Source tracking of *Enterococcus moraviensis* and *E. haemoperoxidus*

Maja Taučer-Kapteijn, Wim Hoogenboezem, Remco Hoogenboezem, Sander de Haas and Gertjan Medema

ABSTRACT

Enterococci were detected occasionally in 100 L samples of water abstracted from a shallow aquifer in a natural dune infiltration area for drinking water production. *Enterococcus moraviensis* was the species most frequently identified in these samples. Because there are no existing reports of faecal sources of *E. moraviensis* and the closely related *E. hemoperoxidus,* this study aimed to find such sources of these two species in the dunes. Faecal samples from various animal species living in the vicinity of abstraction wells, were analysed for enterococci on Slanetz and Bartley Agar. From these samples, enterococci isolates (1,386 in total) were subsequently identified using matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry. *E. moraviensis* was found in the faeces of geese, foxes and rabbits. Also, *E. haemoperoxidus* was isolated from goose faeces. Using hierarchical clustering, the species composition of *Enterococcus* spp. isolated from abstracted water formed one cluster with the species composition found in geese droppings. A sanitary survey supported the indication that feral geese may provide a substantial faecal load in particular parts of this dune infiltration area, close to the water abstraction system. This study confirms the faecal origin of *E. moraviensis* and *E. haemoperoxidus* from specific animals, which strengthens their significance as faecal indicators.

Key words | animal faeces, dune filtration area, enterococci, faecal indicators, geese

Maja Taučer-Kapteijn (corresponding author)
Wim Hoogenboezem
Het Waterlaboratorium,
J.W. Lucasweg 2, Haarlem 2031 BE,
The Netherlands
E-mail: *maja.taucer@hetwaterlaboratorium.nl*

Maja Taučer-Kapteijn
Gertjan Medema
Faculty of Civil Engineering & Geosciences,
Department of Water Management,
Delft University of Technology,
Stevinweg 1, Delft 2628 CN,
The Netherlands

Remco Hoogenboezem
Erasmus University Medical Center,
Wytemaweg 80, Rotterdam 3015 CN,
The Netherlands

Sander de Haas
PWN Drinking Water Supply Company,
Rijksweg 501, Velserbroek 1991 AS,
The Netherlands

Gertjan Medema
KWR Watercycle Research Institute,
Groningenhaven 7, Nieuwegein 3433 PE,
The Netherlands

INTRODUCTION

Removal of micro-organisms during soil passage in dune infiltration areas is often used as one of the treatment steps in drinking water production in the Netherlands. Recovered (abstracted) groundwater is the product of this process and is normally free of faecal indicator bacteria, and therefore considered to be free of faecal-associated pathogenic micro-organisms.

During regular water quality control, enterococci have occasionally been isolated from 100 L samples of abstracted water in the Castricum dune infiltration area (The Netherlands).

Enterococci are bacteria present in the gastro-intestinal tracts of humans and warm-blooded animals and are therefore used as indicators for determining the sanitary quality of water, indicating the possible presence of pathogens. Compared with *Escherichia coli*, the association of *Enterococcus* spp. (all species) with the presence of pathogens is not very well known.

Enterococcus spp. is not only associated with warm-blooded animals, but has also been detected in extra-intestinal habitats like invertebrates (Martin & Mundt 1972; Švec *et al.* 2002), plants (Müller *et al.* 2001), sediments (Grant *et al.* 2001; Le Fevre & Lewis 2003), soils (Fujioka *et al.* 1999), foods (Klein 2003; Foulquie Moreno *et al.* 2006) and water (Švec *et al.* 2001).

Current data on *Enterococcus* species isolated from faecal and non-faecal environments depend upon the identification methods used. Since the number of *Enterococcus* species described is still increasing, greater species diversity can be expected in sources already known. In the past

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decade, matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has increasingly been applied as an identification technique and has also been shown to be suitable for the identification of enterococci in water (Taučer-Kapteijn *et al.* 2013). The introduction of molecular techniques has provided greater insight into the genetic diversity within *Enterococcus* spp. and rapidly accelerated the characterization of new *Enterococcus* species isolated from enteric and extra-enteric environments.

