initial shoulder phase where the bacterial concentration remained stable followed by a linear decline, and a third phase in which the residual concentration remained at a low level of a few cells per mL until the concentration dropped below the detection limit of 3 CFU mL^{-1}. The residual concentration will depend on the inoculation concentration for which is accounted using the ratio of C_{res}/C_0. This persistent bacterial populations poses a risk to cause plant infections if the residence time was too short. However, after 60 days the bacteria were no more detectable in experimental conditions where the bacteria may have either died-off completely or entered the viable but non-culturable (VBNC) state (Elphinstone et al., 1998). It is questionable if VBNC cells in the recovered water from an ASTR system will still pose a hazard in irrigation as they need to restore their viability and remain virulent (Kong et al., 2014).

5

5.2.4.3 Attachment to aquifer sediment

The second and simultaneous natural aquifer treatment process is pathogen removal by attachment to the sand grains which was analyzed using soil column experiments (Eisfeld et al., 2022b). The column material was either clean quartz sand or natural aquifer sand from the pilot site which was obtained during drilling operations. A Hydrus-1D model using an extended advection-dispersion equation accounting for bacterial attachment and detachment was used to fit the bacterial column breakthrough curves. As detachment was orders of magnitude lower than attachment, the detachment parameter can be neglected and removal by irreversible attachment ($z_{attachment}$) to the sediment grains is assumed to be the main mechanism during bacterial transport in the subsurface Schijven et al. (2000):

$$z_{attachment} = \frac{C}{C_0} = e^{x \frac{1-\sqrt{1+4\alpha_L \frac{k_{att}}{v}}}{2\alpha_L}} \quad (3)$$

C/C_0 describes the bacterial removal and x [L] represents the soil passage length determined by the distance between injection and abstraction wells in an ASTR system. α_L is the longitudinal dispersivity [L], ν the average

interstitial water velocity [L T^{-1}] and k_{att} the attachment parameter estimate [T^{-1}]. All input parameters are shown in Table 1. Monte Carlo samples of the attachment parameter were sampled from a truncated normal distribution with the lower limit at zero to avoid the sampling of negative attachment values. Similar to the water die-off, the concentration C_x after the soil passage is the reduction of the source concentration C_s by $z_{attachment}$ which will depend on the soil filtration length. Note, that the water will flow out radially from the infiltration well and when abstracted, the water will have travelled different distances. However, the minimum travel distance will remain the distance between infiltration and abstraction well. Moreover, the aquifer sediment composition will influence the transport velocities and the bacterial removal.

$$C_x = C_s * z_{attachment} \quad (4)$$

In the column experiments, removal was much higher in the natural aquifer sand than in the quartz sand due to a more heterogeneous surface structure and grain size distribution of the aquifer sand which offer more favorable attachment sites. For example, positively charged metal oxides on the grain surface of natural sands will increase bacterial attachment (Johnson et al., 1996). Moreover, the column experiments with the bacterial plant pathogen *Pectobacterium carotovorum* have shown that fine aquifer material (d_{50} = 192 µm) resulted in much higher (31-40 log_{10}) removal per meter than in medium aquifer material (d_{50} = 305 µm; 19 log_{10} m^{-1}). The aquifer material has been selected as it is a good representation of the average medium grain sizes found in the aquifer of the pilot site (Eisfeld et al., 2022b).

The attachment is also influenced by the average interstitial water velocity which is set to the point estimate of 0.246 cm min^{-1} (3.5 m day^{-1}). Different velocities from 1 m day^{-1} and 5.4 m day^{-1} will also be tested in the QMRA. Note, that the column length in the experimental setup was 23 cm while the distance in the pilot ASTR system is about 700 cm. For the QMRA, the infection risks will also be calculated for 70 and 100 cm to study the effects of a longer soil passage on the bacterial removal. A soil passage of 100 cm was chosen as this would be the minimum distance which is technically feasible. Longer soil passages could not be simulated due to high removals in aquifer sand which caused too low infection risks. This will be further elaborated in the results and discussion. Although a longer soil passage is desirable to achieve high pathogen removal the recovery efficiency of freshwater from a brackish aquifer will decrease as more mixing between the saline groundwater and infiltrated freshwater will occur (Maliva et al., 2006).

Moreover, the longitudinal dispersivity will increase with the soil passage length by 1-10% and needs to be scaled accordingly (Gelhar, 1986). Therefore, the ratio between tested upscaled filtration length (e.g., 100 cm) and the column length (23 cm) was used to increase the value of the longitudinal dispersivity which in the example would result in 100/23 = 4.4.

5.2.4.4 Exposure dose

The exposure of a potato plant to *R. solanacearum* is given as the dose D [CFU]:

$$D = \frac{C_s}{R} * z_{die-off} * z_{attachment} * V_{irr} \qquad (5)$$

The dose is derived by multiplying the Monte Carlo samples of the bacterial concentration in the source water C_s [CFU mL^{-1}] divided by the recovery rate *R*, with the removal by water die-off or attachment and the irrigation volume V_{irr} [L]. The recovery rate relates to the effectiveness of the detection method to recover the bacteria from an environmental sample. Here, dilution plating on selective medium was used to recover the bacteria from soil or water described in Pradhanang et al. (2000). In this study, the recovery efficiency of *R. solanacearum* using semi-selective South Africa agar medium was 88-97% which was used in the QMRA to describe *R*. $z_{die-off}$ and $z_{attachment}$ (values between 0 and 1 [-]) refer to the natural treatments during ASTR. The irrigation volume and frequencies are strongly dependent on the prevailing climatic conditions and the water requirement of the potato plant during the cropping season. In potato production, irrigation is essential to ensure a high tuber yield and potatoes are specifically sensitive to water stress during tuber initiation (Alva, 2008). Within the "Spaarwater" project, drip irrigation volumes were monitored during the growing seasons of three consecutive years (2016-2018) and ranged between 53-176 mm (Acacia Water, 2019a). The variation in irrigation volume reflects the differences in climatic conditions. Per irrigation event, about 3 mm of water was supplied via drip irrigation. These irrigation volumes are also given in traditional sprinkler irrigation. In a conservative scenario for the QMRA, the highest total irrigation volume of 176 mm is used as point estimate to calculate the dose. This volume was supplied during the dry season in 2018 and relates to the expected future drought events as a consequence of climate change.

5.2.5 Dose-response analysis

The exact beta-Poisson dose-response model was used to calculate the infectivity of *R. solanacearum* when contaminated irrigation water is applied by soil-soak inoculation to potato crops simulating drip-irrigation (Eisfeld et al., 2022a).

$$P_{inf}(D|\alpha,\beta) = 1 - {}_1F_1\,(\alpha, \alpha+\beta;\ -D) \tag{6}$$

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${}_1F_1$ is the confluent hypergeometric function and α and β are the infectivity parameters which are Monte Carlo sample pairs (joint distribution), reflecting uncertainty and variability of infectivity (Table 1). In the greenhouse experiments, two potato cultivars (Kondor and HB) were analyzed. Cultivar Kondor was less resistant to *R. solanacearum*, therefore, the dose-response parameters of this experiment are used in the QMRA which also reflects a more conservative approach. The infection risk is calculated for one potato plant. It is assumed that 5 potato plants grow on 1 m^2 (Beukema and Zaag, 1990) which will receive at total irrigation of 176 mm which results in a volume of about 35 L per plant during a whole cropping season.

In a second step, the probability of having symptomatic plants (illness) within the group of infected plants can be calculated with the hazard model of illness dose response where *r* and η are the illness parameters.

$$P_{ill|inf}\,(cV) = 1 - \left(1 + \frac{cV}{\eta}\right)^{-r} \tag{7}$$

In the dose-response model, there is a differentiation between risk of infection and risk of illness. Infected plants may not show disease symptoms (latent infections), whereas infection is conditional for illness which relates to visually symptomatic plants. However, *R. solanacearum* is a quarantine organism for which currently a zero tolerance applies including latent infections. Therefore, the risk assessment is only executed calculating the risk of infection but not illness due to the zero tolerance policy. An infected plant without symptoms cannot by recognized during field inspection but the bacteria can move within the plant and infect the progeny tubers which may be detected during seed testing. For the seed testing, a random sample of 200 tubers is selected per 25 tons of harvested potato tubers (Directive, 1998). It is assumed that each tuber originates from a different plant.

5.2.6 Sensitivity analysis

The sensitivity analysis can help to identify the input parameters which mostly influence the infection risk and therefore, the most critical treatment step within ASTR. Of each parameter used to calculate the dose D (Equ. 5), the variance is calculated and divided by the variance of the respective infection risk (Equ. 6):

$$Sensitivity = \frac{variance(log(parameter))}{variance(log(infection\,risk))} \quad (8)$$

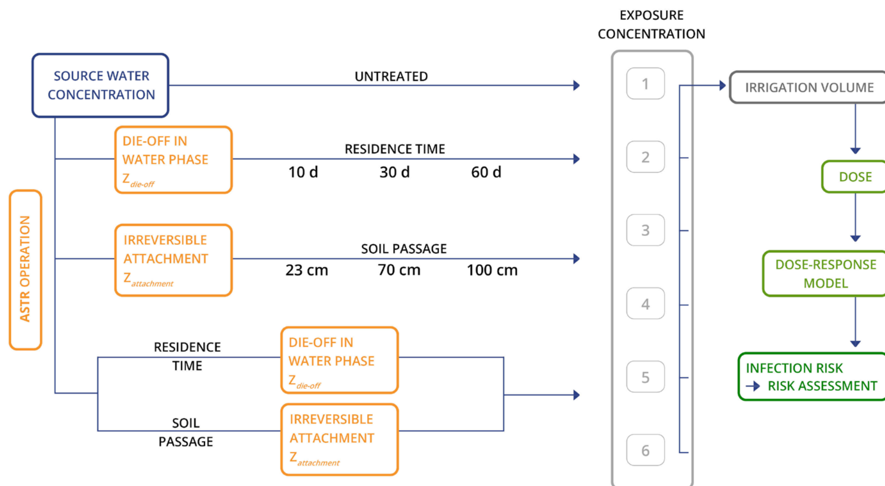
5

Note, the parameters have an equal effect on the final dose through their linear relationship. In the sensitivity analysis, the variances of the parameters impacting the variation in infection risk are compared. Therefore, different ASTR configurations and the impact of residence time and soil passage length can be analyzed. Comparison of the different scenarios will give an insight about which treatment step mostly contributes to the variance of the infection risk.

5.2.7 Risk characterization

Figure 3 shows the steps of the QMRA with an overview of different scenarios and possible ASTR configurations like the required soil passage length and the optimal residence time. The baseline scenario 1 refers the infection risk when potato plants are irrigated with untreated surface water using the 'NAK' dataset which detected *R. solanacearum* during yearly surveys in the summer (Figure 2). Scenario 2 only considers bacterial die-off in the water phase as treatment of the source water which depends on the residence time. In comparison, scenario 3 only considers bacterial removal by soil attachment to quartz sand and scenario 5 removal by attachment to aquifer sediment, which both depend on the soil passage length. The comparison of the different scenarios will display which treatment will have the greatest impact on pathogen removal similar to the sensitivity analysis. In scenarios 4 and 6, the combined removal by water die-off and by attachment to quartz or aquifer sand is estimated, respectively. Hence, the comparison of the scenarios also allows to investigate different MAR configurations and compare ASR with ASTR. In ASR, water is pumped into the subsurface with the same well used for infiltration and abstraction. The water will travel away from the infiltration well when the freshwater storage expands where pathogen attachment and removal will occur. However, the exact soil passage length cannot be predicted. Consequently, an ASR system can only rely on die-off in the water phase for a predictable risk estimation. In contrast, an ASTR system uses an infiltration well and a spatially separated abstraction well which guarantees a minimum travel length of the water through the

subsurface. From all scenarios and different AS(T)R operations, the exposure dose after using AS(T)R treated irrigation water is calculated to obtain the infection risk per plant. The MAR operation will be simulated using different residence times of 10, 30 or 60 days. Moreover, soil passages of 23, 70 and 100 cm will be simulated.



▲ **Figure 3** Scheme for the steps of quantitative microbial risk assessment of an agricultural managed aquifer recharge system using aquifer storage, transfer and recover (ASTR) technology. In scenario 1, the exposure of potato plants after using untreated and *Ralstonia solanacearum* contaminated source water is predicted. Then, different ASTR configurations are compared: In scenario 2, the source water concentration is reduced by the die-off in the water phase which depends on the residence time (10, 30 or 60 days). Scenario 3 and 5 consider the natural treatment only by attachment to quartz sand or aquifer sediment, respectively, which depend on the soil passage length (23, 70, 100 cm). Scenario 4 and 6 evaluate the exposure concentration after the combined treatment by water die-off and attachment to either quartz or aquifer sediment. From the different treatment scenarios an exposure concentration of *R. solanacearum* is calculated. Using a dose-response model, the infection risk of a potato plant after irrigation with ASTR treated water is estimated.

5.3 Results

5.3.1 Removal by die-off in the water phase and attachment

Table 2 lists the estimated log_{10} removals by water die-off as a result of different residence times (10, 30 or 60 d). A simulated residence time from 10 to 30 days almost doubled the removal while a further increase to 60 days achieved about the same removal as for 30 days. As stated earlier, the removal is based on lab experiments which observed the die-off from about 10^4 CFU mL^{-1} to about 10^1 CFU mL^{-1}. Therefore, 2.7-log_{10} removal after 60 days residence time is the theoretical maximum although higher removals may be achieved with a longer storage time. The distributions of the log_{10} removals at 10 and 30 days showed a wide range (e.g., 0.7 – 1.9 log_{10} at 10 days) depicting their minimum, maximum and most likely values. They resulted from the Monte Carlo sampling (n=10'000) which included uncertainty and variability in the parameter estimates.

Table 2 also lists the estimated log_{10} removals of *R. solanacearum* during the natural soil passage in the subsurface. In the column experiment, the removal within 23 cm was studied. In the QMRA, the filtration length was upscaled by modifying the soil passage length in the model to 70 or 100 cm which increased the removal. Considering clean quartz sand as aquifer material, the log_{10} removal were much lower (0.3-log_{10} removal at 23 cm, or 1.2 log_{10} removal at 100 cm soil filtration) than in the natural aquifer sand (4.2-log_{10} removal at 23 cm, or 18-log_{10} removal at 100 cm soil filtration).

When upscaling the soil passage length to 100 cm, the longitudinal dispersivity can be adapted linearly. In quartz sand, α_L scaled from 0.038 to 0.17 cm at 100 cm which did not reduce the mean log_{10} removal but influenced the 95% distribution of the results. In aquifer sediment, α_L scaled from 0.28 to 2.0 cm at 100 cm which reduced the mean bacterial attachment from 18 to 14-log_{10}. Moreover, the effect of a changing velocity on the bacterial removal by soil filtration was simulated. A slower velocity of 1 instead of 3.5 m day^{-1} at a soil passage length of 23 cm increased removal from 0.3 to 1-log_{10} in quartz and from 4.2 to 12-log_{10} in aquifer sediment. On the contrary, a higher velocity of 5.4 m day^{-1} reduced the removal to 0.2-log_{10} in quartz and to 3-log_{10}. aquifer sediment.

