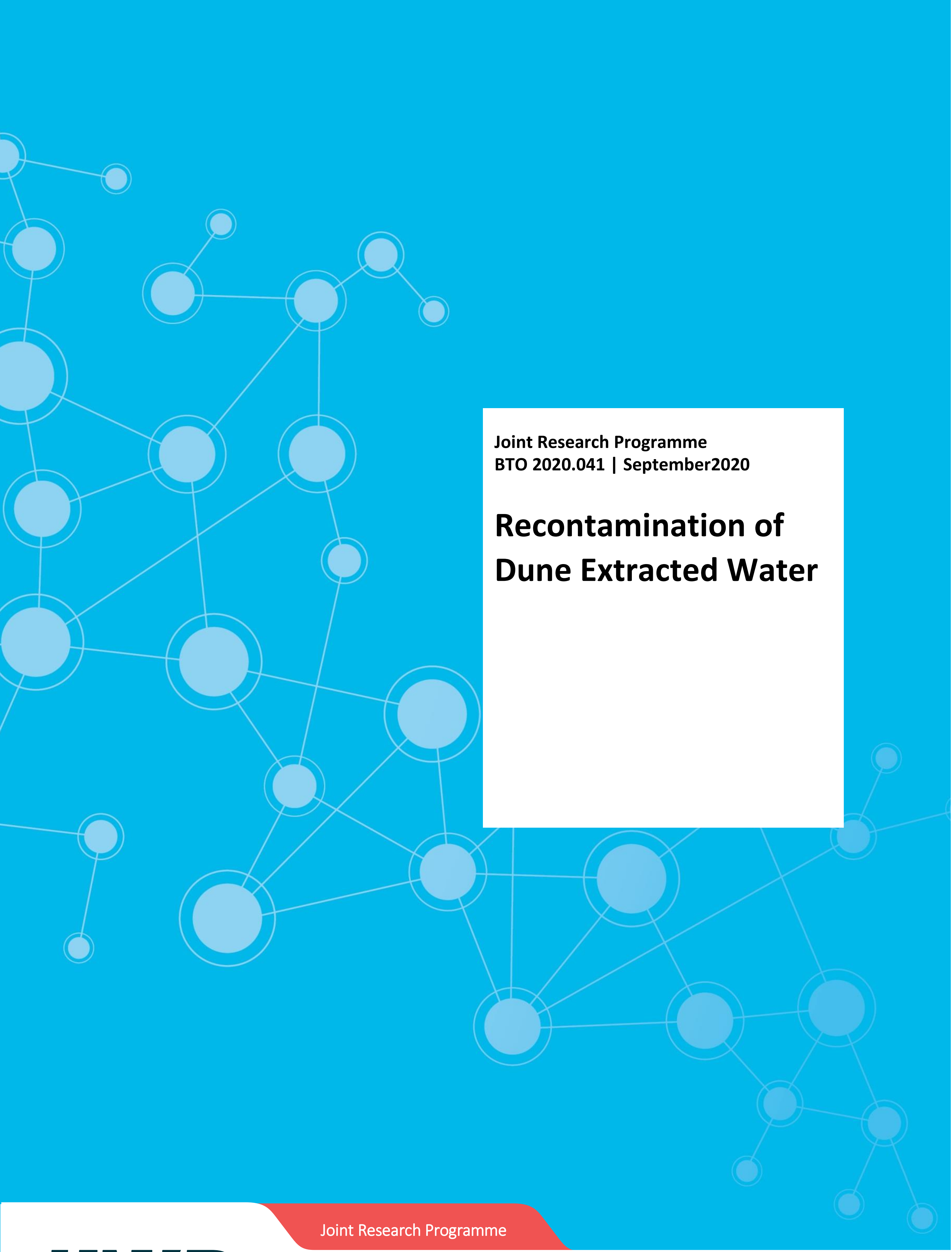

Joint Research Programme
BTO 2020.041 | September2020

Recontamination of Dune Extracted Water

Joint Research Programme

KWR

Bridging Science to Practice

Report

Recontamination Of Dune Extracted Water

BTO 2020.041 | September 2020

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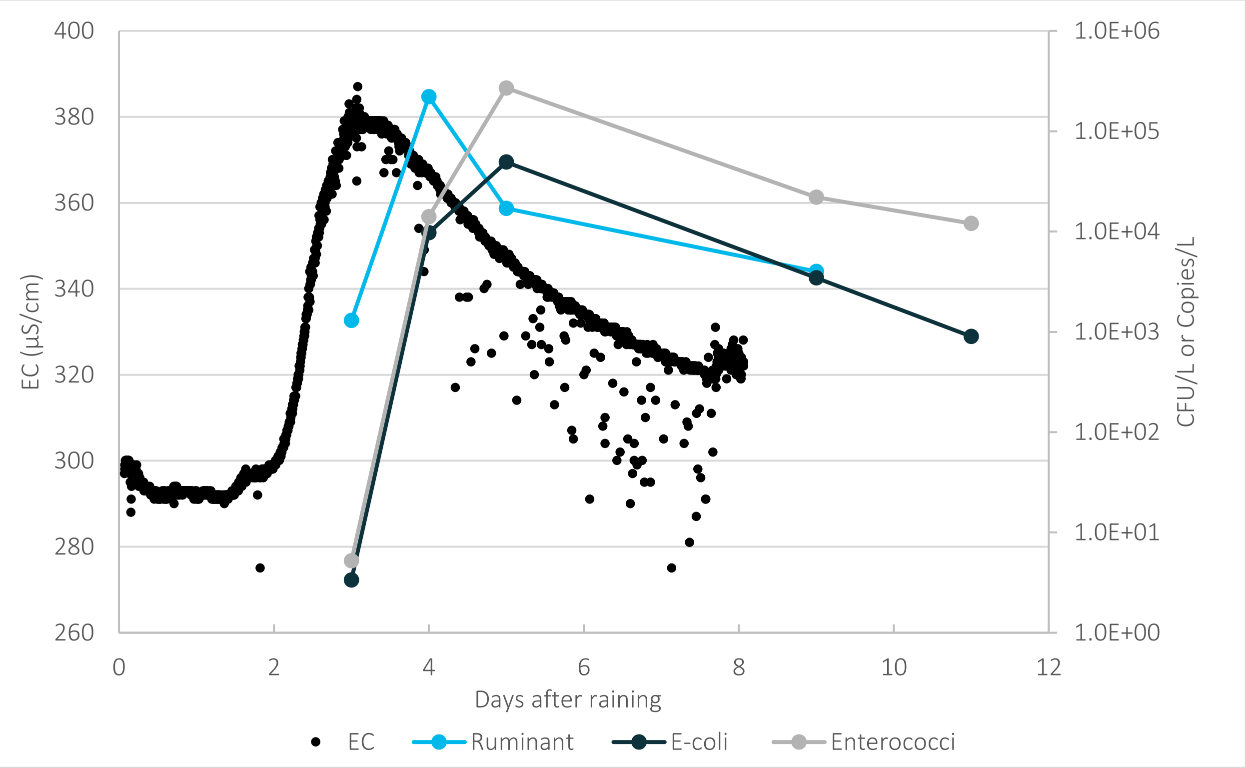
Managementsamenvatting

Dieren vormen een mogelijke bron voor herbesmetting van duinwater bij onttrekkingsputten

Auteur(s) Alex Hockin MSc, dr.ir. Gijsbert Cirkel, dr. Luc Hornstra

Duinpassage is een belangrijke barrière tegen pathogene micro-organismen. In het onttrokken water van de duinen worden echter af en toe fecale indicatorbacteriën (FIB) aangetroffen. De herkomst van deze bacteriën is niet duidelijk, hoewel de veronderstelling is dat deze bacteriën de verzadigde zone vanaf het maaiveld bereiken. Met dit onderzoek is geprobeerd een direct verband te leggen tussen bacteriën in feces op maaiveldniveau en de mogelijke detectie van deze bacteriën in het water in de put, en om vervolgens te bepalen van welke diergroepen deze bacteriën afkomstig zijn. Dit onderzoek toont aan dat uitwerpselen van dieren, zoals grazers een mogelijke bron vormen voor herbesmetting van duinwater bij onttrekkingsputten van Dunea.