In 2001, two new species of enterococci, *Enterococcus moraviensis* and *E. haemoperoxidus* were isolated from surface water and described by Švec *et al.* (2001). *E. moraviensis* has been observed as the most frequently identified species in water samples abstracted from the dunes. Laboratory experiments have shown that *E. moraviensis* is able to multiply under non-enteric circumstances in the presence of dune plant material at 15 °C (Taučer-Kapteijn *et al.* 2016). The observation that certain strains of *Enterococcus* spp. may be able to survive and replicate in non-enteric environments – for instance, *E. casseliflavus* in submerged aquatic vegetation (Badgley *et al.* 2010) and *E. casseliflavus*, *E. faecalis*, *E. faecium*, *E. hirae*, *E. mundtii*, *E. sulfureus* and many other strains resembling *E. faecalis* isolated from forage crops (Cai 1999; Müller *et al.* 2001; Ott *et al.* 2001) – strongly supports the existence of plant-associated enterococci (Byappanahalli *et al.* 2012). Furthermore, some enterococci species have been shown as able to grow and persist under non-enteric conditions (Mundt *et al.* 1962; Whitman *et al.* 2003; Badgley *et al.* 2010; Taučer-Kapteijn *et al.* 2016). These findings challenge the suitability of *Enterococcus* species for the indication of faecal pollution.

Until now, there have been no reports of faecal sources of *E. moraviensis* and *E. haemoperoxidus*. This study aimed to determine if *E. moraviensis* and *E. haemoperoxidus* are present in animal faeces in the dune infiltration area. Since this area is used for recreational purposes, human faecal samples were also included in this study. To gain an overview of the *Enterococcus* species associated with various animal species living in the vicinity of abstraction wells in the infiltration area, a series of faecal samples from these animals was analysed. In order to track the possible origin of the observed contamination of abstracted water, this study additionally focused upon similarities between species distributions in both abstracted water samples and the faeces from the different animals. Information on the sources would establish the reliability of *E. moraviensis* and *E. haemoperoxidus* as indicators of faecal pollution, help to interpret the presence of these enterococci in abstracted water and help in the development of effective preventive measures.

METHODS

Faecal samples

To find a faecal source of *E. moraviensis* and *E. haemoperoxidus*, and to determine the abundance of various enterococci species in faecal samples, animal faecal samples from highland cattle (*Bos taurus*), red foxes (*Vulpes vulpes*), dogs (*Canis lupus familiaris*), greylag geese (*Anser anser*), sheep (*Ovis aries*) and rabbits (*Oryctolagus cuniculus)* were collected between March and October 2014 in the Castricum infiltration area (The Netherlands). Additionally, faecal samples from 20 healthy persons ranging in age from 3 to 66 years were analysed for enterococci. The numbers of faecal samples per animal host are indicated in Table 1.

Preparation of faecal samples and isolation method

Faecal samples were collected in a sterile plastic jar and analysed within 24 hours after collection. Each sample was divided into two parts using two sterile forceps, with the

Table 1 | Number of isolates belonging to *Enterococcus* spp. isolated in faecal samples from different animal hosts

Host species	No. of faecal samples	*Enterococcus* spp. isolates
Red fox (*Vulpes vulpes*)	20	384
Rabbit (*Oryctolagus cuniculus*)	9	108
Dog (*Canis lupus familiaris*)	10	144
Goose (*Anser anser*)	20	231
Human	20	342
Sheep (*Ovis aries*)	11	126
Highland cattle (*Bos taurus*)	11	51
Total	101	1,386

inner part taken for the analysis in order to exclude contamination from other sources (sand, grass, etc.). An amount of 0.5 g of faecal material was placed in a sterile container with 3 mm glass beads (Boom, The Netherlands) and suspended using 9 ml of sterile drinking water. Dilution series (10^{-1}–10^{-5}) were then prepared. An amount of 1 ml of each dilution was filtered using a 0.45 μm cellulose nitrate filter (Sartorius Stedim) and incubated on Slanetz and Bartley Agar (SBA) for 48 hours at 37 °C (as per ISO 7988-2:2000). After incubation, the total number of characteristic colonies was counted. Moreover, a maximum of 20 single colonies per sample was used to make pure cultures on SBA, which were subsequently identified using MALDI-TOF MS (Biotyper, Bruker) in accordance with the manufacturer's instructions.