▼ **Table 2** Log_{10} removals during aquifer storage transfer and recovery (ASTR)

-log_{10} removal by die-off in the water phase (scenario 2)						**Infection risk**			
Residence time [days]		mean	5%	50%	95%	mean	5%	50%	95%
10		1.3	0.7	1.3	1.9	1.4×10^{-2}	1.2×10^{-4}	2.8×10^{-3}	6.6×10^{-2}
30		2.5	2.0	2.7	2.7	1.6×10^{-3}	8.6×10^{-6}	1.6×10^{-4}	5.3×10^{-3}
60		2.7	2.6	2.7	2.7	1.0×10^{-3}	7.2×10^{-6}	1.3×10^{-4}	3.5×10^{-3}
-log_{10} removal by attachment to quartz sand (scenario 3)									
Soil passage [cm]	α_L [cm]	mean	5%	50%	95%	mean	5%	50%	95%
23		0.3	7.5×10^{-3}	0.1	1.1	5.8×10^{-2}	1.2×10^{-3}	2.9×10^{-2}	2.2×10^{-1}
70	0.038	0.9	2.3×10^{-2}	0.2	3.3	4.3×10^{-2}	1.7×10^{-5}	1.4×10^{-2}	1.9×10^{-1}
100		1.2	3.2×10^{-2}	0.3	4.7	3.9×10^{-2}	7.7×10^{-7}	1.0×10^{-2}	1.8×10^{-1}
		Upscaled longitudinal dispersivity							
100	0.17	1.2	3.2×10^{-2}	0.3	4.6	3.9×10^{-2}	9.1×10^{-7}	9.6×10^{-3}	1.9×10^{-1}
200	0.33	2.4	0.1	0.6	9.0	3.0×10^{-2}	4.3×10^{-11}	4.3×10^{-3}	1.6×10^{-1}
	v [m day^{-1}]	Upscaled flow velocity							
23	1	1.0	2.6×10^{-2}	0.3	3.7	0.04	5.9×10^{-6}	0.01	0.19
23	5.4	0.2	4.8×10^{-2}	4.7×10^{-2}	0.7	0.06	1.9×10^{-3}	0.03	0.23
-log_{10} removal by attachment to aquifer sand (scenario 5)									
Soil passage [cm]	α_L [cm]	mean	5%	50%	95%	mean	5%	50%	95%
23		4.2	3.2	4.1	5.5	8.4×10^{-5}	8.1×10^{-8}	4.2×10^{-6}	2.1×10^{-4}
70	0.28	13	10	12	17	5.2×10^{-11}	$<1.8 \times 10^{-14}$	1.8×10^{-14}	2.0×10^{-11}
100		18	14	18	24	1.1×10^{-14}	$<8.9 \times 10^{-16}$*		8.9×10^{-16}
		Upscaled longitudinal dispersivity							
100	2.0	14	10	14	20	2.5×10^{-11}	$<2.5 \times 10^{-11}$*	1.4×10^{-3}	1.9×10^{-11}
200	3.9	24	16	22	37	1.2×10^{-15}	$<1.2 \times 10^{-15}$*		
	v [m day^{-1}]	Upscaled flow velocity							
23	1	12	9	12	17	3.2×10^{-10}	$<1.4 \times 10^{-13}$*	1.4×10^{-13}	4.1×10^{-10}
23	5.4	3	2	3	4	8.9×10^{-4}	2.7×10^{-6}	8.0×10^{-5}	3.3×10^{-3}

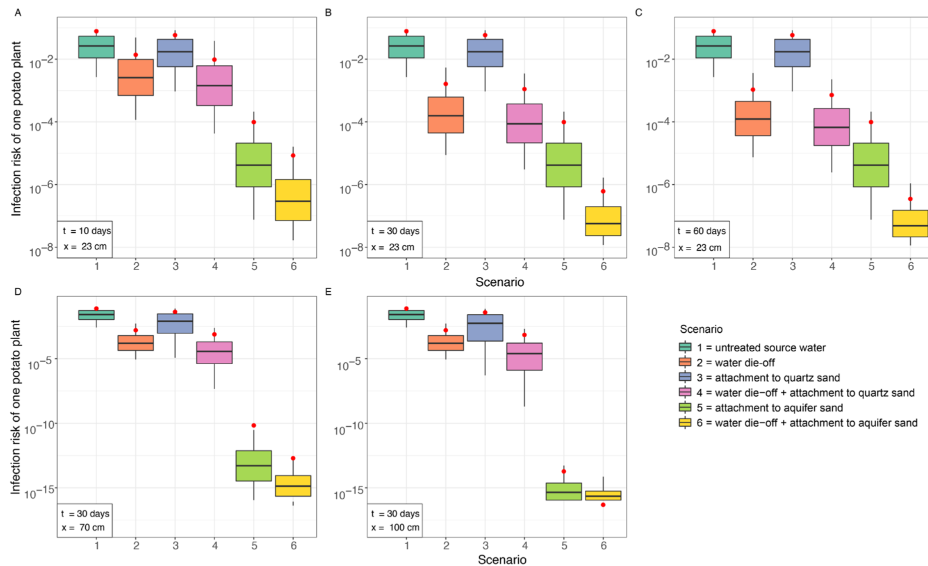
*the high removal resulted in very low exposure concentrations and in very low infection risks that were so small that R program mostly showed zero values in the 10000 Monte Carlo samples

5.3.2 Infection risks

Table 2 also lists all related infection risks which were calculated with the depicted log_{10} removal rates. Note that filtration through aquifer sediment at a soil passage >70 cm resulted in high pathogen removals of at least 13-log_{10} and therefore, a very low exposure concentrations. Consequently, the calculated infection risks resulted in very small numbers near zero which could not be computed by the R program anymore and most of the 10'000 Monte Carlo sample distributions contained zeros. Therefore, it was not possible to upscale to 7 m to simulate the soil passage in the pilot ASTR site. The resulting infection risks were shown as zeros when simulating filtration through aquifer sediment at lengths of >1 m as the removal was predicted to be so high. Figure 4 visualizes the infection risks of a single potato plant after irrigation with ASTR treated water that may have still contained *R. solanacearum*. Graphs A-E in Figure 4 relate to different ASTR operations (residence time, soil passage length) that influenced the infection risk. In each graph, scenarios 1-6 relate to different treatment scenarios of the source water by die-off in the water phase, attachment to the sand or a combination of both. In graphs A-C, an increase in residence time (10, 30, 60 d) was simulated with a constant soil passage of 23 cm. Graphs D+E simulated an increase in soil filtration length (70, 100 cm) at a constant residence time of 30 days.

The infection risks of scenario 1 in Figure 4A-E were all the same as the untreated source water (*R. solanacearum* contaminated surface water) was used for irrigation. An increase in residence time resulted in a higher log_{10} removal by water die-off (Table 2) and also reduced the infection risk as shown in scenario 2 (Figure 4A-C). Scenario 3 simulated water flow through an aquifer consisting of clean quartz material. The removal by quartz filtration was very low and the infection risk remained higher than after removal by water die-off. Even a longer soil passage length of 100 cm through quartz sand (Fig. 4E, scenario 3) resulted in an infection risk of 4% per 1 potato plant, while a 30 day residence time reduced it to 0.2%. Scenario 4 shows the infection risks of a combined treatment by water die-off and quartz sand removal (30 d, 100cm), which was lower (0.07%) than the individual treatments. In contrast, removal by attachment to the natural aquifer sand alone (scenario 5) reduced the infection risk by six order of magnitude in comparison with the untreated source water (Fig. 4A-C). Increasing the filtration length to 100 cm (Fig. 4E) further reduced the infection risk by magnitudes to about 10^{-14} per one potato plant. The lowest infection risk (3.3 x10^{-17}) was achieved by scenario 6 in Fig. 4E, simulating a residence time of 30 days and a 100 cm natural aquifer sand soil passage. The mean values of

the Monte Carlo samples are located between the 75% and 95% percentile with the exception of Fig. 4E, scenario 6. As stated earlier, the removal by die-off and filtration through aquifer sediment was so high that the resulting sample distribution contained many zero values which cannot be displayed on the log_{10} scale.

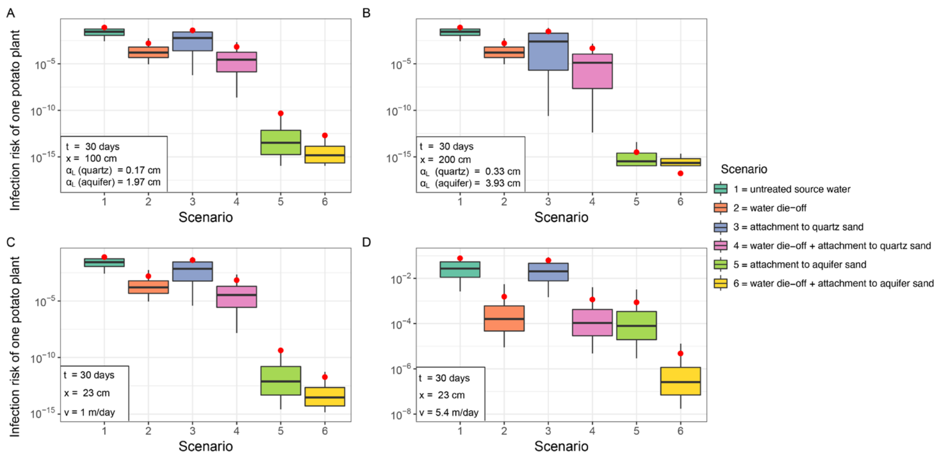


▲ **Figure 4** Infection risks by *Ralstonia solanacearum* per one potato plant after irrigating with water treated through aquifer storage transfer and recovery – ASTR. Box-whisker plots describe the distribution of the data and its 5-95% confidence interval. The bottom and top of the box represent the first and third quartiles (25th and 75th percentile values) and the red dot indicates the mean value. Each graph shows the effect on the infection risk depending on the different treatments during ASTR (scenario 1-6). Scenario 1 in all graphs shows the infection risk if untreated source water is used in irrigation (no residence time or soil passage). The treatment depends further on the characteristics of the ASTR operation. A-C: increase in residence time, from 10 d (A), to 30 d (B) and 60 d (C). D+E: Increase in soil passage length from 70 cm (D) to 100 cm (E). The operational characteristics of the ASTR site are described in the left corner of each graph specifying the simulated residence time (t) and the soil passage length (x).

5.3.3 Upscaling velocity and dispersivity

Infection risks were also calculated for variations in longitudinal dispersivity which was scaled linearly with the soil passage length. Results are shown in Fig. 5A+B. The ASTR configuration in Figure 5A and 4A were the same and resulted in slightly higher infection risks when the dispersivity was scaled

with the soil passage length. The scaling was also increased to a soil passage of 200 cm and in both cases, the infection risks of one plant remained at very low values ($<10^{-10}$). Furthermore, we also simulated different velocities and their effect on the infection risks (Figure 5C+D). Scenario 1 or 2 were both not affected by changes in dispersivity or velocity as it is only considered in the calculation of (Eq. 3). These results should be compared with Figure 4B where the same ASTR configuration regarding residence time and soil passage length was used. A slower velocity of 1 instead of 3.5 m day^{-1} decreased the infection risk of scenarios 3-6 while a faster velocity of 5.4 m day^{-1} increased infection risks.

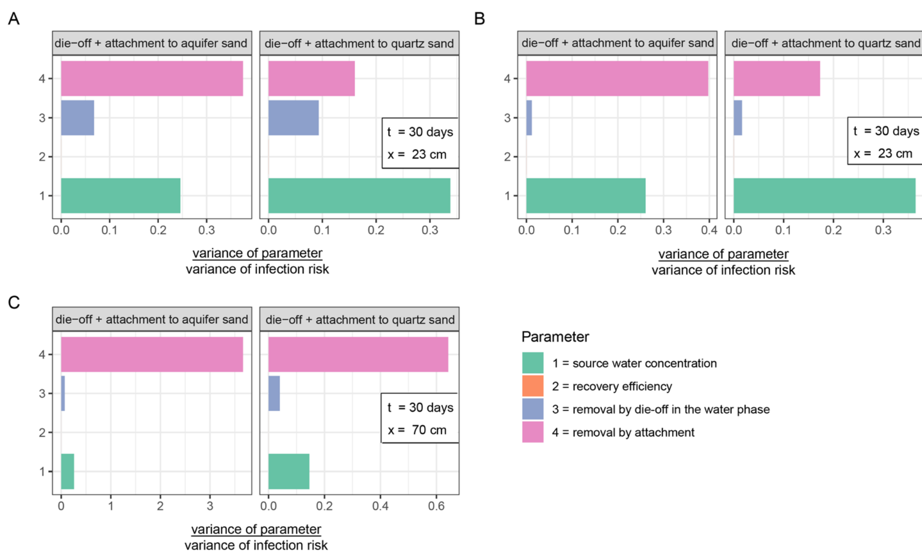


▲ **Figure 5** Infection risks by *Ralstonia solanacearum* per one potato plant after irrigating with water treated through aquifer storage transfer and recovery – ASTR. Upscaling of longitudinal dispersivity (A+B) or velocity (C+D) was simulated. Box-whisker plots describe the distribution of the data and its 5-95% confidence interval. The bottom and top of the box represent the first and third quartiles (25th and 75th percentile values) and the red dot indicates the mean value. Each graph shows the effect on the infection risk depending on the different treatments during ASTR (scenario 1-6). Scenario 1 in all graphs shows the infection risk if untreated source water is used in irrigation (no residence time or soil passage). The treatment depends further on the characteristics of the ASTR operation which are described in the left corner of each graph specifying the simulated residence time (t), soil passage length (x) and longitudinal dispersivity (α_L) or velocity (v).

5.3.4 Sensitivity analysis

Figure 6 shows the results of the sensitivity analysis and graphs A-C compared different ASTR operations, while 1-4 describe the different input parameters which were used to calculate the dose. The resulting value of the ratios of variance of the parameter to variance of the risk is irrelevant but allows to

compare the different parameters with each other. The left panel of each group describes ASTR with bacterial removal by die-off in the water phase and filtration through aquifer sand while the right panel describes ASTR with removal by die-off in the water phase and filtration through quartz sand. Under all conditions, the recovery efficiency had a negligible impact on the infection risk. In all simulations with aquifer sand (Fig. 6A-C, left panels), the variance in removal by attachment to aquifer sand had the greatest impact on the infection risk. In contrast, the variance in removal by attachment to quartz sand had less impact on the infection risk (Fig. 6A+B, right panels). Here, the variation in source water concentration was the most critical parameter to influence the infection risk. However, when increasing the soil passage length to 70 cm (Fig. 6C, right panel), the variance of the removal parameter had the greatest impact on the infection risk. The sensitivity analysis showed that variation in source water concentration and removal by attachment will have the greatest impact on the infection risk.



▲ **Figure 6** Sensitivity analysis to analyze the impact of parameters on the infection risk. A-C: Analysis of different ASTR operations as described in the box on the right side of each graph. Left panel always analyses ASTR with pathogen removal by die-off in the water phase and attachment aquifer sand, right panel analyses ASTR with pathogen removal by die-off in the water phase and attachment quartz sand. Scenario 1-4 relate to the different input parameters that impact the final infection risk. The value of the ratios is irrelevant but allows to compare the different parameters with each other.