Doorbraakcurves van de zouttracer (EC), E. coli, Enterococcen en DNA-markers van herkauwers (Ruminant) uitgezet tegen het aantal dagen na het begin van de beregening. Metingen afkomstig van een testopstelling waarbij na een zouttracerproef gebiedseigen dierlijke fecaliën rondom de put zijn gedeponeerd en beregend. E. coli en Enterococcen zijn weergegeven als CFU/l en de Ruminant source tracking marker als het aantal kopieën per liter.

Belang: herkomst herbesmetting duinwater bij onttrekkingsputten achterhalen

Bij het terugwinnen van water na duinpassage treft Dunea incidenteel indicatoren voor fecale verontreiniging aan. Dit kan het gevolg zijn van herbesmetting in en/of nabij de onttrekkingsputten. Uit eerder onderzoek blijkt dat terreininvloeden zoals hellingen, vegetatie, heterogeniteit en waterafstotendheid van de bodem een rol kunnen spelen bij kortsluitstromen, wat tot een versneld transport van microorganismen vanaf maaiveld naar het grondwater leidt. Daarnaast is aangetoond dat belangrijke Fecale Indicator Bacteriën ofwel FIB (E. coli en Enterococcen) lang kunnen overleven in de onverzadigde zone. Deze indicatoren kunnen door regenval worden geremobiliseerd en getransporteerd naar het grondwater en uiteindelijk het onttrokken water. Dit betekent dat het microbiologisch veilige water dat door de duinpassage wordt gezuiverd, van bovenaf, weer wordt verontreinigd met besmet infiltrerend regenwater. In dit project is ter verificatie onderzocht of uitwerpselen van dieren een waarschijnlijke bron kunnen zijn van deze herbesmetting.

Aanpak: FIB en DNA-analyses om effect dierlijke uitwerpselen te meten

Op een kwetsbare plek in de duinen is een put en een peilbuis gedurende vijf maanden met groot volume op FIB gecontroleerd. Daarnaast zijn in een aanvullend veldexperiment uitwerpselen van dieren die in het duingebied voorkomen (koeien, paarden, schapen en vossen) in de buurt van een ondiepe peilbuis gedeponeerd. Vervolgens zijn zware regenbuien gesimuleerd, zodat bacteriën en DNA-markers vanuit de uitwerpselen uitspoelen en in de bodem infiltreren. De watermonsters van het tweede experiment, genomen in de ondiepe peilbuis, zijn onderzocht op FIB en door middel van sourcetracking met Q-PCR onderzocht op de aanwezigheid van fecale DNA-markers van hond, mens, herkauwer, rund, paard, vogel en varken.

Resultaten: FIB uit uitwerpselen gevonden in onttrokken water

En lage concentratie Enterococcen is aangetroffen in één van de vijf monsters uit de peilbuis terwijl, E. coli is niet aangetroffen in de monsters uit de putten in het kwetsbare gebied.

Bij de peilbuis waar dierlijke uitwerpselen waren neergelegd, werd drie dagen na de start van de beregening een piekconcentratie van zout gemeten, met een transportsnelheid van 0,39 m/dag. De piekconcentraties van E. coli en Enterococcen (respectievelijk 2,7x105 en 4,92x104 CFU/L) traden vijf dagen na het begin van de beregening op. Alleen van herkauwers werd DNA kopieën in de watermonsters aangetoond, met een piekconcentratie van 2,2x105 kopieën/l na vier dagen beregenen.

Implementatie: uitsluiten van dieren in kwetsbare locaties voor microbiële veiligheid

Dit onderzoek toont aan dat op kwetsbare plaatsen in de duinen FIB detecteerbaar zijn in het ondiepe grondwater. Daarnaast is aangetoont dat uitloging uit mest en infiltratie en transport door de onverzadigde zone naar het grondwater relatief snel kan gaan. Dit kan leiden tot herbesmetting van infiltratiewater aan het einde van de duinpassage. Het is belangrijk op te merken dat de hier gepresenteerde resultaten afkomstig zijn uit een ‘worst-case scenario’ en het is onwaarschijnlijk dat extractieputten in de praktijk onderhevig zullen zijn aan de hoge fecale en regenbelasting zoals in de experimenten. Bovendien duiden FIB alleen op fecale besmetting, maar vormen ze zelf wel een risico voor de menselijke gezondheid. Het is echter belangrijk om alle fecale verontreiniging in de duingebieden te voorkomen. Fecale verontreiniging in de nabijheid van putten vormt dan ook een risico, en het advies is om uit voorzorg grazers, zoals paarden, koeien en schapen, te weren bij de onttrekkingsputten en aangrenzende hellingen.

Rapport

Dit onderzoek is gerapporteerd in een rapport Recontamination Of Dune Extracted Water (BTO-BTO 2020.041).Belang: Microbiële verontreiniging van het duinwater

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Summary

Dune passage in an important barrier against pathogenic microorganisms. However, faecal indicator bacteria (FIB) are occasionally found in the extracted water from the dunes. The origin of these bacteria is not clear, though the assumption is that these bacteria reach the saturated zone from ground level. In this research we investigated whether faecal deposition at ground level is the cause of the FIB occasionally found in extracted water from Dunea. Water samples at the wells are examined for FIB and by source tracking with faecal markers from specific animal groups. The aim was to establish a direct link to bacteria at ground level in this way and the possible detection of these bacteria in the water at the well, and to determine from which animal groups these bacteria originate. Wells were monitored from a vulnerable location in the dunes for FIB over a 5 month period. Low concentration of Enterococci were found in a monitoring well in January.

In addition, a second field experiment was conducted, where faeces from dune animals (cow, horse, sheep and fox) was brought to a shallow monitoring well and a heavy rain event was simulated. The peak concentration of salt was measured 3 days after the start of raining, with an infiltration (vertical) velocity of 0.39 m/day. Based on previous experiments with approximately the same rainfall intensity and duration, the infiltrating velocity is likely homogenous and not preferential flow. The peak concentrations of *E. coli* and Enterococci ($2.7x10^5$ and $4.92x10^4$ CFU/L respectively) were measured five days after the start of raining. Ruminants were the only positively measured DNA marker in the water samples, with the peak concentration ($2.2x10^5$ copies/L) measured after 4 days of raining. This research clearly demonstrates that FIB can be detected in vulnerable locations within the dunes and that FIB from animal faeces at the ground surface can be transported to groundwater relatively quickly and result in the recontamination of purified dune water close to the wells.

It is important to note that the results presented here are from a worst-case scenario and it is unlikely that extraction wells in practice would be subject to the high faecal and rain loads as in the experiments. Furthermore, the FIB measured in and of themselves do not present a risk for human health, as they are only indicators of faecal contamination. The highest risk to human health remains to be human faecal contamination in the dunes, for example from the defecation of visitors to the dune areas in or close to the well fields. As a precautionary measure though, all faecal contamination should be prevented. Our results support the recommendation that grazing animals, including horses, cows and sheep, be excluded from the vicinity of extraction wells and adjacent slopes as a precautionary measure to prevent any risk from recontamination of the extracted water in the dune area.