Abstracted water samples

A total of 195 abstracted water samples (14 of 1 L and 181 of 100 L) were filtered at locations in the Castricum infiltration area between July 2012 and August 2014. A total of 5,117 enterococci colonies were isolated from these samples using the filtration method (ISO 7988-2:2000) and 381 selected isolates (7.4%) were identified using MALDI-TOF MS (Biotyper). The number of randomly chosen identified isolates varied from one to eight per sample.

Hierarchical clustering

From the unprocessed measurements, seven *Enterococcus* species were selected. These were all observed in the water samples and in at least one of the faecal samples. Bacterial species that were unique to one of the animal classes or the water class were discarded since they do not convey information concerning the animal class of origin in the water samples. The rabbit measurements were also discarded, since we had only two *Enterococcus* species. Since determining the number and bacterial species for all animal and water samples is labour intensive and expensive, not all *Enterococcus* colonies were identified at species level. In this experiment, we assumed that the samples from the same class were independent and originated from the same underlying distribution. To improve numerical granularity and statistical power, the empirical bootstrap was used. For the smaller classes (the animal classes), all possible combinations were made using half of the number of samples per combination. For the larger water class, 10^5 random permutations were drawn using half the number of water samples for each permutation. The probability of drawing the same combination twice is practically zero. All combinations and permutations were averaged and normalized, such that the sum over all seven *Enterococcus* species for all combinations and permutations equals one. Referring to the combinations and permutations as our bootstrap dataset, this is a seven-dimensional dataset (seven *Enterococcus* species). The only difference is that the number of samples per class is much higher and that each element is probably statistically more robust. To determine how the different classes relate to each other based upon their *Enterococcus* species composition, hierarchical clustering was used. The distance measure used for hierarchical clustering was the Mahalanobis distance (Mahalanobis 1936), which assumes normal distributions. The resulting dendrogram was generated using MATLAB (version 7.10).

Simpson's index (*D*)

As a measure for the diversity of *Enterococcus* species within animal hosts, Simpson's index D was calculated using the formula $D = \Sigma\ n(n-1)/N(N=1)$, where $n =$ the total number of enterococci of a particular species and $N =$ the total number of enterococci of all species (Simpson 1949).

Faecal load contributed by feral geese

During the sanitary survey in the Castricum infiltration area, faecal sources in the vicinity of abstraction wells were recorded. Because it was observed that the number of geese and geese droppings in particular parts of this area were much higher than those of and from other animal hosts, the faecal load of geese was estimated.

Two areas of the same size (c. 340 m^2) at different locations (A and B) 400 m from one another, both in the immediate vicinity of abstraction wells, were chosen for counts of droppings in order to estimate the faecal load contributed by geese in June 2014. Randomly chosen dropping samples ($n = 15$) were weighed and measured (length). The

average number of enterococci (cfu/m^2) was calculated from the quantity of geese droppings per square metre and the average enterococci density (cfu/g faeces) measured in geese droppings.

RESULTS

Animal and human faecal samples (101 in total) were analysed for enterococci. A total of 1,386 isolates were identified as *Enterococcus* species (Table 1).