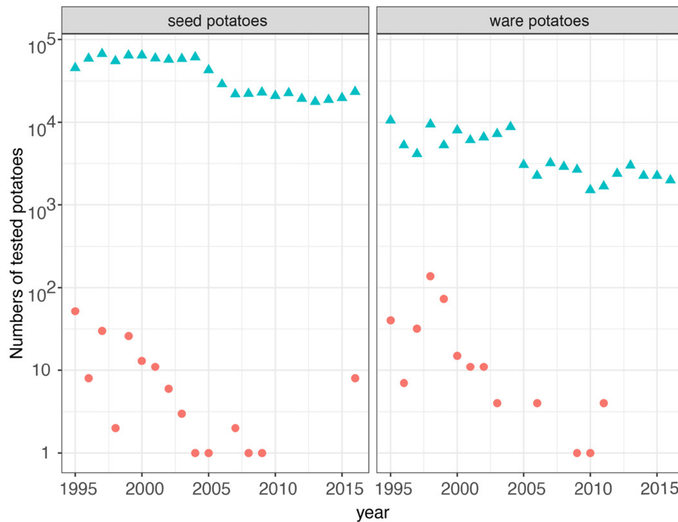
5.4 Discussion

5.4.1 Upscaling to 5 ha potato field

The presented infection risks (Figure 4) were determined for the irrigation of a single potato plant with ASTR treated water. Using a more applicable scenario, the number of infected plants on a 5 ha field with 250 thousand potato plants was calculated using the infection risk of a single plant as input. The average parcel size for seed potato production in the Netherlands is about 5 ha (CBS, 2022). Simultaneously, the current risk of brown rot incidences in the Netherlands was analyzed using the results of the mandatory seed tuber testing. There, the harvested tubers (200 tubers per 25 ton lot) intended as seed tubers are tested for the presence of *R. solanacearum.* Testing of ware potatoes is voluntary and only required if an infection is suspected (Commission, 2017). Figure 7 shows the total number of tested tubers together with the numbers of positive (infected) tubers. In the mid 1990's, the number of positive tested tubers peaked but declined to zero or few cases until 2005 as a result of the implemented eradication scheme (Janse, 2012). From the ratio of positive tested seed tubers out of the total number of tested seed tubers we defined the baseline risk of infection which exists although hygiene measures are followed (e.g., tested seed tubers, ban on surface water irrigation). From the baseline risk and the infection risks calculated in this study (Figure 4) we estimated the number of infected plants on a 5 ha field. Results are shown in Table 3.

The baseline risk of infection for seed tubers was 1.9×10^{-4} which would predict 47 infected plants on a 5 ha potato field. Furthermore, it was estimated that 13% of a 5 ha field (6.2×10^{4} plants) would be infected after irrigation with untreated surface water. Similar high numbers can be expected if the MAR treatment would solely rely on water die-off. Consequently, an ASR system where water is stored no longer than 30 days will still result in too high infection risks. An increase in residence time to 60 days would still result in about 919 infected plants. Equally, ASTR treatment which uses quartz sand filtration (23-100 cm) would produce irrigation water of insufficient water quality as many infected plants are predicted ($>10^{4}$ infected plants on 5 ha field). We even simulated a quartz sand soil passage of 700 cm length which still resulted in a high number of infected plants (2.4×10^{4}). However, it is not expected to find such a sand type in a natural setting as biological activity and weathering will change the surface structure. In our simulations, only the combination of attachment to quartz sand (x = 700 cm) with water dieoff (t = 30 days) would reduce the number of infected plants to 96. Therefore, newly installed slow sand filters (SSF) filled with commercial

'clean' sand used for water treatment require aging of the sand layer to develop a better pathogen removal. Previous research has shown that SSF is an effective method to reduce plant pathogens by magnitudes of order (Prenafeta-Boldú et al., 2017). SSF could also be considered as additional pre-treatment for the infiltrated water and improve an existing ASR system. The active biological layer in a SSF also reduces chemical agropollutants such as pesticides (Majsztrik et al., 2017) which showed little sorption in aquifer injection experiments (Kruisdijk et al., 2022b).



▲ **Figure 7** *Ralstonia solanacearum* infected potato samples detected during yearly surveys (1995 - 2016) of the domestic potato production in the Netherlands (Commission, 2017). Per sample, 200 randomly selected tubers from a 25 ton potato tuber lot are tested for the presence of *R. solanacearum*. The total number of tested tubers is shown in blue triangles and the positive ones are shown in orange circles. Left panel shows the tested potatoes for seed production where all tuber lots have to be tested. Right panel shows tested ware potato lots, testing of lots is voluntarily.

In contrast, attachment to aquifer sand (23 cm) alone would predict about the same number of infected plants (n = 56) as the baseline risk (n = 47). A longer soil passage of 70 cm would already reduce the total of infected plants to a negligible number (4.7×10^{-6}). Finally, an ASTR operation with 30 days residence time and a soil passage of 100 cm (aquifer sand attachment) would estimate almost zero (<6.3×10^{-10}) infected plants when using ASTR treated irrigation water. Therefore, the ASTR treatment predicts lower number of brown rot

infections than the current disease incidences where all hygiene measures area followed. In this context, the ASTR system provides irrigation water of sufficient quality as it would not increase the baseline risk.

▼ **Table 3** Estimated number of infected potato plants after irrigating a 5 ha field (250 thousand potato plants) with untreated or ASTR treated surface water.

Baseline risk[1]			47					
	scenario							
	1	untreated source water	6.2 x10^{4}					
ASTR operation		**residence time [days]**	**10**	**30**	**60**			
	2	water die-off	1.8 x10^{4}	1.4 x10^{3}	919			
		filtration length [cm]	**23**	**70**	**100**	**700**		
	3	attachment to quartz sand	5.4 x10^{4}	4.8 x10^{4}	4.5 x10^{4}	2.4 x10^{4}		
	5	attachment to aquifer sand	56	4.7 x10^{-6}	6.3 x10^{-10}	-		
		residence time [days]	**10**	**30**	**60**	**30**	**30**	**30**
		filtration length [cm]	**23**	**23**	**23**	**70**	**100**	**700**
	4	water die-off + attachment to quartz sand	1.3 x10^{4}	866	585	594	504	96
	6	water die-off + attachment to aquifer sand	4	0.18	0.12	8.1 x10^{-6}	<6.3 x10^{-10}	-

[1]risk of brown rot infections while all hygiene measures are followed. *risk cannot be calculated by the model as the dose and risk are too low.

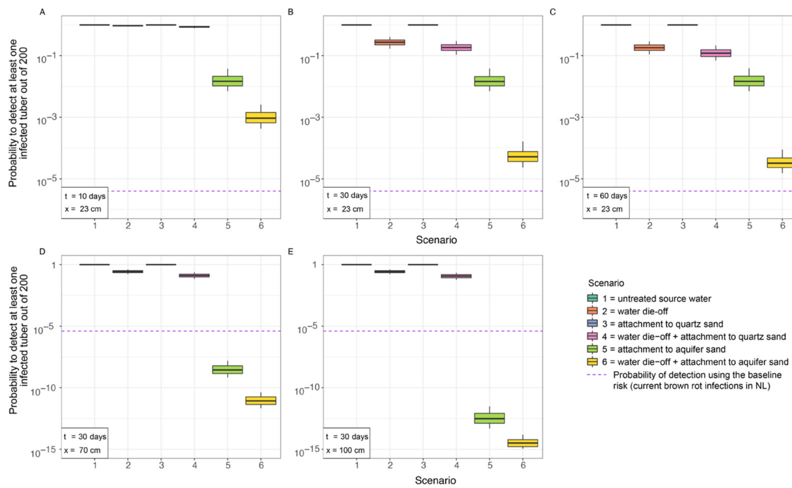
5.4.2 Probability of detection

Seed tuber testing is an important regulations to avoid the transmission of latently infected seed material. In practice, 200 tubers are selected per 25 ton tuber lot and analyzed for the presence of *R. solanacearum* in the tuber material. Given a disease incidence in a tuber lot (P_i), the probability to detect (P_d) at least one infected tuber when sampling 200 randomly selected tubers is calculated as:

$$P_d = 1 - \prod_{i=1}^{n=200}(1 - P_i) \qquad (9)$$

The sample number (n = 200) is based on detecting at least one infected tuber with a 95% probability, assuming a disease incidence of 1.5% within a tuber lot of infinite size (Janse and Wenneker, 2002). Here, we equated the disease incidence with the infection risks of a single potato plant (P_i) calculated in this study. The same ASTR configuration and scenarios were

analyzed to calculate the probability of finding at least one infected tuber in a 200 tuber sample. For comparison, the baseline risk of brown rot incidences in the Netherlands has been used to calculate the probability of detection of at least one infected tuber (P_d = 4.0x10^{-6}). Results are shown in in Figure 8 and the use of untreated surface water had the highest risk of infection and resulted in 100% probability to detect an infected tuber (P_d = 1). The same result was observed when simulating attachment to quartz sand regardless the considered soil passage length (Fig. 8A-E). The resulting distributions have a very narrow range as high infection risks result in a high probability of detecting the disease. Moreover, the distributions show a smaller sample size (n = 200). Bacterial removal by dieoff in the water phase (60 d) reduced the probability of detecting at least one infected tuber to about 7% (Fig. 8C). After removal by attachment to aquifer sand (23 cm), the probability to detect and infected tuber reduced by ten times lower to about 0.7% (Fig. 8A). The combined removal by water die-off and attachment to aquifer sand reduced the probability of detecting an infected tuber to a negligible value. However, only a soil passage of at least 70 cm resulted in lower probabilities of detection than the probability calculated with the baseline infection risk. In general, this demonstrated that the current testing scheme of 200 tubers has a low chance of detecting infected tubers given the few brown rot incidences that are still observed. A bigger sample size could increase the probability of detection but may not be reasonable regarding the cost-benefit of changing the current testing strategy (Breukers et al., 2008). To conclude, both, the upscaling of our infection risks to a 5 ha potato field and the probability to detect at least one infected tuber, aimed to place the obtained results within a practical frame and compare the results with the current legislation. The current policies do not consider irrigation with ASTR treated water. Nevertheless, the results of the QMRA and the comparison with the existing risks demonstrated that irrigation with ASTR treated water did not increase infection risk.



▲ **Figure 8** Probability of detection of at least one infected tuber by *Ralstonia solanacearum* within a sample of 200 randomly selected tubers coming from a potato lot of infinite size and given an infection risk $P_{i.}$ Box-whisker plots describe the distribution of the data and its 5-95% confidence interval. The bottom and top of the box represent the first and third quartiles (25th and 75th percentile values). Each graph shows the probability of detection depending on the different treatments during ASTR (scenario 1-6). The treatment depends further on the characteristics of the ASTR operation. A-C: increase in residence time from 10 d (A), to 30 d (B) and 60 d (C). D+E: Increase in soil passage length from 70 cm (D) to 100 cm (E). The operational characteristics of the ASTR system are shown in the left corner of each graph specifying the simulated residence time (t) and the soil passage length (x). The dashed purple lines show the probability of detection calculated with the baseline risk resulting from the occurrences of brown rot infection in the Netherlands (NL).

5.4.3 ASTR to produce irrigation water

Currently, the use of surface water for the irrigation of seed potatoes is prohibited as disease outbreaks have been linked to contaminated irrigation water (Janse, 1996). The results of this QMRA support this regulation as even low levels of bacteria (max. 65 CFU mL^{-1}) may cause an infection if high irrigation volumes are applied. However, as ASTR is highly effective in removing bacterial pathogens the regulations on water reuse for irrigation should be adapted. From this QMRA, minimal allowable concentrations of the bacterial pathogens could be formulated by decision makers similar to drinking water guidelines (e.g., WHO (2011)). Furthermore, the QMRA allowed to compare infection risks when using an ASR or ASTR system. Considering only die-off in the water phase, an ASR system was insufficient to remove the concentrations of *R. solanacearum* found in surface water

as the risk assessment still estimated high infection risks after irrigation. Only if the source water would contain a lower concentration (accidental contamination) or is known to be free of pathogens, treatment by water die-off can be regarded as sufficient. In this case, the regular monitoring of the source water for the pathogen of interest would be a prerequisite. Note, the recharge water within an ASR will still move through the porous medium of the aquifer when the freshwater lens is expanding in the subsurface. Then, recharged water with a longer residence time will have travelled further away from the infiltration well while freshly infiltrated water will have a short travel distance. In order to predict the water flow within an ASR, and thereby bacterial removal by attachment, requires precise knowledge about the hydrological and geological composition of the aquifer. This was also discussed by Page et al. (2015) who analyzed the aquifer treatment of either ASR or ASTR after stormwater infiltration and the related human health risks. The authors concluded that ASR may also be an option considering the application purpose e.g., when using the recovered water for irrigation in parks. Nevertheless, they argued that ASR may deliver less uniform treatment as residence time and travel distance of the recovered water are not certainly predictable which can result in fluctuating pathogen levels. In contrast, a MAR system designed as an ASTR system with a soil passage is highly effective in removing bacterial pathogens as shown in previous work (Schijven et al., 2000; Pang, 2009). In our study, a soil passage length of >70 cm in combination with water die-off due to a longer residence time resulted in negligible infection risks even when upscaling to a 5 ha potato field. This is in agreement with the multi-barrier approach established in aquifer recharge in drinking water production (Aertgeerts et al., 2003). It suggests that water treatments with multiple treatment steps are safer to produce water as each of the barriers reduces pathogen concentrations. Moreover, the barriers should act independently to compensate the failure of one of system (Haas and Trussell, 1998). Here, the soil passage of the ASTR depicted an additional treatment to the water die-off. Furthermore, the sensitivity analysis confirmed that removal by attachment to aquifer sand was the most critical parameter to impact the infection risk. To conclude, the QMRA illustrated that aquifer treatment of an ASTR system showed higher pathogen removal than an ASR system.

5.4.4 Limitations of QMRA for agricultural ASTR

The QMRA was based on several assumptions to simplify and evaluate a complex set of sequential processes. However, the limitations due to simplifications were opposed by conservative assumptions. First, concentrations of *R. solanacearum* found in surface water during summer were used as conservative scenario instead of measurements of the TDW where the concentrations are expected to be zero or lower. Nevertheless, the results demonstrated successful removal of bacterial pathogens by ASTR treatment even if TDW mixes with contaminated surface water as it may happen during storm events and flooding, or if contaminated surface water is directly used for infiltration. Furthermore, the presence of *R. solanacearum* in surface water depends on the water temperature. In temperate climates, a seasonal fluctuation of the pathogen concentration in surface water is observed and bacteria are only present at very low concentrations during winter or remain undetectable (Wenneker et al., 1999). Consequently, infiltration of surface water depicts another safety measure to prevent contamination of the aquifer. Next, the aquifer treatment is based on laboratory studies where the pathogen removal could be studied under well controlled conditions (Eisfeld et al., 2021; Eisfeld et al., 2022b). Most QMRAs which analyzed aquifer treatment mostly measured pathogen concentration in the infiltrated and abstracted water to derive a total $\log_{10}$ removal rate that did not differentiate between removal by water die-off or attachment; a general removal rate is selected to consider both processes during aquifer treatment (e.g., Ayuso Gabella (2015); Masciopinto et al. (2020). Our study allowed to analyze the individual results of die-off in the water phase or removal by attachment, and a combination of both processes. As this requires the experimental data for both processes of all pathogens of interest other studies collected existing removal rates from literature especially when different microorganisms (virus, bacteria, protozoa) needed to be considered in the QMRA. The experimental conditions of the literature data might not always simulate the aquifer treatment faultlessly. For example, most bacterial die-off in water phase is described by linear die-off models even though the die-off graph often follows a non-linear pattern. As for *R. solanacearum*, the bacterial population undergoes morphological changes during the die-off and different subpopulations may exhibit different resistances to the environmental conditions (Elsas et al., 2001). The die-off experiments with *R. solanacearum* showed that a persistent population exists that remains viable at a low concentration which may pose a risk in aquifer treatment if the water is recovered too early. Even fewer studies investigated the transport of bacterial (plant) pathogens in saturated porous media although our study demonstrated the great potential of

aquifer recharge to remove *R. solanacearum*. Therefore, future studies should study the transport of pathogens in different media. However, the complexity of a natural system cannot be fully pictured in lab experiments although natural water and aquifer sand from the MAR study site have been used in the experiments.