Contents

1 Introduction

Dune passage is an important barrier against pathogenic microorganisms. However, faecal indicator bacteria (FIB) are occasionally found in the extracted water from the dunes. The origin of these bacteria is not clear, though the assumption is that these bacteria reach the saturated zone from ground level. Previous research has shown that terrain influences such as slopes, vegetation and heterogeneities in the soil can play a role in short-circuit flows (Cirkel et al., 2019; Hornstra & Cirkel, 2018). Research at Dunea (2018) showed that (rain) water runoff from (hydrophobic) dune slopes can infiltrate more quickly near the well row and may be a cause of rapid transport to groundwater. The microbiologically safe water purified by the dune passage is contaminated again in the last phase, close to the well. Furthermore, Hornstra and Cirkel (2018) found that though *Escherichia coli* and *Enterococcus moraviensis* die-off in the dune sand, the process is very slow. As a result, bacteria that are transported to the unsaturated can survive for months at a time. The unsaturated zone therefore becomes a reservoir for these bacteria, which may be remobilized and transported to extracted water, a potential cause for the occasional detection of FIB in dune abstracted water.

In this research we investigated whether it was possible to substantiate the assumption that faecal deposition at ground level is the cause of the FIB occasionally found in extracted water from Dunea. This was done by two separate experiments. In the first experiment, water from the top layer of groundwater in a vulnerable infiltration areas was examined monthly for FIB. While it is known that FIB can occasionally be found in extracted water from the dunes, those results are from composite water samples from different depths. The hypothesis tested in the first experiment is that FIB travel through the unsaturated zone and are most likely to be found on the surface of the groundwater. . Vulnerable areas in the dunes are characterized by a) limited thickness of the unsaturated zone (often <1 m) and where seasonal changes in groundwater levels can occasionally result in the groundwater level being above the ground surface, b) terrain influences described above, such as slopes and vegetation resulting in pooling of run-off water around the extraction wells and c) the presence of domesticated and wild animals from the dunes which can defecate in close proximity to the extraction wells.

In the second set of experiments, a monitoring well was installed well away from drinking water wells. Faeces was collected from animals living in the dune area and the faeces was spread on top of a 1m^2 soil layer, with the monitoring well located in the centre. Subsequent initiated rain events allows the transport of bacteria from the faeces through the unsaturated zone to the saturated zone. This experiment simulates a worst case scenario, with the highest change of finding bacteria in the abstracted water. The aim of both experiments was to establish a direct link to bacteria in faeces at ground level in this way and the possible detection of these bacteria in the water at the well, and to determine from which animal groups these bacteria originate. When bacteria are found, it is shown that faecal droppings are a possible source of faecal bacteria in extracted water, and that measures can then be initiated to keep animal groups away from vulnerable extraction locations.

Research Questions

1. Can FIB be found in the top layer of groundwater extracted from vulnerable wells in the dunes?
2. Can FIB from faeces at ground level reach the saturated zone and how quickly are FIB transported through the unsaturated zone?
3. If FIB are found in the extracted water from faeces placed at ground level, from which animal group(s) do the FIB originate?

2 Methods

2.1 Water samples from vulnerable extraction wells in area FH

Two wells were monitored from a vulnerable location (FH) in the dunes; an existing monitoring well next to a production well (WME-WFH-VEFF), and a new monitoring well (WME-WFH-PB-651, filter screen 1.25-2.25 mbgs), which was installed for this project between the production well line and the infiltration pond, located approximately 10 m upstream of the existing monitoring well (Figure 1). WME-WFH-VEFF is a collection point of all wells of FH. The location FH was chosen as there is an infiltration pond close to the wells and the thickness of the unsaturated zone is limited; during certain periods of the year the groundwater table is very close to, and sometimes above, the ground surface. In addition both domesticated and wild animals visit the area.

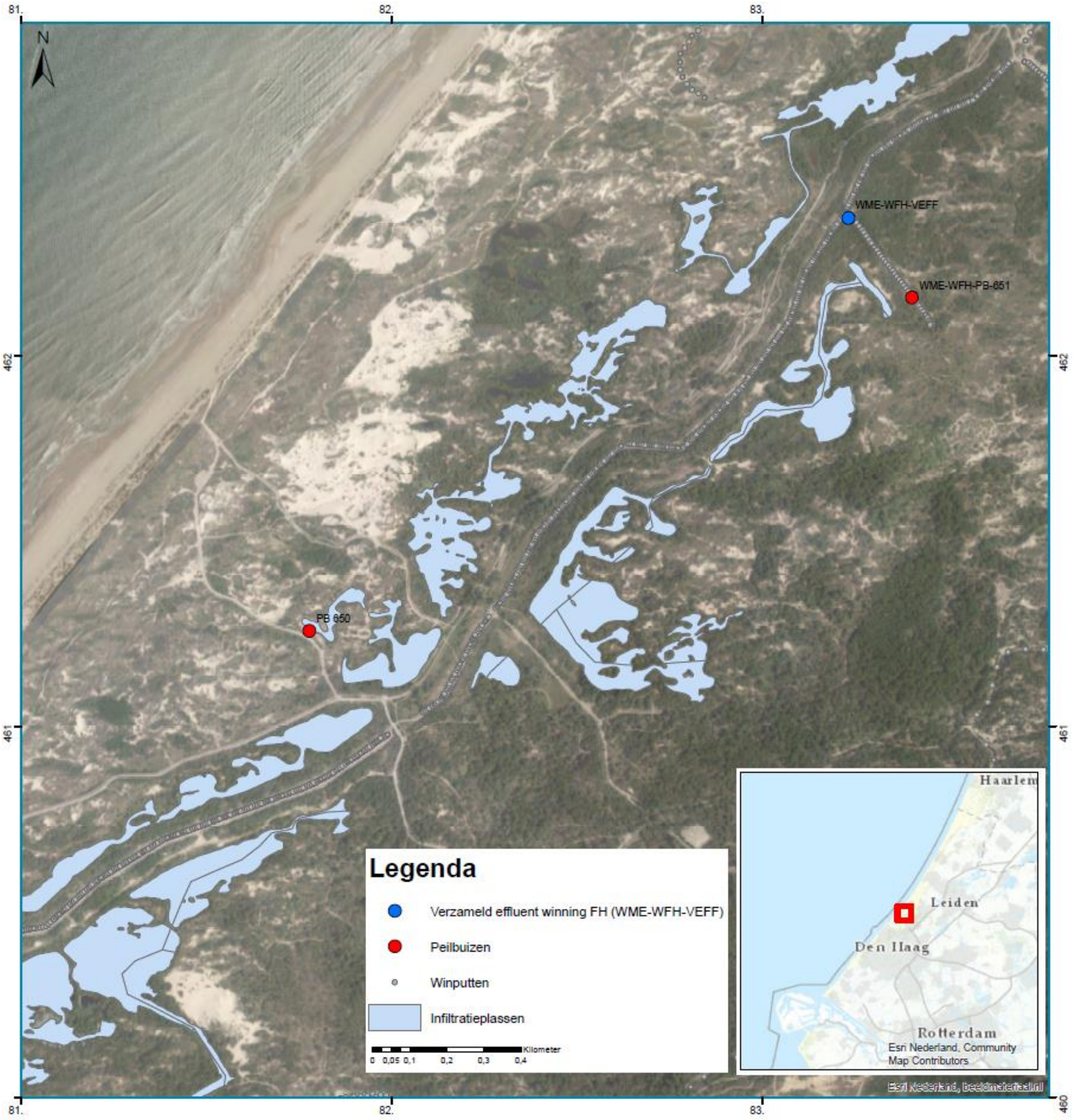

Figure 1 Locations for water samples from vulnerable wells (WME-WFH-VEFF and WME-WFH-PB-651) and the location of the field experiment (PB 650) in the Meijendel dune area. Image courtesy of Dunea.