The relative distribution of *Enterococcus* species among selected host species and in abstracted water samples is shown in Table 2. Considerable variation in species composition was found between faecal samples and abstracted water samples. *E. faecalis* was the enterococcal species most frequently identified in faecal samples, with the exception of those from sheep. The second most common species was *E. faecium,* with its highest frequency observed in humans (35.1%). *E. faecium* was not found in any faecal sample from rabbits or sheep. It is also noteworthy that a very high percentage of isolates from rabbits were identified as *E. gallinarum* (98.1%). While *E. faecium* was one of the most frequently represented species in human faeces, it was only sporadically isolated from abstracted water samples (3.9%). Ten *Enterococcus* species found in faecal samples were not isolated from any abstracted water. *E. phoeniculicola* was isolated from water, but not found in any of the animal hosts. *E. moraviensis* was most abundant in droppings from geese (23.8%), but also present in droppings from foxes (0.9%) and rabbits (0.3%). *E. haemoperoxidus* was isolated from geese (11.3%) as the only carrier of this species. These results demonstrate a faecal origin for *E. moraviensis* and *E. haemoperoxidus*.

Higher numbers of *E. moraviensis* and *E. faecalis* isolates were found in water samples and in geese droppings. Moreover, species distributions in water samples and geese droppings were similar. Seven species isolated from water samples corresponded with species found in droppings from geese; this is higher than the number of corresponding

Table 2 | Relative (%) distribution of different *Enterococcus* species among selected hosts in faecal samples and in abstracted water samples

Enterococcus spp.	Red fox	Rabbit	Sheep	Highland cattle	Dog	Human	Goose	Abstracted water
E. faecalis	39.6	0.9		35.3	54.2	27.2	29.4	30.0
E. faecium	23.7			5.9	11.8	35.1	7.8	3.9
E. hirae	25.5		69.0	5.9	18.1	23		1.8
E. durans	4.9		2.4		8.3	3.8		
E. casseliflavus	1.0		5.6	52.9	2.1	2.6	5.2	12.9
E. gallinarum		98.1	9.5		1.4	2.0	3.9	
E. mundtii	2.9		13.5		0.7	5.8	8.7	3.9
E. moraviensis	0.3	0.9					23.8	44.2
E. haemoperoxidus							11.3	0.5
E. avium					2.8	18.7		
E. gilvus	0.3						4.3	
E. termitis							3.5	2.6
E. saccharolyticus						2.3		
E. silesiacus							2.2	
E. aqamarinus	1.0							
E. thailandicus					0.7			
E. malodoratus	0.5							
E. sulfurens	0.3							
E. phoeniculicola								0.3

species in other animal hosts. In order to verify these similarities, statistical methods were applied.

As shown in Figure 1, the relationships between different classes (animal faecal samples and abstracted water samples), which are based upon their *Enterococcus* species composition, confirm the existence of strong similarities between the *Enterococcus* species composition in abstracted water samples and in geese droppings. Using Mahalanobis distance as a measure, these two classes have been determined as one cluster. Relationships between this cluster and those for other animal hosts were more distant. Omnivores like dogs, red foxes and humans formed one cluster, which was also related to the sheep cluster. Highland cattle were determined as a separate cluster related more to dog, red fox, human and sheep than to abstracted water or goose.

Additionally, the diversity of *Enterococcus* species (D) was calculated for each animal host and for water samples using Simpson's index. The highest diversity was found in geese ($D = 0.17$), followed by humans ($D = 0.24$), red foxes ($D = 0.28$), water samples ($D = 0.30$) and dogs ($D = 0.34$). The lowest diversity was observed in rabbits ($D = 0.96$).

To enumerate enterococci in different animal hosts, the average total number of enterococci (cfu/g) in faecal samples was calculated for each host species (Figure 2). Higher numbers were observed in omnivores (dogs 1.6×10^6/g, humans 7.7×10^5/g and red foxes 4.4×10^5/g) and geese (3.1×10^5/g), whereas lower numbers were observed in herbivorous mammals: sheep (1.3×10^3/g), rabbits (2.1×10^2/g) and highland cattle (2.9×10^1/g).