Predicting pathogen removal in the field from lab column studies has to be done with caution as lab experiments can overestimate the removal (Pang, 2009; Oudega et al., 2021). Therefore, in this study, we also accounted for the effect of increasing dispersivity which scales linearly with the tested filtration length. From column experiments, dispersivity was determined by monitoring the transport of a non-reactive salt tracer solution through a 23 cm column. To account for the scaling effect, the column-derived longitudinal dispersivity was multiplied with the ratio of the soil passage length field/column. The resulting higher dispersivity values decreased the $\log_{10}$ removal rates in quartz and aquifer sand but calculated infection risks remained still very low. Although the change in dispersivity should not be neglected it only had a very minor effect considering a sandy aquifer of fine to coarse pore structure. More influence on the bacterial removal had a changing velocity to simulate different pumping rates within the aquifer. While lower velocities increased removal and can be neglected, the higher simulated velocity decreased removal in quartz and aquifer sand. Less bacterial attachment in saturated porous media caused by a higher velocity has been shown on column (Hendry et al., 1999) and field scale (Hijnen et al., 2005; Oudega et al., 2022). Consequently, the effect of flow velocities should be closely examined as high pumping activity may influence removal. However, these studies also indicated that the overall effect on removal at higher velocities remained negligible. This was also confirmed in our study when simulating a higher flow velocity. Lastly, attachment of bacteria to sand grains is a complex process by itself which may even depend on the bacterial species with regard to velocity changes Hendry et al. (1999). Plus, differences in bacterial transport were also observed in our column study using the same porous media where removal varied depending on the species (Eisfeld et al., 2022b).

The dose-response model used in the QMRA analyzed the effect of contaminated irrigation water using a single inoculation event and experiments had to be done in a greenhouse due to the quarantine status of *R. solanacearum*. Therefore, the infection situation in the field may differ. In the field, plants may be irrigated several times with contaminated

water using smaller volumes. Here, a single irrigation event with the water volume of a whole cropping season was simulated as conservative scenario. Moreover, the irrigation method (drip, sprinkler) may influence the infection risks (Dixon, 2015; Café-Filho et al., 2018). The effects of irrigation frequency and method on disease incidences should be analyzed in future studies. The dose-response model was essential to determine the final infection risk. Two different potato cultivars could be tested in the dose-response experiments. Potato cultivar Kondor was more sensitive to irrigation with *R. solanacearum*. Hence, this dose-response model was selected for a more conservative risk estimation in comparison to the more resistant cultivar. Nevertheless, only one host-pathogen interaction could be studied as these experiments are expensive (especially when working with quarantine organisms) and laborious. In the water die-off and soil column experiments, two other plant pathogenic bacteria were analyzed (Soft Rot *Pectobacteriaceae*) but they could not be included in the QMRA as dose-response models are lacking which has to be addressed in future research.

Although the risk assessment contains several uncertainties due to simplification of certain processes, the overall effect of ASTR treatment on the improvement of water quality was clearly identifiable. The attachment to aquifer sand alone could minimize infection risks if ASTR treated water was used for irrigation. Moreover, conservative assumptions (e.g., *R. solanacearum* concentration in the source water) were used in the risk assessment. Plus, uncertainties of the input parameters were including during the Monte Carlo sampling. The resulting infection risks were presented as distribution where the 95% quantile should be used as conservative value in decision making processes. The results indicate that storage time is not needed as the soil passage causes high removal. However, a residence time and thereby removal by water die-off would give an additional safety to the farmer to guarantee pathogen removal. To conclude, the QMRA demonstrated that ASTR is a robust system to store excess tile drainage water in the subsurface and reuse it for potato irrigation. The QMRA predicted that the soil aquifer treatment removes bacterial plant pathogens in order to recover the water for irrigation without risking crop infections.

5.5 Conclusion

The QMRA presented in this research can help to answer questions regarding microbial water quality during MAR where critical treatment steps of the MAR operation were analyzed. MAR systems designed as ASR or ASTR system

both store fresh water in the subsurface where bacterial pathogens can be removed before the water is reclaimed for irrigation. An ASR system will only depend on the residence time and bacterial die-off in the water phase which was slow resulting in low log-removal. Consequently, if the infiltrated water contains levels of *R. solanacearum* as found in surface waters it may require several months of storage time to reduce the bacterial concentrations sufficiently. On the contrary, an ASTR system has an additional soil passage of known length which adds a second treatment barrier where bacteria were removed effectively by attachment to the aquifer sand. The processes during ASTR are complex but QMRA helped to understand the individual treatment steps and their effect on the water quality of the recovered water used for irrigation (or other applications). As consequence, our results demonstrated that a residence time is not required because of the high log-removal by attachment during the soil passage alone (> 1 m). However, an additional residence time makes the system's potential to improve water quality more robust. The remaining risks to infect potato plants after using ASTR treated irrigation water were very low or negligible depending on the residence time and soil passage length. This is the first QMRA that focused on plant health with the aim to prevent plant infections. As for drinking water production, QMRA can be used in decision making processes to evaluate water reclamation projects for agriculture. Then, the risk of a plant infection after using ASTR treated irrigation water should be compared with the risk of crop losses due to insufficient water quantity as consequence of droughts. To conclude, QMRA can serve as a valuable tool for risk managers to examine the suitability of MAR with the aim to provide safe irrigation water.

5.6 Appendix D

The supplementary data contains the R code which was used to perform the steps of the QMRA analysis.

5.7 References

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Chapter 6 **Synthesis**

Agriculture is the biggest fresh water consumer as crop production often depends on irrigation. But water scarcity jeopardizes food security as result of water overuse, groundwater exploitation and decrease in water quality. A MAR system designed as ASTR can store excess fresh water in the subsurface from where it is available for later reuse for irrigation or other end-purposes while simultaneously improving water quality during the storage. The infiltrated source water may be of a quality that is unfit for irrigation, containing agropollutants and plant pathogenic organisms. If plant pathogens would persist during water storage, they pose a risk to cause plant diseases when the recovered water is used in irrigation. Earlier studies on microbiological water quality during MAR only investigated the removal of human pathogens (bacteria, viruses, protozoa) and analysed the impact on human health (e.g., Ayuso-Gabella et al. (2011); Page et al. (2015); Masciopinto et al. (2020)). In contrast, no study investigated the fate of plant pathogens during MAR and studies on the persistence of the selected plant pathogens in water and soil are scarce. Therefore, the removal of three bacterial plant pathogens was studied in lab experiments that simulated aquifer conditions. The selected pathogens were the potato brown rot causing *R. solanacearum* and the two Soft Rot *Pectobacteriaceae* (SRP) *D. solani* and *P. carotovorum* causing soft rot to many crops including potato. To determine the required level of removal of these pathogens in ASTR, a dose-response function was established and a QMRA was developed from the experimental results to assess the natural treatment during ASTR and compare it with the removal efficiency of an ASR system, in support of design of ASTR systems that produce water that is safe for irrigation with respect to these plant pathogens. This was the first study analysing the fate of plant pathogens during MAR and assessing the risks of reusing drainage water for agricultural irrigation with the aim to protect plant health.

6.1 Summary of scientific findings

The results of this study contribute to the knowledge on microbiological water quality changes during ASTR. At first, the residence or storage time has been investigated as natural treatment in ASTR or ASR where the pathogen concentration will reduce over time through die-off (**Chapter 2**). This was the first study that analysed the die-off of SRP in natural waters and the

die-off of *R. solanacearum* in anoxic water. Initially, we hypothesized that the pathogen die-off will be faster under anoxic conditions as the absence of oxygen depicts a less favourable condition for survival. Surprisingly, the conditions found in the aquifer, anoxic and low temperature (10 °C), led to a slower die-off of the bacterial pathogens compared to the die-off in TDW at 10 °C and 25 °C. In anoxic aquifer water, a 3-log_{10} removal within 60 days was observed. A more active microbiota present in the TDW due to the higher temperature or the presence of oxygen may have contributed to the faster pathogen die-off in TDW. From the results, a non-linear die-off model was developed which is flexible as it can also be used to predict log-linear kinetics. Thereby, it allowed to predict the bacterial die-off of several species influenced by different environmental conditions and depending on the residence time. Previous research on the die-off of *R. solanacearum* in water only reported first-order kinetics although the curve shape indicated non-linear die-off (Elsas et al., 2001). However, this might underestimate the pathogen's survival in the environment because persisting pathogens at low concentrations are not considered by first-order die-off.

Simultaneously to the die-off in the water phase, bacteria will be removed by natural processes, including attachment to soil grains, die-off on the solid surfaces of the soil grains, and straining, when water moves through the aquifer of an ASTR system. The bacterial transport and removal was studied in soil column experiments where the bacterial breakthrough was tested in saturated porous medium of either clean quartz or natural aquifer sand (**Chapter 3**). Little removal was observed in clean quartz sand columns (max. 2.7 log_{10}/m) as the sand surface offered poor attachment conditions for the bacteria. In contrast, high bacterial removal was achieved in columns filled with natural aquifer sediment (18-40 log_{10}/m). The transport of the three bacterial plant pathogens was compared with *Escherichia coli* WR1 which may have been used in later field testing. However, its removal was higher than the plant pathogens making it an unsuitable surrogate. The experiments also investigated the effects of oxygen on the transport of the bacteria. Contrary to the expectation that the presence of oxygen may lead to oxidation of metal oxides which creates more attachment sites (Johnson et al., 1996), the removal was slightly higher under anoxic conditions. Future research is needed to elucidate the role of oxygen and other parameters on plant pathogen transport as the existing literature does not give a clear answer to this topic yet. Using the column results, the bacterial removal during ASTR can be predicted which depends on the length of the soil passage, set by the distance between infiltration and abstraction well.

To account for bacterial pathogens that still may persist the treatment during aquifer recharge and be present in the reclaimed water, dose response experiments were conducted. In two greenhouse experiments, the effect of *R. solanacearum* contaminated irrigation water on potato plants was assessed in dose-response experiments (**Chapter 4**). The plants were irrigated with water containing different concentrations of *R. solanacearum* to determine the relation between bacterial concentration in irrigation water and an infection or visual disease symptoms. The lowest inoculation concentration of 500 CFU/mL caused one latent infection (plant infected, but without symptoms) in 15 tested plants while visual disease symptoms were only observed when irrigating with at least $5x10^5$ CFU/mL. These were, to our knowledge, the first experiments that analysed the doseresponse relationship of *R. solanacearum* and potato plants including low inoculum concentrations. The effect of an even lower inoculum was tested in *in vitro* plants where a dose of 0.5 CFU/plant was sufficient to cause wilting after invasive stem-inoculation. Although these *in vitro* experiments did not represent natural conditions for infections they demonstrated the virulence of *R. solanacearum*. A dose-response model was developed for the greenhouse and *in vitro* experiments, respectively. They describe the quantitative relationship of bacterial dose and the risk of infection or illness. Similar to dose-response models of human pathogens (e.g., Teunis et al. (2004)), the *R. solanacearum* dose-response model can be employed in risk assessments with the aim to prevent plant infections.

Finally, all experimental results from die-off, soil-column and dose-response experiments have been used as input data in a QMRA in **Chapter 5**. There, infection risks of potato plants after the irrigation with ASTR treated surface water were calculated. The QMRA allowed to compare different operations of an ASTR and formulate recommendations for the safe reuse of drainage water for irrigation. Moreover, Monte Carlo sampling was used to characterize the distribution of the infection risks given the uncertainty of the input parameters. In the simulations, surface water contaminated with *R. solanacearum* (max. 65 CFU/mL) was regarded as source water for the aquifer recharge. The natural treatments during aquifer storage are a combination of the bacterial die-off in the water phase and their removal by soil filtration governed by the distance between infiltration and abstraction well. Different residence times and soil passage lengths have been tested to analyse their effects on pathogen removal. A residence time of 60 days alone was not sufficient to decrease the pathogen concentrations due to persisting pathogen populations. However, adequate removal was achieved when including the bacterial removal by soil passage as additional treatment.

This was the first QMRA that studied the fate of plant pathogens during MAR and related risks to plant health. QMRA is an established tool to analyse and improve water treatment systems, and to set guidelines in order to protect human health (Alcalde-Sanz and Gawlik, 2017). Hence, it can also serve in the decision-making and legislative processes to support the installation of ASTR systems to produce irrigation water of required quality and tackle water scarcity. Therefore, legislations on water reuse in agriculture should be adapted and include target concentrations for plant pathogens in reclaimed water. As a result of this PhD work, an ASTR system can be considered a safe natural treatment to remove bacterial plant pathogens depending on the initial concentration in the source water, the residence time and soil passage length. We consider it safe as plant pathogenic bacteria are very effectively removed during the recharge and the natural soil treatment. Thereafter, we demonstrated negligible infection risks for potato plants if using ASTR treated water in irrigation.

6.2 Agricultural ASTR produces safe irrigation water

The results of this study demonstrated that MAR designed as an ASTR system can produce safe irrigation water. Nevertheless, the results of the lab studies illustrated some remaining treatment challenges in the form of persisting or detaching pathogen populations. In the following, the related risks will be discussed and the advantages of the multibarrier approach presented.

First, a persisting subpopulation was observed during the die-off in the anoxic aquifer water at 10 °C (Chapter 2). Their persistence caused a long tail shape in the die-off curve (about 15-20 days at the end of the 60 days monitoring period). Second, the filtration through the soil columns showed high removal by attachment (Chapter 3). However, some bacteria detached and were detectable in the column effluent even though the concentration of detached bacteria was magnitudes lower. Both, the persisting population from the water die-off and the detaching bacteria, require attention. They pose a risk if they are still present at low levels (< 100 CFU/mL) in the reclaimed water used for irrigation. Irrigation of a 5 ha field with untreated surface water containing about 65 CFU/mL predicted a high infection risk (Chapter 5). Furthermore, low bacterial concentrations may infect under favourable conditions. For example, colonization is facilitated when the bacteria get in direct contact with the plant roots after irrigation and root openings (wounding, growing root) are present. Moreover, the plant may be continuously exposed to a low number of pathogens through repeated irrigation which can lead to a pathogen build-up until the infection threshold is reached.

The persistence of pathogenic bacteria, such as *R. solanacearum*, is also linked with their potential to form viable but non-culturable (VBNC) cells. The VBNC state is a survival strategy of many bacteria, where the bacteria exhibit very low metabolic activity and cannot be detected by culturing on media anymore (Oliver, 2010). This state has also been observed for *D. solani* induced by oxidative stress (Przepiora et al., 2020), while *R. solanacearum* is known to enter the VBNC state when encountering prolonged cold stress (Elsas et al., 2001). Recent research demonstrated that resuscitation of *R. solanacearum* from cold 4 °C water samples was achieved by a temperature shift to 24 °C, 40 days post inoculation. Moreover, the cells remained virulent when stem-injecting tomato plants (Álvarez et al., 2022). However, bacteria in the VBNC state will reach a phase in which resuscitation is not possible anymore and bacteria remain non-culturable. Kong et al. (2014) observed this state of *R. solanacearum* after 30 days in artificial soil microcosms at 4 °C while Álvarez et al. (2022) monitored this transition in environmental waters at 4 °C only after at least 40 days. Consequently, a sufficient residence time can overcome the risk of undetectable VBNC cells which may regain pathogenicity under favourable conditions. The scenario of a temperature shift is relevant for a MAR system where water is stored in the subsurface at groundwater temperature (about 10 °C) and recovered for irrigation in the summer months when atmospheric temperatures are higher. Therefore, to overcome the risk of persistent cells, VBNC cells, or detaching pathogen populations, water treatments should consist of multiple barriers to reduce the pathogen concentrations and related risks to a minimum (Haas and Trussell, 1998).