The two wells were monitored monthly between November 2019 and March 2020 (n=5). Samples of 100L were extracted from the wells, filtered (Hemoflow) and analysed for coliforms, *E. coli* and Enterococci. Samples were taken from the top layer of groundwater (~20 cm) by using two pumps, with the top pump extracting the water at the top of the well (which is representative of the top groundwater) and the bottom pump extracting the water that flows at greater depths (Figure 7 in Appendix). The depth of the groundwater during extraction was 1mbgs, the rate of sample extraction for the top layer of the groundwater was 0.83 L/min (extracted water tested for FIB) and the rate of extraction of the bottom pump was 4.16 L/min. Both pumps were operated continuously.

2.2 Field experiment

As passage of FIB through the unsaturated zone may depend on specific conditions, a ‘worst-case scenario’ experiment was conducted, where faeces from dune animals were brought to a shallow monitoring well and a heavy rain event was simulated. During the experiment, the monitoring well was continuously pumped by a peristaltic pump on solar energy. From this experiment it was determined: 1) whether FIB from faeces could be transported through the unsaturated zone, 2) how quickly the bacteria could be transported through the unsaturated zone, and 3) from which animal group(s) the FIB originated.

2.2.1 Salt Tracer test

Figure 2 Field equipment set-up in the tracer and faeces trials. Solar panels provided power for the groundwater and the air-pumps.

Equipment to simulate the rain events was used from previous research (Cirkel et al., 2019). Artificial rain was simulated over an area of 1 m^2 aroufind a monitoring well using 203 1.1 L/h Supertif drippers (Rivulis irrigation) and a nitrogen gas-powered Teflon membrane pump (Almatec) to pump water through the drip setup (Appendix Figure 8). Nitrogen gas (Linde) at a pressure of 2 bar was used to power the gas-pump. The large number of drippers on the equipment ensured the tracer solution was homogeneously applied over the surface area.

A salt tracer test was performed using a newly installed monitoring well (located next to well PB 650, Figure 1) for this trial, with a well screen 0.45-1.45 mbgs (Figure 2). The groundwater level was located at approximately 1.23 mbgs during the experiments. For each of the rain-events, a salt-solution was prepared with final concentration of 1 g NaCl/L. The salt solution was rained on the area around the monitoring well for a period of ca. 3.5 hours, using a 30 seconds on, 7 minutes off schedule, for a total volume of 50 L per m^2 (50 mm rainfall event). In the trial, the salt solution was used for each of the three rain events (March 31-April 2, 2020). Electrical conductivity (EC) was measured using a portable EC meter (WTW Cond 3310) and flow-through cell. The EC and temperature were logged every 5 minutes for a period of 9 days. The monitoring well was pumped continuously during the tracer test at a rate of 12L/hr.

2.2.2 Faeces Collection and Analysis

Faeces from dune animals were collected on two days, April 2 and April 14, 2020. With the help of a duinwachter from Dunea, fresh faeces (<1 day old) from cow, horse, fox, sheep, rabbit and deer were collected in sterile plastic bags. The faeces were weighed on site using a portable scale (accuracy to 0.01 kg) and a small sample was collected in sterile plastic pots and brought back to KWR for analysis. Faeces sample were tested for presence and concentration of coliforms, *E. coli* and Enterococci before the experiment. The remainder of the faeces were stored out of sight in the dunes for the faeces raining trial.

2.2.3 Faeces raining and water samples from faeces trial

All faeces was used from the second round of testing (21 kg, see Appendix Table II), including cow, horse, fox and sheep faeces. The faeces was laid out evenly around the newly installed monitoring well within the perimeter of the 1 m^2 rain equipment (Figure 3). Fresh water was rained on the area around the monitoring well using the same schedule as outlined above the for salt trial (50L over 3 hours per day, 30 seconds on, 7 minutes off, repeated over 3 days for a total volume of 150L). Water samples (~40L) were taken, using the same two pump system as described before, before the start of the trial (Day 0, blank) and then after 3, 4, 5, 9 and 11 days after the start of the raining. Water samples were filtered on site (Hemoflow) and tested at KWR for *E. coli* and Enterococci. Furthermore, DNA was isolated for microbial source tracking using PCR markers for dog, human, ruminant, bovine, horse, avian helicobacter and swine (Heijnen, 2015). No DNA markers for fox and specifically sheep are available. The retardation factor (R_f) for each *E. coli*, Enterococci and source tracking markers was calculated as the ratio of the velocity groundwater flow (V_{water}) to the velocity of the microorganism ($V_{bacteria}$): $R_f = V_{water}/V_{bacteria}$ (Foppen et al., 2006).

Figure 3 Set up of faeces within the perimeter of the rain equipment.

3 Results

3.1 Water samples from vulnerable extraction wells

Both wells (PB-651 and VEFF) were positive for coliforms in the November 26, 2019 sample (0.29 & 0.05 CFU/L respectively, Table I). In January, Well VEFF was positive for Enterococci (0.4 CFU/L). In November and December unidentified colonies (not Enterococci) were found on selective S&B medium for Enterococci. Most likely these unidentified colonies have no faecal history, but the appearance was not anticipated.

Table I Water samples from monitoring and production wells analysed for coliforms, E. coli *and Enterococci. Positive samples in bold.*

Sample Date	Volume Analysed (L)	Coliforms (CFU/l)	E-coli (CFU/l)	Enterococci (CFU/l)
Monitoring well (WME-WFH-PB-651)				
26-11-2019 / 18-11-2019	20 direct / 21.04 HF	**0.29**	< 0.05 direct / < 0.05 HF	< 0.06 / < 0.07[1]
12/17/2019	47.25	< 0.02	< 0.02	< 0.02
1/20/2020	45	< 0.02	< 0.02	**0.4**[2]
2/17/2020	45	< 0.02	< 0.02	na[4]
3/30/2020	41.77	< 0.02	< 0.02	< 0.02
Production well (WME-WFH-VEFF)				
26-11-2019 / 18-11-2019	36.38	**0.054**	< 0.03	< 0.1
12/17/2019	na[3]	na[3]	na[3]	na[3]
1/20/2020	46.05	< 0.02	< 0.02	< 0.02
2/17/2020	35.51	< 0.03	< 0.03	Na[4]
3/30/2020	45.37	< 0.02	< 0.02	< 0.02

[1]15L direct/17.8L Hemaflow (HF),[2] Unidentified bacteria also present in addition to Enterococci, na[3] = not sampled, na[4] no results due to human error

3.2 Field experiment

3.2.1 Faeces Samples

The concentration of *E. coli* and Enterococci in cow and horse faeces varied considerably between samples, ranging six order of magnitude for both *E. coli* and Enterococci in cow samples (Figure 4) and three and five orders of magnitude in horse samples. The concentrations varied less for rabbit, sheep and deer faeces, likely as a result of the aggregation of faeces from more than one animal in the samples (due to the dropping size). Foxes consistently had high concentrations of *E. coli* and Enterococci in all samples.

Fox faeces had the highest concentration of *E. coli* (on average 3.7x10^{6} ± 1.8x10^{6} CFU per gram faeces (pgf)) and Enterococci (6.2x10^{7} ± 3.0x10^{5} CFU pgf) of all the dune animal faeces samples (Figure 5). After fox, cows had the second highest concentration of both *E. coli* and Enterococci (2.5x10^{5} ± 4.4x10^{5} and 1.5x10^{5} ± 4.2x10^{5} CFU pgf, respectively) while horse faeces had lowest *E. coli* and low Enterococci concentrations (2.3x10^{3} ± 2.5x10^{3} and 3.0x10^{4} ± 6.7x10^{4} CFU pgf, respectively). In the second sampling round, no deer faeces were found, and no fresh rabbit faeces were found. Though fox faeces had the highest concentration, it was also the smallest sample size, at

< 0.01 kg per dropping. Cows and horses on the other hand had an average dropping size of 2.2 kg each. Rabbit, deer and sheep all had faeces samples on the order of 0.01-0.02 kg each.