Faecal load contributed by the geese population

During a sanitary survey in the vicinity of abstraction wells, it was observed that, in a particular area of the dune

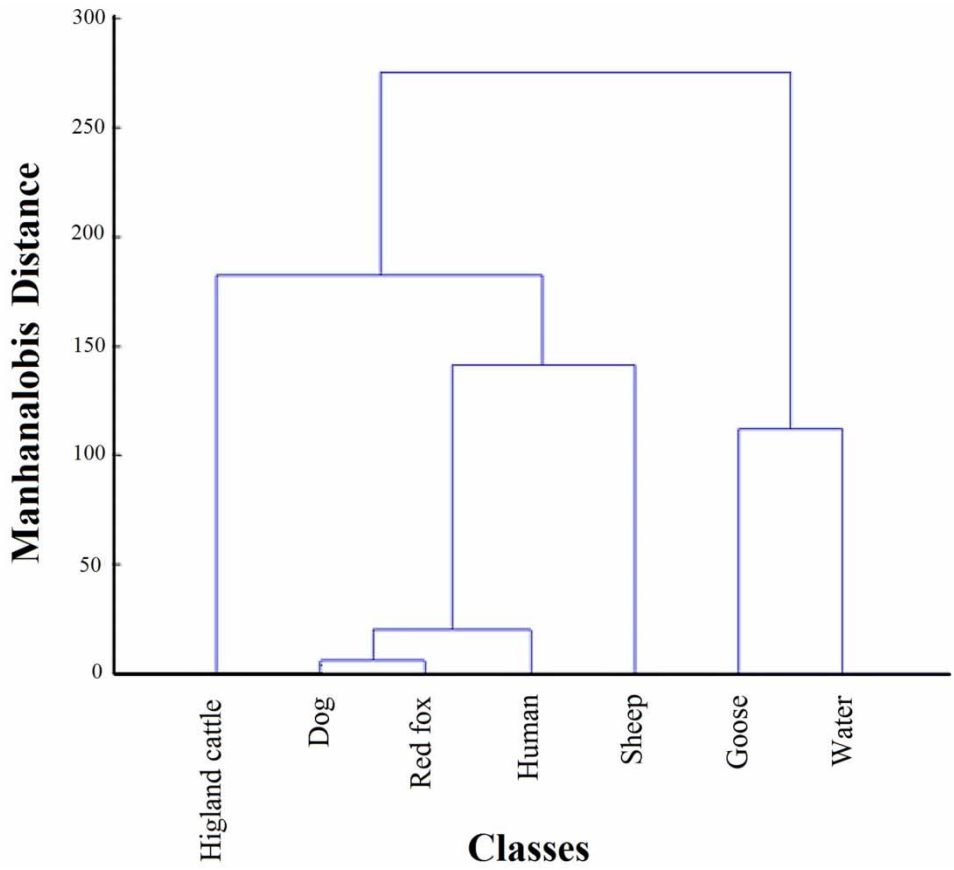

Figure 1 | Relationships between different classes (animal hosts and abstracted water) based upon their bacterial composition, using Mahalanobis distance (MATLAB).