Multi-barrier approach

The concept of multiple barriers is an established approach in aquifer recharge and also applied in drinking water production to ensure pathogen removal (Aertgeerts et al., 2003). The barriers should act independently to compensate the failure of one system. As evaluated in the QMRA (Chapter 5), the comparison of ASR and ASTR demonstrated that the additional treatment by soil filtration is highly effective in removing pathogens. More importantly, soil passage was required in order to remove *R. solanacearum* sufficiently. As discussed earlier, persisting bacterial concentrations may pose a threat to crop production if they are present in the recovered water. However, the persisting subpopulation after die-off can be removed during the sand filtration. Equally, detaching bacteria during the transport will die-off after time. Existing MAR systems which only rely on one treatment barrier (e.g., residence time) could enhance their water treatment by adding barriers to remove pathogens. By installing a separate abstraction well, an ASR system could be extended to an ASTR to create a soil passage. If this

is not possible, the recovered water could go through additional water treatment using chemical (e.g., ozone, chlorine, UV) or physical methods (e.g., membrane filtration, heat) before reusing in irrigation (Raudales et al., 2014). These methods are cost-intensive and only justified if a high water quality is needed for crop production, e.g., in soilless horticulture. A slow-sand filtration unit depicts a cheaper but still effective pre-treatment of the source water (Ferreira et al., 2012; Oki et al., 2016). By doing so, not only pathogens but also other agropollutants such as pesticides will be removed (Vandermaesen et al., 2019). This reduces the risk of recovering pathogens while simultaneously decreasing contamination of the aquifer. The alternative to a multi-barrier approach may be the consequent monitoring of the source or recovered water for the hazard(s) of interest. While on-line monitoring exists for chemical components such as nitrate (e.g., Drolc and Vrtovšek (2010)), the monitoring of pathogens only exists for certain pathogens or does not have the required detection limit (Li et al., 2020). Monitoring of plant pathogen by traditional methods such as cultivation on selective media or by molecular methods are timely and costly and allows to analyse only a few samples. In contrast, the multiple-barrier approach would render frequent sampling unnecessary if the treatment guarantees negligible pathogen concentrations in the reclaimed water as shown in the studied ASTR system.

6.3 Water quality of MAR

This study focussed on the microbiological water quality during agricultural MAR designed as ASTR with focus on plant pathogenic bacteria. Nevertheless, the recovered water also needs to compile with chemical water quality standards for irrigation water. In the following, the some relevant chemical water quality challenges are discussed.

Source water quality

As shown in the QMRA in Chapter 5, source water quality is an important component when analysing a MAR scheme. When rain water percolates through the topsoil, agropollutants can leach with the pore water into the drains. As a result, the collected drainage water for infiltration may contain increased concentrations of fertilizers and pesticides. On the one hand, this might be beneficial if they could be recycled when reclaiming the stored water for irrigation. On the other hand, this would require an exact prediction of the quantity of chemicals leaching into the drain water and knowledge about their fate in the aquifer which will depend on the water chemistry and the overall aquifer reactivity. This scenario would necessitate frequent sampling

of the infiltration and recovered water and the selection of a representative group of chemicals for monitoring, together with a good model to predict the degradation pathway. The PhD research of Emiel Kruisdijk as part of the AGRIMAR project aimed to investigate the fate of agrochemical pollutants during aquifer recharge and the change in chemical water quality. His research showed that fertilizer components such as nitrate was degraded within a few days in a sandy anoxic aquifer while pesticides were hardly removed (e.g., degradation half-lives of minimum 54 days for Boscalid and up to 209 days for Bentazon) (Kruisdijk et al., 2022a; Kruisdijk et al., 2022b). If recycling is not feasible and low degradation of a certain pollutant is expected the source water needs to be pre-treated in order to compile with water infiltration regulations that prevent contamination of aquifers. This is for example laid down by the groundwater directive of the European Union (Directive, 2006). In MAR, aquifers can be recharged with source waters of different origins and possible negative effects on the groundwater should be considered beforehand. For example, the recharge with TDW will have a different impact on aquifer reactivity than recharge with treated wastewater consisting of a higher nutrient and pathogen load. Yet, the impact of a higher nutrient load in the source water can be beneficial for the present microbial aquifer community. Denitrification at a MAR site was increased by installing a carbon-rich permeable reactive layer (Gorski et al., 2019). On the contrary, high nutrient load in the source water may prolong the survival of bacteria including pathogens present in the water (Gordon and Toze, 2003). Yet, an active bacterial population stimulated by nutrients can also compete with the pathogens and contribute to their removal. To conclude, when looking at water quality of MAR systems not only the biological water quality needs investigation but also chemical water quality. They are both interlinked and influence each other For a successful MAR implementation, the source water should be well characterized and possible fluctuations of its composition should be taken into account.

Characterization of the aquifer

Next to the source water quality, the aquifer itself will influence the quality of the recovered water. The results of the column experiments in Chapter 3 have shown the effects of different sediment types and grain size distributions on the bacterial removal. The clean quartz sand had much less removal capacity than the natural aquifer sediment which had a more heterogeneous surface structure and a wider grain size range. The results also showed that finer aquifer sediment removed the pathogens better than the medium aquifer sand. Consequently, a test drilling to analyse the geological layers of an aquifer is a relevant step in aquifer characterization. Moreover, reaction processes within the aquifer of selected pollutants

can be directly measured using push-pull tests. There, water with a known concentration of specific contaminants or nutrients is injected via a well into an aquifer. Then, water samples are recovered from the same well over time and analysed for the reactants. In combination with reactive transport modelling it has been proposed as a useful method to describe aquifer reactivity, investigate different redox processes, or precipitation or dissolution of minerals (Kruisdijk and van Breukelen, 2021). Dissolution of the aquifer matrix and the release of geogenic contaminants can result in adverse health effects if the reclaimed water is not treated after recovery. For example, carcinogenic arsenic is one of the most challenging contaminants for MAR as it is ubiquitously present in aquifer sediments in many regions of the world and is toxic at trace level concentrations (Fakhreddine et al., 2021). Consequently, the initial characterization of the aquifer using test drilling or having available a relevant database about geology, hydrology and chemistry of the aquifer is a prerequisite to avoid adverse effects on human and plant health and a successful MAR implementation.

Microbial community

While studies on physical and chemical processes during MAR are abundant, the description of the MAR microbial community and its metabolic functions is only evolving in the past ten years (Ginige et al., 2013; Barba et al., 2019; Schrad et al., 2022). This is also due to the methodological and technical advancement necessary to study microbial communities. In the studied MAR, oxic tile drainage water is infiltrated which changed the redox status of the aquifer and the microbial community (Kruisdijk et al., 2022a). Therefore, the effect of oxygen on the die-off and the transport of the bacterial pathogens was studied in Chapter 2 and 3. Oxygen can increase the metabolic activity of the present bacteria which may have led to a faster die-off in the water phase in comparison to the anoxic water microcosms. In the column experiments, the oxic infiltration water may have increased the biomass of the present bacteria which can block attachment sites and reduce pathogen removal. However, the effect of oxygen in the infiltration water on pathogen removal did not yield clear results and requires future research. Other studies on MAR microbial communities that investigated the infiltration of oxygen-rich water suggested an increase in biomass leading to enhanced biochemical processes (Ginige et al., 2013; Ma et al., 2020). However, effects on pathogen removal were not reported. Future research should explore the effect of aquifer microbial communities on pathogen removal or the degradation of pollutions with the aim to predict changes in water quality more precisely.

6.4 Recommendations for future research

While this research helped to answer questions about the fate of plant pathogenic bacteria during AS(T)R, new questions arose for future research or could not yet be answered.

- Based on the quantitative data in Chapter 2 and 3, a risk assessment could also be performed for *D. solani* and *P. carotovorum*. However, a dose-response model for these bacteria as done in Chapter 4 for *R. solanacearum* is currently lacking but could be developed based on results from for example Kastelein et al. (2020).

- As discussed in Section 6.2, bacteria can form VBNC cells which may pose a risk if they are present in reclaimed water used for irrigation. Yet, research should identify if low concentrations of VBNC cells actually cause infections by specifically studying non-invasive methods to simulate irrigation.

- *E. coli* WR1 was included in the column experiments in Chapter 3 as possible surrogate for field experiments. However, it showed higher removal than the plant pathogens making it an unsuitable surrogate. In general, surrogates are only mentioned in studies with human pathogens but currently no surrogates are established in the field of plant pathogens. In the case of *R. solanacearum*, this is necessary as the bacterium cannot be used in field experiments in the Netherlands due to its quarantine status. Even experiments in the greenhouse had to be conducted in certified quarantine greenhouses, as done in Chapter 4, which complicates studies with this pathogen due to restrictions and high costs involved. As alternative surrogates, a non-virulent strains of the selected pathogen could be used in field experiments. Moreover, DNA coated silica particles or DNA encapsulated in silica particles are currently explored for their potential as field tracers although their applicability has still to be further developed (Pang et al., 2020; Chakraborty et al., 2022).

- Field experiments could not be conducted in this research but, as discussed in Chapter 4 and 5, the extrapolation from lab to field scale has to be done with caution to not overestimate removals. Therefore, a field experiment in combination with a column study should deliver valuable insights when studying pathogen removal due to the complex processes occurring within a MAR system.

- Due to the conservative assumptions in the risk assessment in Chapter 5, it was concluded that an ASR system would be insufficient to remove the bacterial pathogens if only the die-off in the water phase is considered. However, the water will flow through the aquifer where removal by soil filtration will occur. Future research should address this by using a modelling approach combined with lab or field experiments to elucidate the potential of ASR instead of ASTR to remove pathogens.

- Lastly, this research analysed the fate of bacterial plant pathogens but as for human pathogens, different organism groups such as viruses, protozoa, fungi and nematodes are also threatening crop production (Hong and Moorman, 2005; Dixon, 2015). Fungal-like oomycetes and in particular *Phytophothora* species present in irrigation water pose a high risk to cause waterborne plant diseases (Hausbeck and Lamour, 2004). They produce thousands of zoospores during their reproduction cycle which can infect host plants. Nevertheless, a column study (12.4 cm length, 1 cm inner diameter) with *Phytophthora capsici* zoospores in saturated porous media achieved up to 99% removal (Jeon et al., 2016). This indicates that they also will be sufficiently removed during aquifer recharge especially due to their big size (< 10 μm) which favours straining. As with human pathogenic viruses, plant viruses are the smallest microorganisms causing plant diseases. MAR studies about the fate of two bacteriophages showed less removal (6-$\log_{10}$) within the first 8 m soil passage compared to *E. coli* WR1 (7.5-$\log_{10}$) (Schijven et al., 2000). Due to their small size, phages or viruses are more likely to be transported over longer distances with the pore water. Interestingly, the Pepper mild mottle virus has been employed as surrogate for human viruses and thereby served to study the efficiency of wastewater treatments (Verbyla et al., 2016). This demonstrates that plant viruses potentially persist aquifer treatment which should be investigated in the future.

6.5 Outlook and opinion

Economics of a MAR system

An agricultural MAR system requires a high initial investment starting with the assembly of a consortium of experts and companies from different fields (hydrologists, engineers, plant scientists, risk modellers). Then, test drillings may be necessary to characterize the site followed by the installation of the MAR system by placing the infiltration and abstraction well system. The cost estimation should include a monitoring concept where water

samples are regularly analysed for the hazard(s) of interest, plus the costs if system parts break down. For example, costs may arise when a broken pump needs repair or replacement. Furthermore, well-clogging is a common problem which can stall MAR operation for a long time (Jeong et al., 2018). The total investment for an ASTR system (infiltration wells, installation of a new drainage system) was estimated to be around 800 €/ha as presented in the Spaarwater report. However, this estimation did not include monitoring costs or costs of replacement material (Acacia Water, 2019). In the end, the investment costs for MAR need to outweigh the costs caused by drought related yield losses. The cost-benefit analysis of a MAR system located in Southern Spain suggested that MAR is a financially positive method as the irrigation water is essential in the intensive agricultural production of this arid region. Simultaneously, the MAR system would contribute to the restoration of the ecosystem by replenishment of aquifers within 5-9 years (Rupérez-Moreno et al., 2017). MAR may not always be financially beneficial for a farmer by just considering the provision of irrigation water. In contrast, the ecological benefits of a MAR system should also be taken into account which includes the restoration of aquifers. Furthermore, the ASTR systems could help to improve the surface water quality to comply with the European groundwater directive (Directive, 2006). In the Netherlands, many surface waters exceed the allowed nitrate levels of 50 mg/L and also contain plant pathogens such as *R. solanacearum*. In coastal regions, long-term benefits of MAR like the prevention of salt water intrusion and groundwater salinization or minimizing land subsidence to reduce flood risks are positive effects to be considered (Alam et al., 2021).

Management of plant pathogens - Integrated pest management on a warming planet

Irrigation water has been recognized as inoculum for waterborne plant disease outbreaks (Hong and Moorman, 2005). However, plant diseases are also introduced via different entry points such as infected seed material (as main contributor to international spread), infested soil or machinery, and even insects. Moreover, rising temperature as consequence of climate change facilitates the establishment of new species in areas that have been free of specific pathogens (Richard et al., 2022). Skelsey et al. (2018) predicted that the capacity of *Pectobacterium* and *Dickeya* species to macerate tuber tissue will increase in the future by 1-19% under different emission scenarios in Great Britain due to a rise in temperature. The current agricultural production system needs to adapt to the challenges posed by plant pathogens and climate change which requires a holistic approach as proposed in integrated pest management (IPM) (Richard et al., 2022). Similar to IPM, a recent study is proposing the concept of 'one health' used in the

prevention of zoonotic diseases to be applied in the management of plant diseases (Morris et al., 2022). First, the reservoirs for plant pathogens should be identified together with virulence factors that assist the pathogen's survival in the environment. Then, pathways for short and long distance dissemination need to be identified. Lastly, the adaption of the pathogen to changing environmental conditions including climate change should be monitored as they could support pathogen establishment in new habitats. Morris et al. (2022) also recognized that the research of plant pathogen epidemics requires an interdisciplinary approach for successful plant disease management. The removal of pathogens by aquifer recharge contributes to the 'one-health' concept by disrupting pathogen dispersal via irrigation water and combining a multidisciplinary research. Furthermore, irrigation management and the selection of an irrigation method can strongly reduce plant disease outbreaks (Dixon, 2015). Irrigation by overhead sprinklers favours the establishment of pathogens infecting aerial plant organs as they will be in direct contact with the plant leaves if present in the irrigation water. In this case, drip irrigation which provides water to the root zone would be the preferred method to reduce plant infections (Café-Filho et al., 2018). Thereby, the choice of irrigation may also pose a barrier for pathogen entry and contribute to the multibarrier approach. Furthermore, soil biodiversity has been shown to not only be beneficial for plant growth but also in suppressing plant pathogens (Yadessa et al., 2010; Zhan et al., 2015). Yet, soil biodiversity is reduced by intensive agriculture (Tsiafouli et al., 2015). To conclude, plant diseases are manageable if using a holistic approach that considers the prevention of entering pathogens during all crop production steps, such as: tested pest-free seed material, hygiene practices during farming or soil management. Moreover, this research showed that the combined knowledge from different disciplines could propose solutions not only to fight water scarcity using ASTR, but also to address water quality challenges posed by plant pathogens present in irrigation water.

Designing a safe MAR system

Based on the results of this research, recommendations for the design of a safe MAR system planned as AS(T)R can be made. In particular, an ASTR system in North Holland was investigated to provide irrigation water. The characteristics of the source water, aquifer composition and requirements of the reclaimed irrigation water were considered when analysing pathogen removal. Thereby, the results contributed to the knowledge about the environmental persistence of the selected plant pathogenic bacteria. Removal in the aquifer will mostly depend on two mechanisms, die-off in the water phase and removal during the soil passage. In comparison, water die-off predicted a much lower removal (max. $3\log_{10}$ after 60 days) than

attachment to aquifer sediment during soil passage (min. 18$\log_{10}$/m). Although the residence time contributes to pathogen removal, a long storage might be required to achieve similar removal as during soil passage. Consequently, an ASR system is less recommended to produce irrigation water as the improvement in microbiological water quality mostly relies on the residence time. Removal by attachment within ASR is less predictable as no defined soil passage exists. In contrast, a soil passage of 0.7 m in the studied ASTR would be sufficient to produce safe irrigation water (as predicted in 23 cm column length, 3.6 cm inner diameter). However, the existing soil passage of 7 m instead of 0.7 depicts a safer distance to remove the bacterial plant pathogens.