3.2.2 Faeces raining and water samples from faeces trial

The peak of the salt breakthrough curve observed approximately 3 days after the start of raining (Figure 6). The groundwater table was measured at 1.23 mbgs, giving an infiltration (vertical) velocity of 0.39 m/day, or 2.54 days for the tracer to travel 1 m in the unsaturated zone

The tracer test and DNA measurements (ruminant-specific DNA markers, *E. coli* and Enterococci) were taken during two different trials and the results have been superimposed in Figure 6 as a function of the number of days since the start of the rain-events. The peak concentrations of *E. coli* and Enterococci were measured two days later than the peak salt concentration, with a calculated retardation factor of 1.6 for both *E. coli* and Enterococci. Ruminants were the only positively measured DNA marker in the water samples (Table III Appendix). The peak concentration of the ruminant-specific marker was measured only 1 day later than the salt breakthrough, with a calculated retardation factor of 1.23. The peak concentration of *E. coli* was higher than the concentration of Enterococci (2.7x10^5 and 4.92x10^4 CFU/L respectively), while the peak ruminant-specific marker concentration was measured at 2.2x10^5 copies/L. The water samples were all negative for dog, human, bovine, horse, avian helicobacter and pig specific DNA markers.

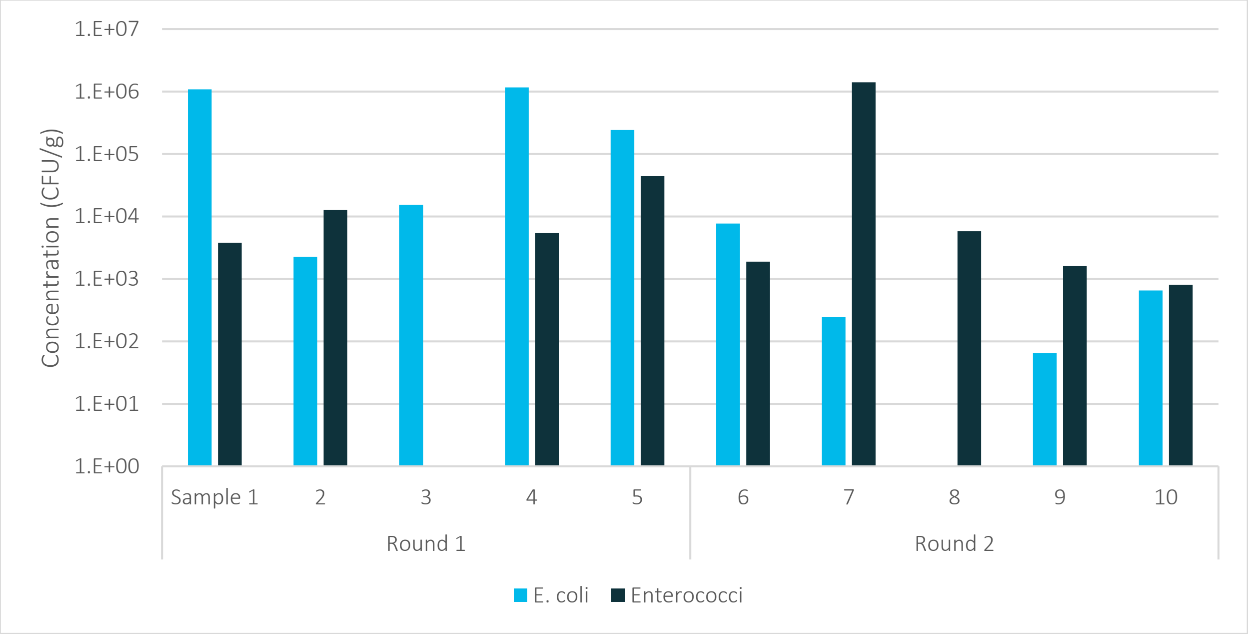

Figure 4 Variability in E. coli and Enterococci concentration in cow faeces samples. E. coli concertation <100 CFU/g in sample 3, round 1 and Enterococci <90 CFU/g in sample 8, round 2.

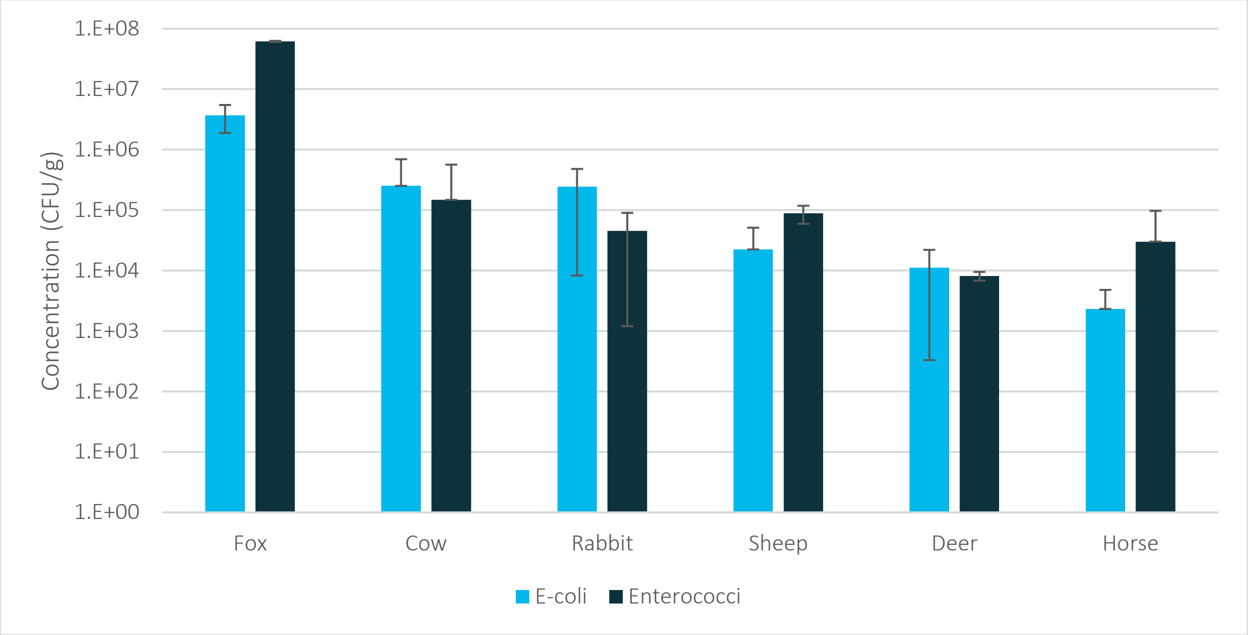

Figure 5 Average concentration of E. coli *and Enterococci in faeces from dune animals collected in round 1 (April 3, 2020) and round 2 (April 14, 2020). Listed in order of decreasing average concentration.*