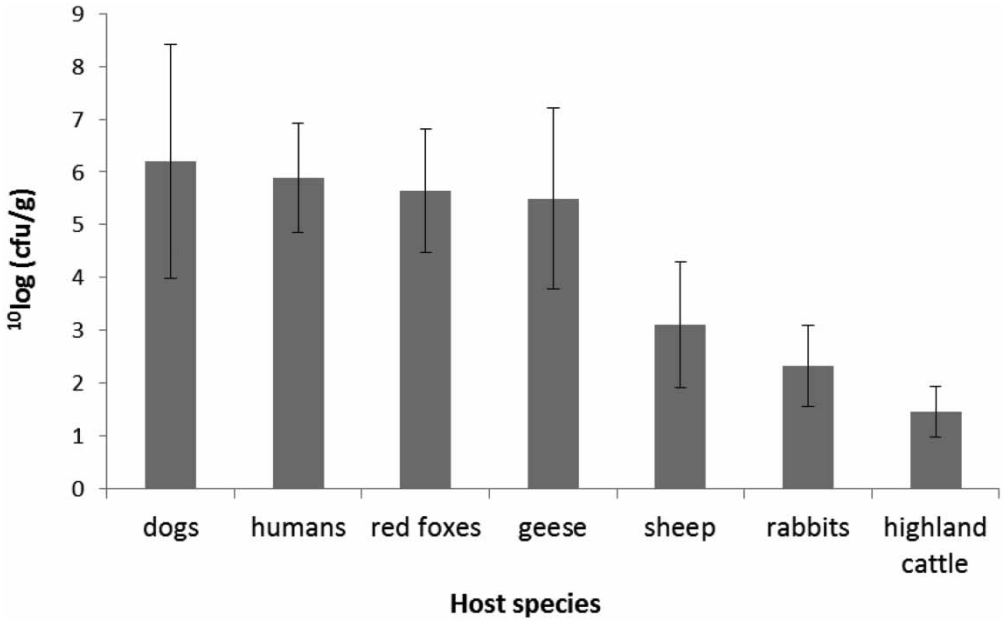

Figure 2 | Average numbers of *Enterococcus* spp. per gram of faeces from selected hosts.

filtration area, numbers of geese droppings were much higher than those from other animal hosts. In recent years, a distinct increase in the feral geese population has occurred near these abstraction wells, especially in the period March–June. Therefore, that population was considered to have made a substantial contribution to the faecal load in particular parts of the area. On average, the amount of enterococci isolated from geese droppings ($n = 20$) was 3.48×10^5 cfu/g. The faecal loads for enterococci at two locations (A and B) were almost the same: 1.9×10^7 cfu/m^2 and 1.8×10^7 cfu/m^2 respectively (as shown in Table 3). Due to the absence of geese in other parts in the dune area, the faecal load from these birds is believed to be much lower in those areas. The same is true for other animals, with their droppings much less frequently present in the vicinity of the abstraction wells.

Table 3 | Faecal load number of geese droppings, geese faeces per square metre and estimated load of *Enterococcus* spp. contributed by the geese population in the immediate vicinity of the abstraction wells

Location	No. of faecal droppings/m^2	g/m^2	Faecal load (cfu/m^2)
A	1.27 ($std = 0.03$)	55.3	1.93×10^7
B	1.19 ($std = 0.07$)	51.8	1.80×10^7

DISCUSSION

This study demonstrates faecal sources of *E. moraviensis* and *E. haemoperoxidus,* which means that occurrence of these two *Enterococcus* species in water samples indicates the possible presence of pathogens. It is not clear if geese, red foxes and rabbits are the only faecal sources of *E. moraviensis* and *E. haemoperoxidus*, because the samples had been diluted by means of membrane filtration and so species present in lower concentrations might have remained undetected. To avoid this disadvantage, the application of molecular techniques specific to these species would be useful. Until recently, *E. moraviensis* and *E. haemoperoxidus* may have been identified as the closely related *E. faecalis*, which – together with *E. faecium* – is the predominant *Enterococcus* species in human faeces and sewage (Murray 1990; Ruoff *et al.* 1990; Manero *et al.* 2002) but is also present in the faeces of non-human animals (Devriese *et al.* 1987; Aarestrup *et al.* 2002; Kühn *et al.* 2003), including wildlife (Mundt 1963). *E. moraviensis* and *E. faecalis* have been shown to be the species most frequently observed in abstracted water, together representing 74.2% of all isolates. Because the same two species were also those most frequently represented (53%) in geese droppings, which were

regularly observed in the vicinity of abstraction wells (specific parts of infiltration area), and because geese have been observed to make a substantial contribution to the faecal load in specific parts of the Castricum infiltration area, especially during warmer periods of the year, it is assumed that geese droppings may be the source of the *Enterococcus* species found in the abstracted water. Also, the bacterial compositions of *Enterococcus* species found in abstracted water samples were much closer to those in geese droppings than those observed in any other animal host. Moreover, since the presence of geese in the area of study coincides with detection of enterococci in abstracted water, molecular techniques could be applied to confirm that the isolates found in geese faeces and in water samples are identical.