The studied conditions for the bacterial pathogen removal are specific for the studied location. If a different location is chosen for aquifer recharge the local conditions need to be considered and if necessary, similar experiments should be conducted in order to safely predict the removal. For example, the recharge of a gravel aquifer probably requires a longer soil passage and residence time. There, the sand material is coarser than in our studied system which can result in preferential flow paths and an overall reduced removal during soil passage (Oudega et al., 2021). However, the removal rates obtained in this study can be translated to other locations for aquifer recharge when the aquifer characteristics are similar. Finally, this research advanced our knowledge about the fate of plant pathogenic bacteria during ASTR. This nature-based solution not only efficiently removes pathogens but also provides a solution to water scarcity in agricultural production.

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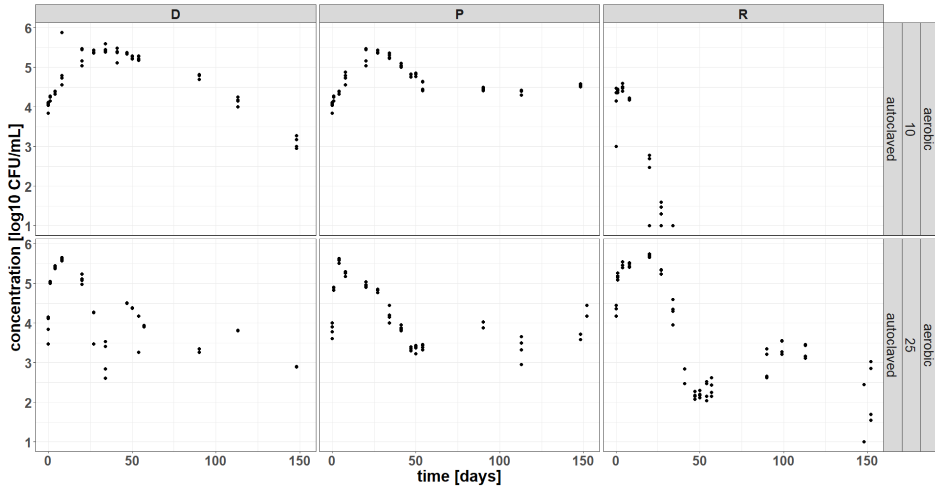
Appendix A



▲ **Figure S1** Die-off of *Dickeya solani* (D), *Pectobacterium carotovorum* sp. *carotovorum* (P), and *Ralstonia solanacearum* (R) in microcosms in autoclaved tile drainage water (TDW) at two temperatures (10 °C, first row, and 25 °C, second row), shown as $\log_{10}$ [CFU/mL] vs. time [days]. Points represent the plate counts in duplicate of two microcosms per treatment.

▼ **Table S1** Enumeration of culturable bacteria in natural waters from a managed aquifer recharge system.

1. Oxic microcosms in tile drainage water

a) Oxic tile drainage water at 10 °C					Oxic tile drainage water at 25 °C				
	days after inoculation*	micro-cosm	medium	CFU/mL		days after inoculation	micro-cosm	medium	CFU/mL
Control	0		TSA	1.00×10^2	Control	0		TSA	2.00×10^2
	16		TSA	9.56×10^3		16		TSA	2.32×10^3
			R2A	9.80×10^3				R2A	1.00×10^4

Total culturable bacteria were also assessed at the end of pathogen die-off period in microcosms of *Dickeya solani* and *Pectobacterium carotovorum* sp. *carotovorum* (the pathogens were no more detectable by plating). The pathogen concentration of *Ralstonia solanacearum* was still 10^2 CFU/mL by plating when this assessment was done.

	days after inoculation*	micro-cosm	medium	CFU/mL		days after inoculation	micro-cosm	medium	CFU/mL
Rsol	12	1	TSA	1.12×10^4	*Rsol*	12	1	TSA	2.00×10^3
			R2A	1.06×10^4				R2A	2.40×10^3
		2	TSA	7.12×10^3			2	TSA	1.74×10^3
			R2A	6.80×10^3				R2A	3.00×10^3
Dsol	16	1	TSA	4.44×10^3	*Dsol*	16	1	TSA	6.20×10^3
			R2A	3.50×10^3				R2A	1.32×10^4
		2	TSA	1.64×10^3			2	TSA	1.48×10^3
			R2A	1.56×10^3				R2A	4.92×10^3
Pcar	16	1	TSA	1.98×10^3	*Pcar*	16	1	TSA	5.92×10^3
			R2A	2.42×10^3				R2A	4.48×10^3
		2	TSA	2.38×10^3			2	TSA	2.46×10^3
			R2A	2.92×10^3				R2A	6.72×10^3

2. Anoxic microcosm in aquifer water at 10 °C

	days after inoculation*	medium	CFU/mL		
			plate 1	plate 2	average
Control (no NO_3)	3	TSA	4.24×10^3	3.16×10^3	3.70×10^3
		R2A	7.56×10^3	4.28×10^3	5.92×10^3
Control (NO_3 added)	3	TSA	3.28×10^3	3.12×10^3	3.20×10^3
		R2A	5.08×10^3	3.64×10^3	4.36×10^3

*Controls are non-inoculated water microcosm and the time indication refers to the start of the experiment when pathogen microcosms were inoculated. The total duration of the die-off experiments can be followed in Figure 3. *Rsol = Ralstonia solanacearum; Dsol = Dickeya solani; Pcar = Pectobacterium carotovorum* sp. *carotovorum*

◀ **Table S1** Enumeration of culturable bacteria in natural waters from a managed aquifer recharge system.

▼ **Table S2** Akaike information criterion (AIC) values; the minimum value among the three models is with a grey background and depicts the model with the best fit.

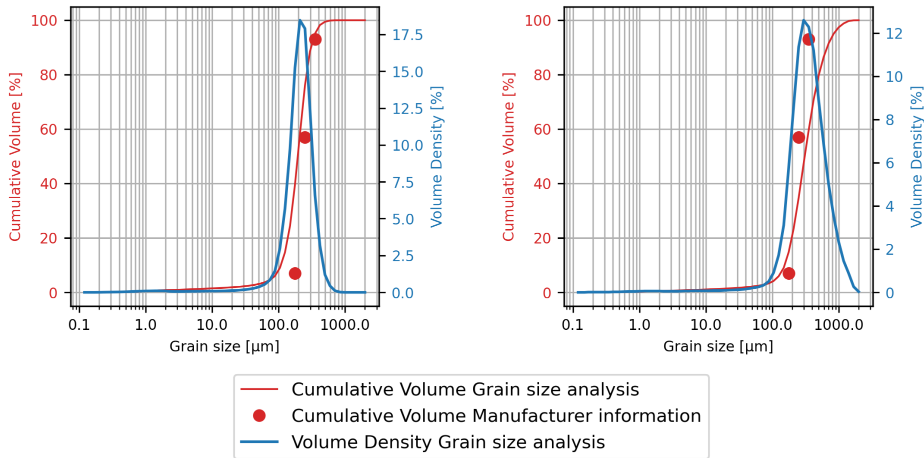
dataset					Model type		
		[°C]	treatment	redox	Weibull + tail	Weibull	Log-linear
Ralstonia solanacearum	R1	10	natural	oxic	53	64	62
	R2	25	natural	oxic	109	108	118
	R3	10	0.22 µm filtered	oxic	-	49	62
	R4	25	0.22 µm filtered	oxic	121	145	157
	R5	10	natural	anoxic	181	224	223
	R6	10	natural + NO_3	anoxic	188	203	204
Dickeya solani	D1	10	natural	oxic	48	53	54
	D2	25	natural	oxic	-	31	33
	D3	10	0.22 µm filtered	oxic	69	79	128
	D4	25	0.22 µm filtered	oxic	-	37	37
	D5	10	natural	anoxic	-	168	199
	D6	10	natural + NO_3	anoxic	111	111	133
Pectobacterium carotovorum sp. carotovorum	P1	10	natural	oxic	-	32	54
	P2	25	natural	oxic	-	11	36
	P3	10	0.22 µm filtered	oxic	73	85	110
	P4	25	0.22 µm filtered	oxic	-	46	51
	P5	10	natural	anoxic	144	148	157

Appendix B

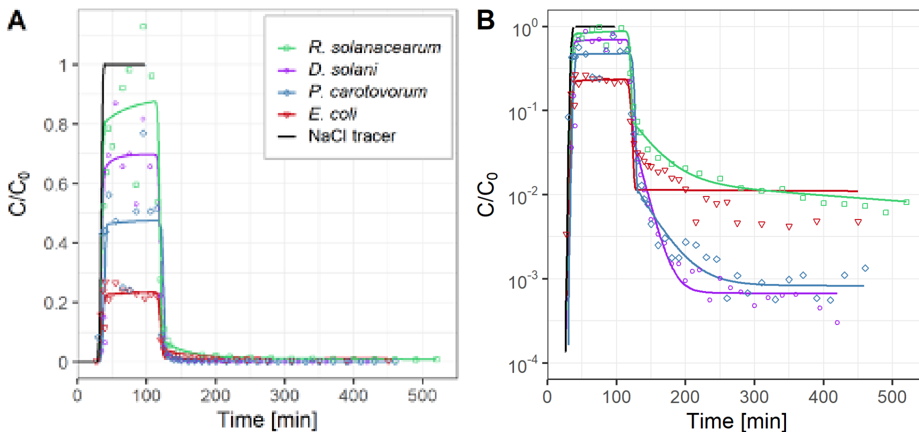
S1: Characterization of bacteria and culture media

R. solanacearum race 3 biovar 2 (phylotype II) strain IPO-1828, *D. solani* IPO-2266 and *P. carotovorum* IPO-1990 were used in this study. These strains were isolated from potato, but strains from the same species were also isolated from surface water in Europe (Potrykus et al., 2016; van Duivenbode, 2021). Their transport was compared with *Escherichia coli* WR1 (NCTC 13167) (Hijnen et al., 2005; Hijnen et al., 2004; Schijven et al., 2000). Strains were kept at -80 °C using the multi-purpose protect cryobeads system (Technical Services Ltd). The strain of *R. solanacearum* used in this study is naturally resistant to rifampicin and was grown on non-selective yeast peptone glucose agar (YPGA) (EPPO, 2004), prepared with 5 g L^{-1} yeast extract, 5 g L^{-1} peptone, 10 g L^{-1} glucose, 15 g L^{-1} agar, and supplemented with rifampicin (50 mg L^{-1}). Liquid cultures were prepared in casamino acid peptone glucose (CPG) broth (Hendrick & Sequeira, 1984) composed of 1 g L^{-1} casamino acids, 10 g L^{-1} peptone, and 5 g L^{-1} glucose. *D. solani* and *P. carotovorum* were both naturally resistant to streptomycin and grown on non-selective tryptone soya agar (TSA; Oxoid; Thermo Fisher Scientific) supplemented with streptomycin (100 mg L^{-1}). *E. coli* WR1 was grown on Chromocult coliform agar (Merck KGaA, Germany). Liquid cultures of these bacteria were prepared in LB (Luria Bertani) medium. Duchefa Biochemie (Haarlem, The Netherlands), Sigma-Aldrich (St. Louis, MO), and Fisher Scientific (Hanover Park, IL) were our chemical suppliers.

Enumeration of the inoculation and bacterial effluent concentration was done by dilution plating on selective media to suppress the growth of background bacteria. *R. solanacearum* was enumerated on semi-selective medium South Africa (SMSA) (Elphinstone et al., 1998b) supplemented with 50 mg L^{-1} rifampicin. *D. solani* and *P. carotovorum* were incubated on the selective double layer-crystal violet pectate (DL-CVP) (Hélias et al., 2012) medium supplemented with 100 mg L^{-1} streptomycin and *E. coli* was enumerated using Chromocult agar (Merck KGaA, Germany).



▲ **Figure S1** Grain size distribution chart of fine (left) and medium (right) grained aquifer sand; the red points in both graphs represents the cumulative volume of the quartz sand (M32, Sibelco, Belgium)



▲ **Figure S2** Bacterial breakthrough curves in quartz sand. The normalized effluent concentration (C/C0) is plotted as a function of time (min) in linear (A) and logarithmic (B) scale. Solid lines represent the fitted models obtained from Hydrus-1D and the symbols represent the corresponding experimental data in the same color. The black solid line represents the fitted chloride BTC from the experiment with *Ralstonia solanacearum*. Square symbol (□) and green solid line represent *R. solanacearum*, open circle (○) and purple solid line *Dickeya solani*, diamond symbol (◊) and blue solid line represent *Pectobacterium carotovorum* and triangle symbol (▽) and red solid line represent *Escherichia coli* WR1.

▼ **Table S 1** Grain size analysis of natural aquifer sands used in column experiments

aquifer sand		fine	medium
%Clay	< 8 µm	1.36	0.97
%Silt	8-63 µm	2.40	1.51
%Very Fine Silt	8-16 µm	0.35	0.31
%Fine Silt	16-32 µm	0.58	0.43
%Coarse Silt	32-63 µm	1.47	0.77
%Sand	63-2000 µm	96.25	97.52
%Very Fine Sand	63-125 µm	10.92	3.45
%Fine Sand	125-250 µm	61.36	28.81
%Middle Coarse Sand	250-500 µm	23.41	44.95
%Coarse Sand	500-1000 µm	0.57	17.71
%Very Coarse Sand	1000-2000 µm	0.00	2.61

▼ Table S 2 Chemical analysis of natural aquifer sands used in column experiments

aquifer sand			fine	medium				fine	medium
Method	Analyte	Unit			Method	Analyte	Unit		
WGHT	Wgt	KG	0.028	0.028	LF100	W	PPM	<0.5	0.7
XF700	SiO_2	%	92.73	88.84	LF100	Zr	PPM	222.7	67
XF700	Al_2O_3	%	2.87	3.91	LF100	Y	PPM	5.2	5.9
XF700	Fe_2O_3	%	0.63	0.88	LF100	La	PPM	6.8	8.5
XF700	CaO	%	0.385	1.61	LF100	Ce	PPM	12.75	15.3
XF700	MgO	%	0.145	0.38	LF100	Pr	PPM	1.54	1.84
XF700	Na_2O	%	0.57	0.82	LF100	Nd	PPM	5.95	7.1
XF700	K_2O	%	0.96	1.28	LF100	Sm	PPM	1.095	1.3
XF700	MnO	%	0.01	0.02	LF100	Eu	PPM	0.235	0.25
XF700	TiO_2	%	0.115	0.11	LF100	Gd	PPM	0.855	1.08
XF700	P_2O_5	%	0.02	0.03	LF100	Tb	PPM	0.15	0.18
XF700	Cr_2O_3	%	<0.01	<0.01	LF100	Dy	PPM	0.86	1.07
XF700	Ba	%	0.025	0.03	LF100	Ho	PPM	0.185	0.22
XF700	LOI	%	0.61	1.63	LF100	Er	PPM	0.605	0.7
XF700	SO_3	%	0.1005	0.057	LF100	Tm	PPM	0.06	0.07
XF700	Sr	%	<0.002	0.003	LF100	Yb	PPM	0.68	0.57
TC000	TOT/C	%	0.205	0.45	LF100	Lu	PPM	0.105	0.09
TC000	TOT/S	%	0.05	0.02	AQ200	Mo	PPM	0.4	0.2
LF100	Ba	PPM	176	244	AQ200	Cu	PPM	2.1	2.3
LF100	Be	PPM	1.5	<1	AQ200	Pb	PPM	1.7	4
LF100	Co	PPM	2.45	2.6	AQ200	Zn	PPM	5	11
LF100	Cs	PPM	0.8	1.4	AQ200	Ni	PPM	5.05	10.3
LF100	Ga	PPM	1.65	3.9	AQ200	As	PPM	1.5	1.8
LF100	Hf	PPM	5.4	1.9	AQ200	Cd	PPM	<0.1	<0.1
LF100	Nb	PPM	2.25	2.3	AQ200	Sb	PPM	<0.1	<0.1
LF100	Rb	PPM	29	43.3	AQ200	Bi	PPM	<0.1	<0.1
LF100	Sn	PPM	<1	<1	AQ200	Ag	PPM	<0.1	<0.1
LF100	Sr	PPM	47.1	85.5	AQ200	Au	PPB	<0.5	<0.5
LF100	Ta	PPM	0.2	0.2	AQ200	Hg	PPM	<0.01	<0.01
LF100	Th	PPM	1.7	2.4	AQ200	Tl	PPM	<0.1	<0.1
LF100	U	PPM	0.5	0.7	AQ200	Se	PPM	<0.5	<0.5
LF100	V	PPM	9.5	11	TC005	C/ORG	%	0.17	0.18

▼**Table S 3** Results of MATH hydrophobicity tests

		Escherichia coli WR1		*Dickeya solani*		*Ralstonia solanacearum*		*Pectobacterium carotovorum*	
OD600 before adding hexadecane)		0.439	% of bacteria that partitioned into hexadecane phase	0.247	% of bacteria that partitioned into hexadecane phase	0.171	% of bacteria that partitioned into hexadecane phase	0.493	% of bacteria that partitioned into hexadecane phase
OD600 after adding hexadecane	1	0.424	3.4	0.267	-8.1*	0.169	1.17	0.391	20.69
	2	0.434	1.1	0.24	2.8	0.187	-9.36*	0.48	2.64
	3	0.394	10.3	0.237	4.0	0.166	2.92	0.476	3.45
Hydrophobicity (%)			4.9		3.4		2.1		8.9

*negative values not considered in hydrophobicity calculation; OD600 = measured at 600 nm.