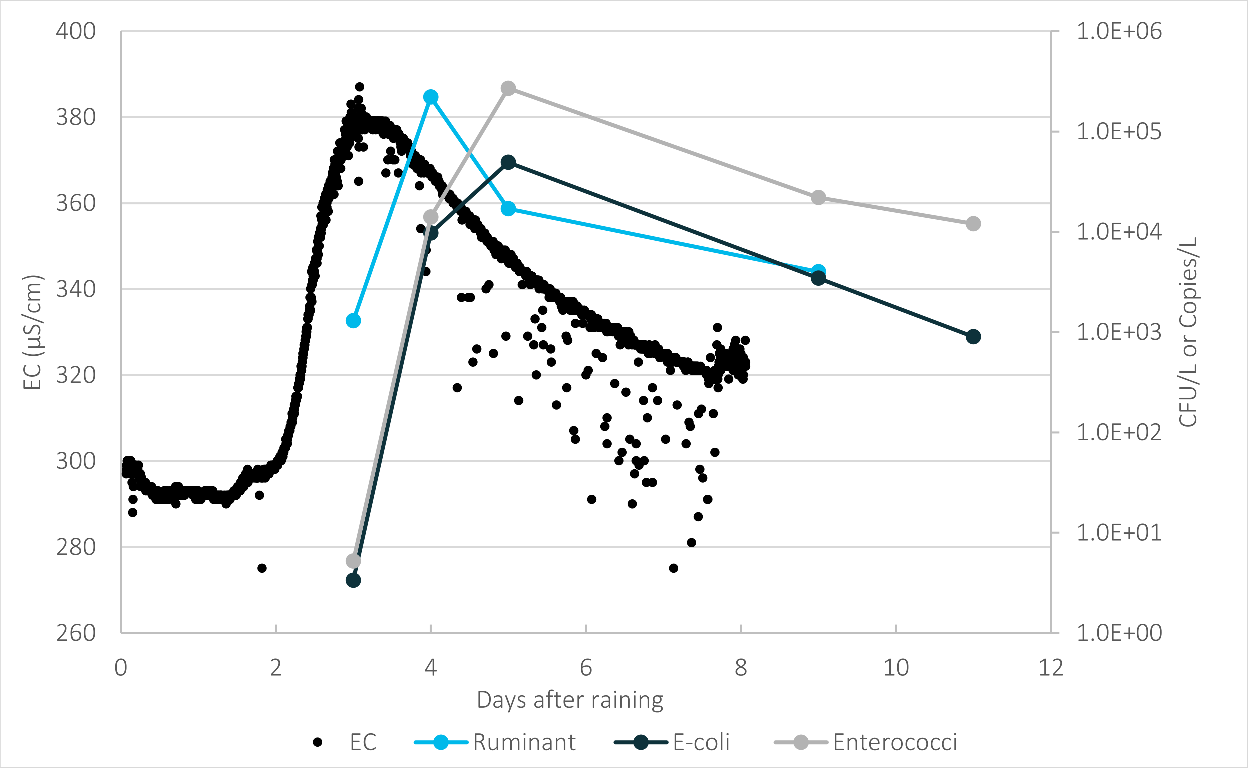

Figure 6 Electrical conductivity (EC) breakthrough curve overlaid with the DNA results from water samples as a function of the days after raining began. E. coli and Enterococci are presented CFU/l and the Ruminant source tracking marker in Copies/L

4 Discussion

The first research question was: *Can FIB be found in the top layer of groundwater extracted from vulnerable wells in the dunes?*

Enterococci were detected in the sample from the newly installed monitoring well in November, confirming the hypothesis that FIB can be found in the top layer of groundwater, albeit in these conditions in low concentrations, and low frequency, in wells in vulnerable areas within the dunes. Notably, although transport to the groundwater has been observed, the number of measurements is too low to make an estimation about the number of FIB that can reach the groundwater in a specific dune area.

It should be noted that the filter of the additional monitoring well placed at the vulnerable location was found to be placed too deep by the contractor upon inspection. As a result, contaminated groundwater may have been missed during the sampling. In addition, unidentified colonies were detected in January in the production well and in November in the newly installed monitoring well. As the origin of these bacteria is not known, it is unlikely that their origin is faecal. However, when future experiments also show the growth of these unidentified bacteria, it is recommended to type the bacteria, in order to know the species and strain. This may bring further insight into the microbial profile of contaminated water.

The second research question was: *Can FIB from ground level reach the saturated zone and how quickly are FIB transported through the unsaturated zone?*

FIB were detected in the monitoring well within 3 days of the start of the trial, with the peak concentration of *E. coli* and Enterococci arriving after 5 days. Based on the infiltrating flow velocity of the tracer i.e. 0.39 m/day, the vertical flow through the unsaturated zone was likely homogenous and not preferential flow. Previous experiments using Brilliant Blue as a tracer, found infiltrating flow rates of ca. 0.35 m/day on flat wettable surfaces, with approximately the same rainfall intensity and duration (Cirkel et al., 2019). In addition, during drilling of the new monitoring well, the soil appeared homogenous with depth and previous research has shown that bare soil has limited hydrophobicity (Cirkel et al., 2019).

The peak concentration of Enterococci were higher than the peak concentration of *E. coli*, which differs to the results found in previous column (Foli, 2019) and field (Hornstra, Cirkel, et al., 2018) experiments. In column experiments with sand from Castricum (PWN), *Enterococcus moraviensis* were more easily bound to the sand grains than *E. coli*, leading to higher retentions of *E. moraviensis* within the column. Microbial removal rates in the subsurface are sensitive to site specific geochemical and geohydrological processes, including the type subsurface media, grain size, saturation level, pH, presence of heterogeneity in the soil and preferential flow paths (Pang, 2009). Also, the experiments at PWN were performed with bacteria suspended in water, while the experiments described in this report were done with bacteria from faeces. Probably bacteria are attached to faecal particles, which may influence the transport characteristics. Finally, the experiment at PWN used bacteria that were cultivated in the laboratory. It is known that lab grown bacteria differ from natural strains in many characteristics, therefore it is difficult to compare removal rates between experiments.

The rain event simulated in this research had a return period of approximately 20-25 years (Beersma et al., 2018). However it is not only the intensity of the rain event, but also the infiltration of the rain into the subsurface and in vulnerable areas there can be rapid infiltration due to preferential flow from heterogeneities, hydrophobicity and/or slopes in the dune area (Cirkel et al., 2019) resulting in increased intensity of infiltrating water compared to the rainfall intensity itself. Furthermore, research has shown that *E. coli* and Enterococci are able to survive for long periods of time at depth (Hornstra & Cirkel, 2018) and can be remobilized (Foli, 2019). Therefore, it is conceivable that smaller consecutive rain events can result in the slow, but steady transport of FIB to groundwater in vulnerable locations.

The final research question was: *If FIB are found in the extracted water from faeces placed at ground level, from which animal group(s) do the FIB originate?*

Source tracking using qPCR was used to determine from which animals the measured FIB originated. Ruminant-specific DNA markers were measured in the water samples, while the samples did not detect markers for bovine and horse though both faeces were present. The negative controls for human, dog, pig and avian helicobacter were all negative as expected. No markers for fox and specifically sheep were available.

The peak concentration of ruminant-specific DNA markers was detected 1 day earlier (after 4 days) than the peak concentrations for *E. coli* and Enterococci (Figure 6). However, the resolution of measurements of 1 sample per day, was fairly low and therefore it is possible that the peak concentration occurred between the daily measurements and was not captured in our samples. Higher resolution measurements (e.g. hourly) would be needed to better comment on the relative retardation of the microbial parameters, however that was outside of the scope of this project and not necessary to answer the research questions at hand.

Concentrations of *E. coli* and Enterococci in faeces were, on average, lower than concentrations measured in previous research from cow and sheep faeces collected from the Amsterdam water supply dunes, though within the range of concentrations measured (Heijnen, 2015). Heijnen (2015) also measured the concentration of ruminant-, and in the case of cow, bovine-specific DNA markers in cow and sheep faeces. The average concentration of bovine-specific DNA markers was 42 times lower than the average concentration of ruminant-specific DNA marker (Heijnen, 2015). Taking this into consideration and given the concentration of ruminant-specific DNA markers found ($2.2x10^5$ copies/L) we would still have expected to measure bovine specific markers in the water samples on the order of 10^3 copies/L. As discussed above, transport of microbes through the subsurface is highly sensitive to geohydrochemical processes as well as specific to the microbe itself. It is possible that bovine specific markers were transported, but not yet detected in the extracted water. More detailed sampling with depth could help to determine the transport of specific microbial parameters in the future.

No research exists that has investigated whether bacteria from different faecal dropping differ in penetration of the soil layer. It might be the case that, depending on the faecal composition, bacteria from faeces from a species can penetrate the soil more easy than from another species. For example, attachment of bacteria to particles can prevent bacteria from entering the soil. Therefore, faecal characteristics might influence the transport of bacteria through the soil, but this is unknown.