The numbers of enterococci isolates and the diversity of *Enterococcus* species found in geese were higher than in other herbivores like sheep or cattle, and comparable with or even higher than those found in humans or dogs (omnivores). Since the diet of geese consists mainly of plant material and is therefore much more monotonous than an omnivorous diet, these results remain unexplained.

When feral geese cause a heavy faecal load near these abstraction wells, the question arises as to whether human pathogenic micro-organisms may be present in geese droppings and so whether contamination from this source poses a risk to human health risk. The geese population in this dune infiltration area consists mainly of greylag geese (*Anser anser*), but also a small number of Canada geese (*Branta canadensis*). Few studies have demonstrated the presence of pathogens in faecal samples from greylag geese, but a high prevalence of *Cryptosporidium* spp. (Chvala *et al.* 2006; Plutzer & Tomor 2009), *Salmonella* (Lillehaug *et al.* 2005) and *Campylobacter* spp. (Colles *et al.* 2008) have been reported. Canada geese have been found to be carriers of *Cryptosporidium* spp. oocysts (Kassa *et al.* 2004; Zhou *et al.* 2004; Moriarty *et al.* 2011), the cysts of *Giardia* spp. (Graczyk *et al.* 1998), *Salmonella* spp. (Fallacara *et al.* 2001) and *Campylobacter* spp. (Pacha *et al.* 1988; Wahlstrom *et al.* 2003; Moriarty *et al.* 2011).

Geese may pollute water by defecating on pasture in the vicinity of abstraction wells, and contamination of groundwater might occur when there is insufficient removal during vertical infiltration through a relatively short unsaturated zone from the surface to the groundwater level. Because it has also been shown that *E. moraviensis* is able to grow on the same plant material (Taučer-Kapteijn *et al.* 2016) as geese feed on, growth of this indicator might also occur in geese faeces. New applications of techniques like whole genome sequencing might have potential as tools to determine whether faecal contamination is recent or comes from a secondary source (environmental growth), and could therefore facilitate the estimation of risks to human health.

The intestinal enterococci group (*Enterococcus faecalis*, *E. faecium*, *E. durans* and *E. hirae*) is described as an indicator of faecal pollution, because these species are typically excreted in the faeces of humans and other warm-blooded animals (World Health Organization (WHO) 2011). This study has shown that high numbers of *E. moraviensis* and *E. haemoperoxidus* can be isolated from the droppings of warm-blooded animals, particularly geese. Since these animals may harbour and excrete human pathogens, it is advisable to revise the guidelines and include *E. moraviensis* and *E. haemoperoxidus* as indicators of faecal pollution, pointing to animal/bird origin of the pollution.

The faecal contamination and load delivered by the geese in the vicinity of the abstraction wells, the presence of *E. moraviensis* and *E. haemoperoxidus* in geese faeces and abstracted water, the similarity of the *Enterococcus* species composition found in geese and abstracted water, and the potential presence of human pathogens in geese faeces were the basis for the water utility to design a preventive measure: fencing of the specific parts of the dune filtration area to keep geese away from the abstraction wells. This resulted in an improvement of the quality of abstracted water in this area.

CONCLUSIONS

In this study, faeces of geese, red foxes and rabbits have been shown to be the source of *E. moraviensis*. Geese have also been found to be carriers of *E. haemoperoxidus*. The *Enterococcus* species compositions in abstracted water samples and in geese droppings were very similar. Although the actual routes of the presumed contamination are not yet

known, large quantities of *E. moraviensis* in geese droppings and frequent identification of *E. moraviensis* in abstracted water, the presence of geese in specific parts of the dune filtration area, and the evidently high faecal load contributed by geese all indicate a probable influence on the quality of the abstracted water.

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