7

▼Table S 4 Comparison of 1-site and 2-site kinetic model fit using R^2 and AIC output parameter from Hydrus-1D

		1-site model		2-site model	
Quartz sand	repetition	R^2	AIC	R^2	AIC
Pectobacterium carotovorum	1	0.904	-231.70	0.919	-235.70
	2	0.712	-134.90	0.766	-135.00
Dickeya solani	1	0.796	-171.30	0.952	-209.20
	2	0.926	-229.90	0.946	-239.60
Ralstonia solanacearum	1	0.926	-168.30	0.989	-221.80
	2	0.523	-112.30	0.710	-135.20
Escherichia coli WR1	1	0.921	-210.60	0.922	-207.10
	2	0.904	-140.50	0.909	-139.70
medium aquifer sand					
anoxic					
Escherichia coli WR1	1	0.712	-186.40	0.906	-223.80
	2	0.275	-68.42	0.628	-99.17
	3	0.519	-182.90	0.837	-219.90
Ralstonia solanacearum	1	0.760	-129.20	0.896	-148.70
	2	0.926	-170.10	0.936	-170.10
	3	0.908	-164.50	0.930	-168.00
Pectobacterium carotovorum	1	0.752	-145.30	0.856	-159.70
	2	0.876	-171.40	0.882	-169.60
	3	0.853	-176.30	0.861	-173.90
oxygenated					
Pectobacterium carotovorum	1	0.962	-213.30	0.962	-209.30
	2	0.804	-155.10	0.813	-152.60
	3	0.685	-136.40	0.685	-132.40
fine aquifer sand					
Pectobacterium carotovorum					
anoxic	1	0.812	-101.40	0.817	-97.91
oxic	2	0.014	-92.86	0.213	-96.50

Table S 5 Total iron content using citrate dithionite extraction after Claff et al. (2010)**;**

	Sample*	replicate	Concentrations in mg L^{-1} Al	Ca	Fe	K	Mg	Mn	Sr	Zn	PO4	Si
Medium aquifer sand	1	a	1	258	20.5	0	2.5	1.5	1	0	3.5	1
sand stored under anoxic		b	1	331	21	0	3.5	1.5	1	0.5	3.5	1
conditions before extraction	2	a	1.5	295	23	0	3	1.5	1	0.5	3.5	1
		b	1	258	19.5	0.5	2.5	1.5	1	0.5	4	1
Fine aquifer sand	1	a	1	33	8	1	1	0	0	0.5	3.5	1
sand stored under anoxic		b	0.5	29	7.5	1.5	1	0	0	0.5	3.5	1
conditions before extraction	2	a	0.5	21	4.5	1	1	0	0	0.5	2.5	0.5
		b	1	29	7	2	1	0	0	0.5	3.5	1
Quartz sand	1	a	0	0	0	1	0	0	0	0.5	2.5	0
Acid-washed sand		b	0	0	0	1.5	0	0	0	0.5	3	0
“negative control"	2	a	0	0	0	2	0	0	0	0.5	2.5	0
		b	0	0	0	2	0	0	0	0.5	2.5	0

*from the collected aquifer sand, two samples were subsamples (1+2) were collected which were again divided in two replicates (a+b)

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Appendix C

The R source code and JAGS model can be found online at https://www.frontiersin.org/articles/10.3389/fpls.2022.1074192/full#supplementary-material.

▲ **Figure S1** Comparison of phenotypes of potato cultivars Kondor (left) and HB (right). Photo of cv Kondor was taken 74 days after planting and of cv HB 54 days after planting. One of the wooden sticks to support the plant is marked red in each graphic as the sticks had the same length and can be used as reference for length comparison. Cultivar HB grew longer than the wooden stick while cv Kondor has about the same length as the stick.

▼ **Table S 1** Weight of plant material collected from potato plants in greenhouse experiments used to re-isolate *Ralstonia solanacearum*.

Samples of different plant material for re-isolation of R. solanacearum, weights in [g]

	stem weight		root weight		number of tubers		Weight of all progeny tubers per plant		cut tuber weight (for re-isolation)		soil weight	
Dose	**Kondor**	**HB**	**Kondor**	**HB**	**Kondor**	**HB**	**Kondor**	**HB**	**Kondor**	**HB**	**Kondor**	**HB**
Control	12.5	8.8	10.0	6.0	11.0	2.2	183.2	48.1	2.9	0.8	2.8	5.7
$5x10^2$	12.3	7.9	9.9	4.6	9.1	2.3	188.6	72.4	2.5	0.8	2.8	6.6
$5x10^3$	12.9	8.5	9.9	5.2	11.0	2.3	190.7	66.7	2.9	0.8	2.8	6.9
$5x10^4$	9.5	8.2	10.0	7.2	8.8	2.2	171.3	65.0	2.6	0.9	2.9	7.4
$5x10^5$	14.1	8.0	11.4	9.0	9.8	2.3	182.5	63.0	2.6	0.9	2.8	6.3
$5x10^6$	10.7	8.0	9.7	9.3	9.6	2.4	183.8	49.5	2.8	0.7	3.0	6.3
$5x10^7$	11.9	9.7	8.8	7.4	9.5	2.6	180.4	56.3	2.8	0.9	3.1	6.7
$5x10^8$	6.8	6.3	10.6	6.7	6.4	0.9	214.2	59.6	1.9	0.8	3.2	6.5
$5x10^9$	8.1	5.4		5.5	3.3	1.0	43.8	34.2	0.5	1.3	3.6	6.4
total average	**11.0**	**7.9**	**10.1**	**6.8**	**8.7**	**2.0**	**170.9**	**57.2**	**2.4**	**0.9**	**3.0**	**6.5**

Appendix D

R code for QMRA to calculate infection risks with *Ralstonia solanacearum* after using ASTR treated irrigation water

```
library(openxlsx)
library(writexl) # to export R data frames to excel
library(gsl)
library(ggplot2)
library(ggtext) # add text to ggplot via geom_label or geom_text
#library(fitdistrplus) # to find out distributions of data
library(truncnorm)    # for z_mar and z_quartz, due to std.error also negativ
library(forcats)      # facet_grid, reversing order of groups in reference
library(scales)
library(ggpubr)
library(patchwork) # to save multiple plots in 1 file, better than ggsave
# function to produce 95% quantiles in boxplot
my_stats <- function(y) {
  r <- quantile(y, probs=c(0.05, 0.25, 0.5, 0.75, 0.95))
  names(r) <- c("ymin", "lower", "middle", "upper", "ymax")
  return(r)}
setwd("C:/.../...") #set working direction
mcnum <- 10000  #number of Monte Carlo simulations
```

INPUT parameters die-off

```
#source water concentration – see Figure 2 of QMRA chapter 5, NAK data (2018-2020)
# using the library(fitdistrplus), the source water concentration was analysed on how the
data is distributed, here you see already the results
mu_cs  <- 0.07932546
sig_cs <- 1.24857498
mc_cs  <- rlnorm(n=mcnum, meanlog = mu_cs, sdlog = sig_cs) #log-normal distribution
quantile(mc_cs, c(0.05, 0.5, 0.95)) # to check MC sample
# recovery efficiency
mu_sens  <- 0.9086002
sig_sens <- 2.4/100
mc_sens  <- rnorm(n=mcnum, mean = mu_sens, sd = sig_sens) #normal distribution

# DIE-OFF in water phase parameters
#alpha
```

```
mu_a   <- 0.0464 # parameter estimate of alpha
sig_a  <- 0.0018 # std. error of parameter estimate alpha
mc_a   <- rnorm (n=mcnum, mean = mu_a, sd = sig_a)
# beta
mu_b   <- 6.6696 # parameter estimate of beta
sig_b  <- 1.9837 # std. error of parameter estimate beta
mc_b   <- rtruncnorm (n=mcnum, a = 0, b = Inf, mean = mu_b, sd = sig_b)
# Cres
mu_cres   <- 33
sig_cres  <- 1.21
mc_cres   <- rnorm(n=mcnum, mean = mu_cres, sd = sig_cres)
#C0
mu_c0     <- 15900
sig_c0    <- 1
mc_c0   <- rnorm(n=mcnum, mean = mu_c0, sd = sig_c0)
```


INPUT parameter ATTACHMENT

```
# QUARTZ sand katt – repetition 1
mu_katt_qu1    <- 0.0041 # parameter estimate of katt1
sig_katt_qu1   <- 0.0146 # std. error of parameter estimate katt1
mc_katt_qu1 <- rtruncnorm (n = mcnum,a = 0,b = Inf,mean = mu_katt_qu1,sd = sig_katt_qu1)
# katt(quartz) from repetition 2
mu_katt_qu2    <- 0.00065394 # parameter estimate of katt1
sig_katt_qu2   <- 0.00069729 # std. error of parameter estimate katt1
# use rtruncnorm() to avoid negative results when drawing the MC sample
mc_katt_qu2 <- rtruncnorm (n = mcnum,a = 0,b = 1,mean = mu_katt_qu2,sd = sig_katt_qu2)
# combine datasets and draw a sample of n = mcnum
mc_katt_qu <- c(mc_katt_qu1, mc_katt_qu2)
mc_katt_qu <- sample(mc_katt_qu, size = mcnum)
#alpha(L) = longitudinal dispersivity unit [cm]
mu_al_qu  <-  0.038
sig_al_qu <-  8.31*10^-3
mc_al_qu  <-  rtruncnorm(n=mcnum,a=0, b=Inf, mean = mu_al_qu, sd = sig_al_qu)
# aquifer sand - katt unit [1/min]
mu_katt_mar1   <- 0.11166 # parameter estimate of katt1 [1/min]
sig_katt_mar1  <- 0.0058658 # std. error of parameter estimate katt
mc_katt_mar1 <- rtruncnorm (n=mcnum, a=0, b=1, mean = mu_katt_mar1, sd = sig_katt_mar1)
# repetition 2
mu_katt_mar2    <- 0.14415 # parameter estimate of katt1 [1/min]
sig_katt_mar2   <- 0.0027505 # std. error of parameter estimate katt
mc_katt_mar2 <- rtruncnorm (n=mcnum, a=0, b=1, mean = mu_katt_mar2, sd = sig_katt_mar2)
```

```
# repetition 3
mu_katt_mar3    <- 0.1074 # parameter estimate of katt1 [1/min]
sig_katt_mar3   <- 0.0111 # std. error of parameter estimate katt
mc_katt_mar3 <- rtruncnorm (n=mcnum, a=0, b=1, mean = mu_katt_mar3, sd = sig_katt_mar3)
# combine datasets and draw a sample of n = mcnum
mc_katt_mar <- c(mc_katt_mar1, mc_katt_mar2, mc_katt_mar3)
mc_katt_mar <- sample(mc_katt_mar, size = mcnum)
#alpha(L) [cm] - aquifer sand - point estimate
mu_al_mar  <-  0.28
sig_al_mar <-  0.41
mc_al_mar  <-  rtruncnorm(n=mcnum,a=0, b=Inf, mean = mu_al_mar, sd = sig_al_mar)
```


INPUT dose-response

```
# DOSE RESPONSE
# read file with alpha and beta values for infection
dr<- read.xlsx("DR.xlsx", sheet=1, startRow = 1, check.names=T )
set.seed(12345)
dr <- sample_n(dr, 10000)
# Dose response parameters
fun_dr <- function(dr) c(rbeta(n = mcdata, shape1 = dr[1], shape2 = dr[2]))
# Hypergeometrice function pinf: infection risk per plant)
fun_pinf <- function(dosis, dr) {
  1 - hyperg_1F1(dr[, 1], dr[, 1] + dr[, 2], -dosis)}
# IRRIGATION volume per 1 plant
irrig <- 176 # unit in [mm] amount given during one full cropping season
vol_irr <- irrig*1000/5 # *1000 to get [mL], /5 to calculate per plant (5 plants per 1 m2 = 50000
plants/hectare)
```

Probability of detection

Current testing scheme after tuber harvesting: 200 tubers per 25 ton tuber lot
What is the probability to detect at least one infected tuber sampling 200 random tubers given the calculated infection risks?

$$P_d = 1 - \prod_{i=1}^{n=200} (1 - P_i)$$

```
# write formula as a function
fun_pinf_test <- function( risk_per_plant, mcnum, number_of_tested_tubers){
  replicate(
```

```
    n = mcnum,
    expr = 1 - prod(
     1 - risk_per_plant[
       sample(
         x = mcnum,
         size = number_of_tested_tubers,
         replace = FALSE ), ],
     na.rm = TRUE   )  ) }
```

Baseline risk

= Brown rot infections are still observed in harvested tubers although all hygiene measures are followed

```
# read data and mutate them to treat them as factors
bl<- read.xlsx("baseline.xlsx", sheet=1, startRow = 1, check.names=T )
bl <- bl %>%
  mutate(
    year = year,
    tested = as.factor(tested),
    number = number,
    type = as.factor(type) )
# plot data and remove data with 0 counts
bl %>% filter(number > 0) %>%
 ggplot( aes(x=year, y=number, color= tested))+
   geom_point()+
   facet_grid(.~type)+
   scale_y_log10(breaks = trans_breaks("log10", function(x) 10^x),
              labels = trans_format("log10", math_format(10^.x)),   )+
   scale_x_continuous(breaks = seq(0,2016, by= 5)  )+
   labs(title = expression(paste("Surveys for ",italic("Ralstonia solanacearum"), "since 1995 on
the domestic potato production") ),
       y = "Numbers of tested potatoes",
       fill = "Potatoes tested") +
  theme(plot.title = element_text(size = 0.5) )+
  theme_bw()+
  theme(legend.position="bottom",
        axis.title = element_text(size=8),
        axis.text  = element_text(size = 11),
        strip.text  = element_text(size = 12) )
```

Velocity

```
 v <- 0.246  # [cm/min] 3.5 m/day - column experiments
```

Infection risk of untreated source water

Risk if source water (surface water) is not stored and treated via MAR and directly used to irrigate (=pinf_1, remains the same in all scenarios)

```
# scenario 1 - surface water used for irrigation - no treatment
mcdata <- data.frame(
  mc_cs,
  mc_sens,
  vol_irr )
dosis    <- with( mcdata, mc_cs / mc_sens * vol_irr)
#####
pinf_1   <- fun_pinf(dosis = dosis, dr = dr)
pinf_1[pinf_1 =="NA"] <- 0
```