The ruminant-specific DNA marker was very common in faecal samples from sheep and the concentration measured in faeces was substantially higher than *E. coli* and Enterococci (754 and 1206 times higher, respectively). Furthermore, the concentration of ruminant-specific DNA markers were, on average, an order of magnitude higher in sheep (10^{11} copies pgf) than in cows (10^{10} copies pgf) (Heijnen, 2015). Therefore, although 14.06 kg of cow faeces and only 0.31 kg of sheep faeces were deposited around the well, we cannot rule out the sheep faeces as a source of the ruminant-specific DNA makers.

5 Conclusions and Recommendations

5.1 Conclusions

- Enterococci were detected in the top layer of groundwater from vulnerable extraction wells in the dune, albeit in low concentrations and with a low frequency. Coliforms were also detected in low frequency, and no *E. coli* were found.

- FIB from faeces at the ground surface can be transported through the unsaturated zone, reach the groundwater table and can be detected in extracted water. The rain event simulated was a 50 mm rainfall in 3.5 hours, corresponding to a rainfall event with a return period in the Netherlands of approximately 20-25 years on a yearly basis (Beersma et al., 2018).

- Ruminant-specific DNA markers were found in the extracted water from the faeces trial, which could have originated from either the sheep or cow faeces deposited on site. The lack of detection of other source tracking markers, however, does not mean we can determine they pose no threat.

- The results from this research build on previous research. Though the highest risk to consumer health remains to be from human faecal contamination (Nobel & Cirkel, 2005), field and column test have demonstrate that faecal contamination from animal faeces can reach groundwater in the dune areas and risk factors include terrain influences, such as slopes, vegetation and soil heterogeneities and ground surface hydrophobicity (Cirkel et al., 2019; Hornstra & Cirkel, 2018). Furthermore, die-off in dune sand has been shown to be a slow, and in some cases, months long process (Hornstra and Cirkel, 2018) and FIB can be remobilized after initial transport (Foli, 2019). Therefore, the animal faeces can recontamination dune areas close to extraction wells, and the unsaturated zone can become a reservoir for these bacteria, which may be remobilized and transported to extracted water, a potential hazard for drinking water consumers. A workshop is being organized for November 2020 with the specific goal of bringing together the research on dune contamination from the last several years into context.

- When considering the results of the experiments presented in this report it is important to keep in mind that the experiment are from a worst-case scenario and it is unlikely that extraction wells in practice would be subject to the high faecal and rain loads as in the experiments. Furthermore, the microbial parameters measured are FIB and therefore do not pose a health risk themselves. Moreover, the risk from faecal contamination from animal faeces is relatively low as microorganisms from animal faeces are removed relatively well in sand filters after dune passage. The highest health risk from faecal contamination in the dunes is from human faeces which contain human pathogenic viruses. Contamination by human faeces can occur, for example, from visitors to the dunes who defecate in or close to extraction well fields. It is therefore imperative that human faecal contamination is prevented, while also preventing other kinds of faecal contamination. Future research could make the link between the results presented here on animal faecal contamination and the risk for consumer health, however it is outside of the scope of this report to perform a risk assessment for faecal contamination in the dunes.

5.2 Recommendations

This research has shown that bacteria from animal faeces at the surface can be transported to groundwater relatively quickly and result in the recontamination of purified dune water close to the wells. Therefore, animals in the dune areas pose a risk for recontamination for FIB and based on this research we cannot rule out that FIB from animals other than ruminants do not pose a risk. As such, we recommend that grazing animals, such as horses, cows and sheep, be excluded from the extraction well fields as a precautionary measure. These animals may be excluded using, for example fencing, while other wild animals, such as foxes, deer and rabbits cannot be realistically excluded from these areas and further research would be necessary to determine what risk these animals pose.

Other options for preventing the recontamination of FIB in the dune are possible, but as of yet unproven. These may include maintaining low groundwater levels to lengthen the unsaturated zone. This could be accomplished through for example recirculation of water or installation of horizontal flow and/or drainage in the dunes to prevent high groundwater levels. Setting minimum limits or guidelines on the depth of the unsaturated zone is another possibility, such that filters in extraction wells must have a minimum depth of unsaturated zone at all times during the year. Finally, additional treatment processes could also be added subsequent to dune passage to disinfect the extracted water. All the above described measures are not yet supported by research, however, and are given only as examples, not recommended practices.

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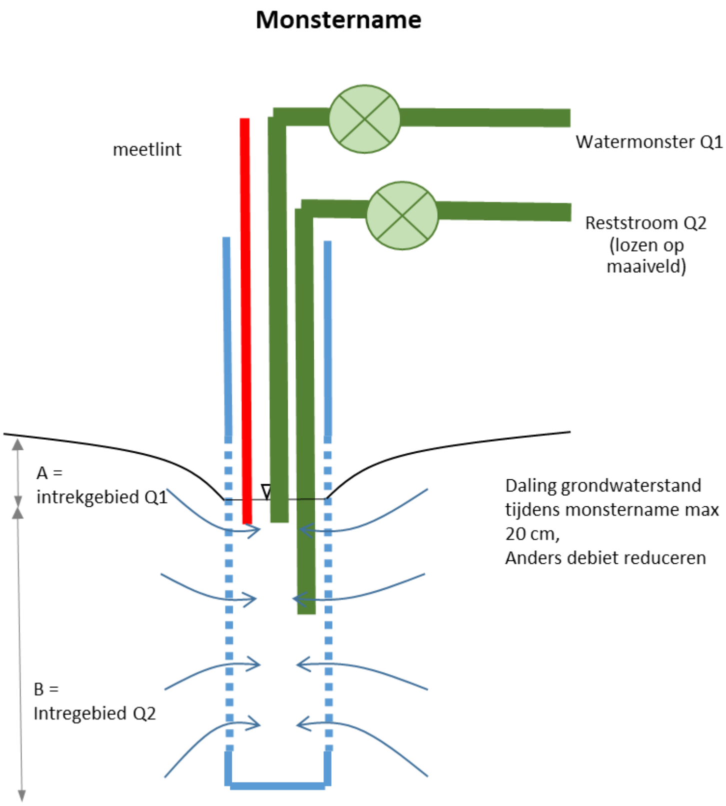
II Appendix



<u>Rekenvoorbeeld: debiet per pomp</u>:
Q1 / Q2 = A / B
Q1 + Q2 = 5 l/min
A = 20 cm
A + B = 120 cm

Q1 = 20/120 * 5 = 0,83 L/min
Q2 = 100/120 = 4,16 L/min

Figure 7 Set-up for sampling top layer of groundwater from vulnerable extraction wells. The top pump (Watermonster Q1) extracts water representative of the top groundwater and the bottom pump (Reststroom Q2) extracts the water that flows at greater depths.