Plot 1, as example; all other plots with different [t] and [x] values are done in the same way

```
t <- 10     # [days]
x <- 23   # [cm]
ratio  <- mc_cres/mc_c0
eterm  <- exp( -mc_a * t)^mc_b
# z_dieoff  - with Weibull + tail model
z_dieoff <- eterm - ratio * eterm + ratio
z_quartz <- exp((x*(1- (1+4*mc_al_qu * mc_katt_qu/v)^(1/2) )/(2* mc_al_qu)))
z_mar    <- exp((x*(1- (1+4*mc_al_mar *mc_katt_mar/v)^(1/2))/(2* mc_al_mar)))
# Scenarios 2-6; different operations of the ASTR system
# Calculate infection risks of all scenarios
# 2  - die-off in the water phase
mcdata <- data.frame(
  mc_cs,
  mc_sens,
  z_dieoff,
  vol_irr )
dosis    <- with( mcdata, mc_cs / mc_sens * z_dieoff * vol_irr)
pinf_2   <- fun_pinf(dosis = dosis, dr = dr)
# 3 – attachment to quartz
mcdata <- data.frame(
  mc_cs,
  mc_sens,
  z_quartz,
  vol_irr )
dosis  <- with( mcdata, mc_cs / mc_sens * z_quartz * vol_irr)
pinf_3 <- fun_pinf(dosis = dosis, dr = dr)
```

```
# 4 - die-off in the water phase and attachment to quartz
mcdata <- data.frame(
  mc_cs,
  mc_sens,
  z_dieoff,
  z_quartz,
  vol_irr )
dosis  <- with( mcdata, mc_cs / mc_sens * z_dieoff * z_quartz * vol_irr)
pinf_4 <- fun_pinf(dosis = dosis, dr = dr)
# 5 - attachment to aquifer sand
mcdata <- data.frame(
  mc_cs,
  mc_sens,
  z_mar,
  vol_irr )
dosis  <- with( mcdata, mc_cs / mc_sens * z_mar * vol_irr)
pinf_5 <- fun_pinf(dosis = dosis, dr = dr)
# 6  - die-off in the water phase and attachment to aquifer
mcdata <- data.frame(
  mc_cs,
  mc_sens,
  z_dieoff,
  z_mar,
  vol_irr )
dosis  <- with( mcdata, mc_cs / mc_sens * z_dieoff * z_mar * vol_irr)
pinf_6 <- fun_pinf(dosis = dosis, dr = dr)
# replace NaN values with 0
pinf_1[is.nan(pinf_1)] <- 0
#
z_dieoff_log10 <- log10(z_dieoff)*-1
z_quartz_log10 <- log10(z_quartz)*-1
z_mar_log10 <- log10(z_mar)*-1

## create data frame; to add mean value on the boxplot
stats_pinf <-   data.frame(
  names = c ("1", "2", "3", "4", "5", "6"),
  mean = c( mean(pinf_1), mean(pinf_2), mean(pinf_3),   mean(pinf_4),
          mean(pinf_5), mean(pinf_6) ),
  quantile_5 =
c( quantile(pinf_1, c(0.05), na.rm=TRUE), quantile(pinf_2, c(0.05)),
   quantile(pinf_3, c(0.05), na.rm = T), quantile(pinf_4, c(0.05)),
```

```
 quantile(pinf_5, c(0.05)), quantile(pinf_6, c(0.05))),
quantile_50 = c( quantile(pinf_1, c(0.5), na.rm=TRUE), quantile(pinf_2, c(0.5)),
 quantile(pinf_3, c(0.5), na.rm = T),quantile(pinf_4, c(0.5)),
 quantile(pinf_5, c(0.5)),quantile(pinf_6, c(0.5)) ),
quantile_95 = c(  quantile(pinf_1, c(0.95), na.rm=TRUE), quantile(pinf_2, c(0.95)),
  quantile(pinf_3, c(0.95), na.rm = T), quantile(pinf_4, c(0.95)),
  quantile(pinf_5, c(0.95)), quantile(pinf_6, c(0.95)) ) )
# data frame of all riks distributions from the 6 scenarios
risk <- data.frame(
 scenario = as.factor (rep(c ("1", "2", "3", "4", "5", "6"), each =mcnum)),
 pinf = c(pinf_1, pinf_2, pinf_3, pinf_4, pinf_5, pinf_6))
#plot – text to be added on the plot
ann_text <- data.frame(x="1", y=10^-7.6, label= paste("t  =", t, "days \nx = ",x, "cm") )

plot1<-   ggplot() +
 stat_summary(data = risk, fun.data = my_stats,
        mapping = aes(  x = scenario, y = pinf, fill = scenario ),
        geom = "boxplot")+
 scale_fill_brewer(palette = "Set2",
        labels = c("1 = untreated source water",
              "2 = water die off",
              "3 = attachment to quartz sand",
              "4 = water die-off + attachment to quartz sand",
              "5 = attachment to aquifer sand",
              "6 = water die-off + attachment to aquifer sand")) +
 labs(y= "Infection risk of one potato plant" , x = " ")+
 theme_bw()+
 geom_point(data= stats_pinf, mapping =aes(x= names, y = mean), size=2,  color = "red")+
 scale_y_log10(breaks = trans_breaks("log10", function(x) 10^x),
        labels = trans_format("log10", math_format(10^.x)),
        limits = c(10^-8, 10^-1) )+
 theme(legend.position="none",
              axis.title   = element_text(size=14),
              axis.text    = element_text(size = 11),
              strip.text   = element_text(size = 16) )+
  geom_label(data = ann_text, aes( x=x, y=y, label=label),
        color="black", size=4,
        hjust = 0,     # to left align text
        nudge_x = -1, # to position added text
        label.padding = unit(0.4, "lines"),
        label.r = unit(0, "lines"))
```

Upscaling infection risk

```
#plot1
ha <- 5 # define size of field in hectare – 50000 plants/hectare
n_pl <- 50000*ha # plants on a x-ha field
n_pl_A <- quantile(pinf_1, c(0.95)) * n_pl
n_pl_B <- quantile(pinf_2, c(0.95)) * n_pl
n_pl_C <- quantile(pinf_3, c(0.95), na.rm=T) * n_pl
n_pl_D <- quantile(pinf_4, c(0.95)) * n_pl
n_pl_E <- quantile(pinf_5, c(0.95)) * n_pl
n_pl_F <- quantile(pinf_6, c(0.95)) * n_pl
base_risk <- bl_risk_seed * n_pl

pl_inf <- data.frame(
  scenario = as.factor(c("A","B","C", "D", "E", "F", "baseline_risk") ),
  infected_plants = c(n_pl_A, n_pl_B, n_pl_C, n_pl_D, n_pl_E, n_pl_F, base_risk) )
# safe date frame as excel table
write_xlsx(pl_inf, C:\\Users\\...\\data.xlsx')
# for plotting, create data frame

pinf_bl_df <- data.frame(bl_risk_seed) # use baseline risk to calculate prop. of detection
bl_tuber <-  fun_pinf_test(
   risk_per_plant = pinf_bl_df,
   mcnum = mcnum,
   number_of_tested_tubers = 200  )
pinf_1_df <- data.frame(pinf_1)
1_tuber <-  fun_pinf_test(
   risk_per_plant = pinf_1_df,
   mcnum = mcnum,
   number_of_tested_tubers = 200  )
pinf_2 <- data.frame(pinf_2)
2_tuber <-  fun_pinf_test(
   risk_per_plant = pinf_2,
   mcnum = mcnum,
   number_of_tested_tubers = 200  )
pinf_3<- data.frame(pinf_3)
3_tuber <-  fun_pinf_test(
   risk_per_plant = pinf_3,
   mcnum = mcnum,
   number_of_tested_tubers = 200  )
pinf_4 <- data.frame(pinf_4)
4_tuber <-  fun_pinf_test(
```

```
    risk_per_plant = pinf_4,
    mcnum = mcnum,
    number_of_tested_tubers = 200  )
pinf_5 <- data.frame(pinf_5)
5_tuber <-  fun_pinf_test(
    risk_per_plant = pinf_5,
    mcnum = mcnum,
    number_of_tested_tubers = 200  )
pinf_6 <- data.frame(pinf_6)
6_tuber <-  fun_pinf_test(
    risk_per_plant = pinf_6,
    mcnum = mcnum,
    number_of_tested_tubers = 200  )
tuber_risk <- data.frame(
  scenario = as.factor (rep(c ("1", "2", "3", "4", "5", "6"), each =mcnum)),
  pinf = c(1_tuber, 2_tuber, 3_tuber, 4_tuber, 5_tuber, 6_tuber) )

ann_text <- data.frame(
  x="1", y=10^-4.7,
  label= paste("t  =", t, "days \nx = ",x, "cm"))

mean_bl_detect <- mean(bl_tuber)

tuber_plot1 <- ggplot() +
  stat_summary(data = tuber_risk, fun.data = my_stats,
             mapping = aes(  x = scenario, y = pinf, fill = scenario ),
             geom = "boxplot")+
  scale_fill_brewer(palette = "Set2",
             labels = c("1 = untreated source water",
                    "2 = water die off",
                    "3 = attachment to quartz sand",
                    "4 = water die-off + attachment to quartz sand",
                    "5 = attachment to aquifer sand",
                    "6 = water die-off + attachment to aquifer sand")) +
  labs(y= "Probability to detect at least infected out of 200 tubers" , x = " ")+
  theme_bw()+
  scale_y_log10(breaks = breaks_log(n=5),
             labels = trans_format("log10", math_format(10^.x)),
             limits = c(10^-5, 1)  )+
  theme(legend.position="none",
             axis.title   = element_text(size=13),
```

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```
            axis.text    = element_text(size = 11),
            strip.text   = element_text(size = 16)   )+
  geom_label(data = ann_text, aes( x=x, y=y, label=label),
           color="black", size=4,
           hjust = 0,     # to left align text
           nudge_x = -1,
           label.padding = unit(0.4, "lines"),
           label.r = unit(0, "lines"))+ # remove round edges
  geom_hline(yintercept= mean_bl_detect, linetype="dashed", color= "purple")
Sensitivity analysis
a <- var(log(mc_cs)    )   / var(log(pinf_4))
b <- var(log(mc_sens)  )  / var(log(pinf_4))
c <- var(log(z_dieoff) )  / var(log(pinf_4))
d <- var(log(z_quartz) ) / var(log(pinf_4))

e <- var(log(mc_cs) )      / var(log(pinf_6))
f <- var(log(mc_sens))    / var(log(pinf_6))
g <- var(log(z_dieoff))  / var(log(pinf_6))
h <- var(log(z_mar))    / var(log(pinf_6))
#make a data frame of all ratios a-h – for plotting
var <- data.frame(
 name  = as.factor( c("1","2","3", "4", "1", "2", "3", "4") ),
 value = c(a, b, c, d, e, f, g, h),
 treat = as.factor (rep(c("die-off + quartz sand", "die-off + aquifer sand"), each = 4) ))

ann_text <- data.frame(
   x="1", y=0.25, label= paste("t  =", t, "days \nx = ",x, "cm"))

sens1 <- ggplot()+
  geom_bar(data = var, aes (x= name, y= value, fill = name), stat = "identity")+
  coord_flip()+
  scale_fill_brewer(palette = "Set2",
         labels = c("1 = source water concentration",
                   "2 = recovery efficiency",
                   "3 = removal by water die-off
                   "4 = removal by attachment to aquifer sand"))+
  labs(title = " ", x = " ", y = " (variance of parameter)/(variance of infection risk)",
      fill = "Parameter")+
  theme_bw()+
  facet_grid(.~treat,  scales = "free_x")+
  geom_label(data = ann_text, aes( x=x, y=y, label=label),
```

```
      color="black", size=4,
      hjust = 0,     # to left align text
      label.padding = unit(0.4, "lines"),
      label.r = unit(0, "lines") )

# combine the different plots and save as TIFF or PDF
plot1 + plot2 + plot3 + plot4 + plot5 + plot6+
  plot_layout(guides = 'collect') + plot_annotation(tag_levels = 'A')
# Probability of detecting an infected tuber in a 200 tuber sample
tuber_plot1 + tuber_plot2 + tuber_plot3 + tuber_plot4 + tuber_plot5 +
  plot_layout(guides = 'collect') + plot_annotation(tag_levels = 'A')
# sensitivity analysis
sens1+sens2 + sens3+plot_layout(guides = 'collect', ncol = 2, nrow = 2) +
  plot_annotation(tag_levels = 'A')
```

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To my family, thank you for your constant encouragement, love, and understanding. Your unwavering support and motivation have sustained me through the ups and downs of this challenging journey, and I am truly grateful for your presence in my life. The calls with Amilia surely brightened up my day. Lastly, thanks to my partner Rafael who 'brought' me to the Netherlands and always supports me and believed in me, whatever I do.

Curriculum Vitae

Carina Eisfeld was born on May 20 in 1990 in Traunstein (Germany) and grew up in Kössen (Austria), a small village in the beginning of the Tirolean Alps. She completed her high-school diploma in 2009 at the 'Staatliches Landschulheim Marquartstein', Germany. Afterwards, she started her first work experience abroad. She lived with a family in France and took care of their two children, while getting to know a foreign culture and language. This period not only allowed her to grow personally adapting to the new environment but also gave her time to formulate her study choice. Growing up in the mountains she naturally developed her love for nature and passion for the protection of our environment. Therefore, Carina began a Bachelor of Science in Biology at the Ludwig-Maximilians Universität in München which she successfully completed in 2013. During her studies she worked as a research assistant at the genetics department where she gained a lot of hands-on working experience in the laboratory when analysing the influence of arbuscular mycorrhizal fungi on plant growth. After her bachelor, Carina started her master studies of biology at the Technische Universität München. She focussed on the topics of ecology, plant sciences and microbiology and wrote her master thesis in the field of water remediation after oil spills. There, she analysed the degradation of methylnaphthalene by anaerobic bacteria. Before obtaining her Master degree, Carina went on a five months Erasmus+ research internship at the Bioengineering department of i3S, University of Porto. She studied the production of exopolysaccharides in the cyanobacterium *Synechocystis* by studying genetic mutants that different in their production of a certain protein and its effect on the bacteria's metabolism. In August 2016, Carina obtained her diploma as Master of Science in Biology and decided to move to the Netherlands where she first started a position as a sales assistant. She continued on searching for a job in her field and got the opportunity on doing a PhD in the Department of Water Management at TU Delft. From July 2017 until begin 2023 she conducted her research on the removal of plant pathogenic bacteria during managed aquifer recharge to provide irrigation water in agriculture. Using her skills and knowledge as environmental microbiologists she studied the conditions within an aquifer which mostly influence pathogen removal and improve water quality. In June 2023, she will start a new position as Researcher Microbial Ecology at Normec Groen Agro Control where she will support applied research projects to improve plant protection.

List of publications

Eisfeld, C., van Breukelen, B.M., Medema, G., van der Wolf, J.M., Velstra, J. and Schijven, J.F. (2023), QMRA of *Ralstonia solanacearum* in potato cultivation: risks associated with irrigation water recycled through managed aquifer recharge, *Science of the Total Environment (accepted with minor/moderate revisions)*

Eisfeld, C., Schijven, J.F., Kastelein, P., van Breukelen, B.M., Medema, G., Velstra, J., Teunis, P.F.M. and van der Wolf, J.M. (2022a). Dose-response relationship of *Ralstonia solanacearum* and potato in greenhouse and *in vitro* experiments. *Frontiers in Plant Science* 13. doi: 10.3389/fpls.2022.1074192.

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