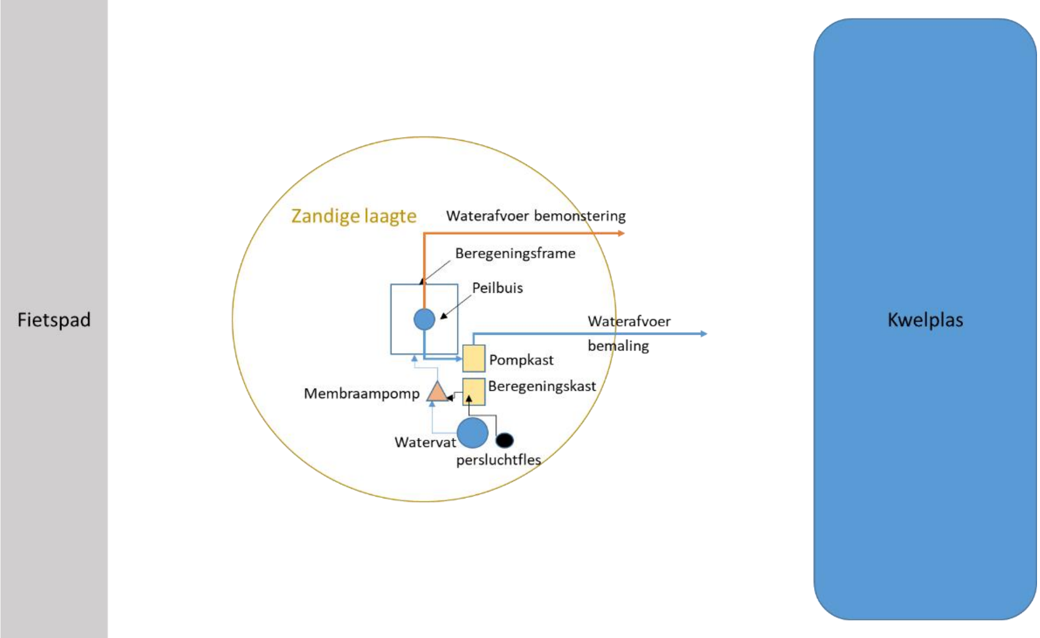


Figure 8 Conceptual diagram of the set-up of the field trial

Table II Faeces sampling round 2 (April 14, 2020) with the weight, location and sample code.

Animal	Animal-code	Weight (kg)	Location
Cow	Koe 1	3.59	Vogelbos
Cow	Koe 2	3.97	Vogelbos
Cow	Koe 3	2.1	Vogelbos
Cow	Koe 4	2.05	Vogelbos
Cow	Koe 5	2.35	Vogelbos
Fox	Vos 1	<0.00	Lopert
Fox	Vos 2	<0.00	Lopert
Fox	Vos 3	<0.00	SprangA
Horse	Paard 1	1.87	Lopert
Horse	Paard 2	2.67	Lopert
Horse	Paard 3	2.12	LV
Sheep	Schaap 1	0.31	Meijendaal

Table III Results of source tracking (PCR) on water samples collected during the faeces raining trial. All concentrations expressed as copies/L. Positive samples in bold.

Sample Date	Days after start of rain	Dog	Human	Ruminant	Bovine	Horse	Avian Helicobacter	Swine
31-3-2020	Day 0 (Blank)	<2900	<2900	<2900	<2900	<2900	<14000	<14000
4/22/2020	Day 3	<20	<20	<20	<20	<20	<100	<100
4/23/2020	Day 4	<80[1]	<80[1]	**1300[2]**	<80[1]	<80[1]	<400[1]	<400[1]
4/24/2020	Day 5	<26	<26	**220 000**	<26	<26	<130	<130
4/28/2020	Day 9	<42	<42	**17 000**	<42	<42	<210	<210
4/30/2020	Day 11	<32	<32	**4000**	<32	<32	<160	<160

[1] Not present (Efficiency 10-20%), [2] Present but not quantitative (Efficiency 10-20%)

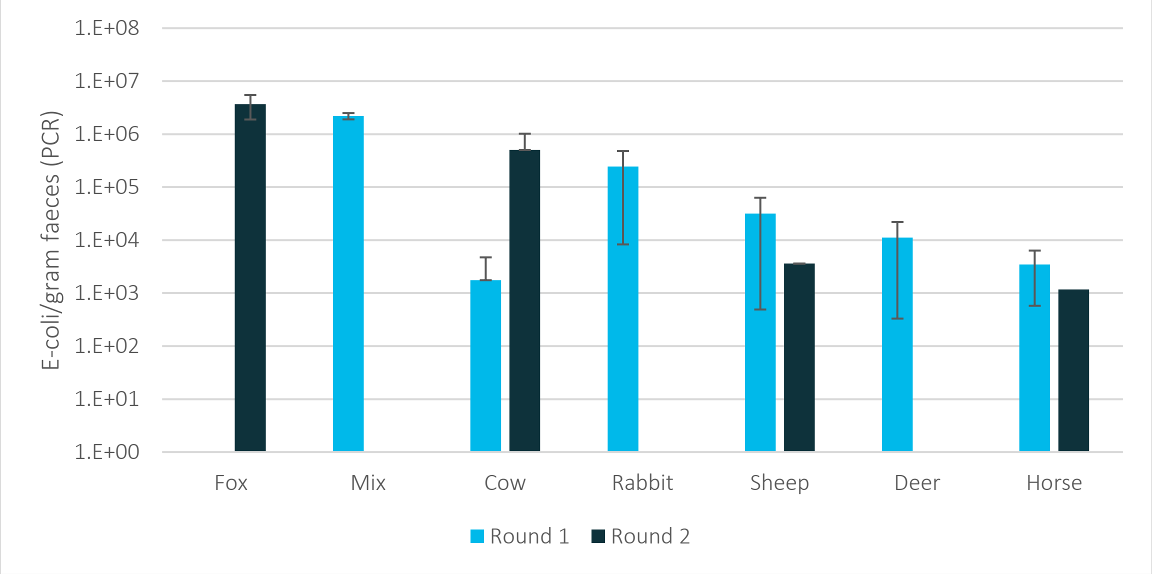


Figure 9 Concentration of E. coli *in faeces from dune animals collected in round 1 (April 3, 2020) and round 2 (April 14, 2020). Mix = mix of small animal faeces (sheep, fox, rabbit). Listed in order of decreasing average concentration (round 1 and round 2).*

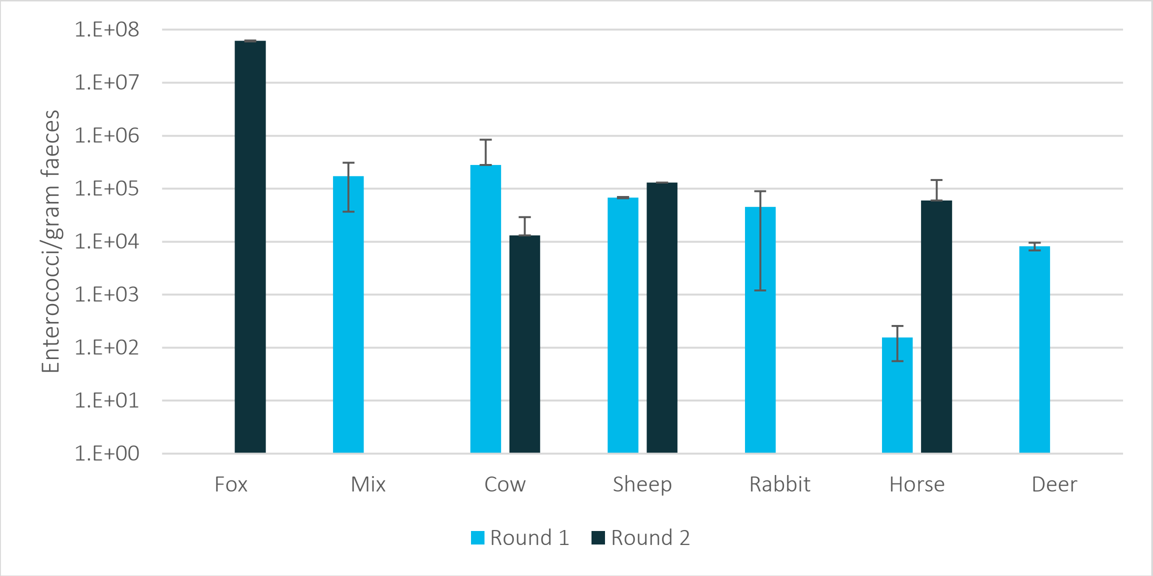


Figure 10 Concentration of enterococci in faeces from dune animals collected in round 1 (April 3, 2020) and round 2 (April 14, 2020). Mix = mix of small animal faeces (sheep, fox, rabbit). Listed in order of decreasing average concentration (round 1 and round 2).