

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. *E. 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. haemoperoxidus*, 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 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, Velsbroek 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; Foulque 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 decade, MALDI-TOF mass spectrometry (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 Casticum 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 <i>Enterococcus</i> spp. isolates	
Red fox (<i>Vulpes vulpes</i>)	20	384
Rabbit (<i>Oryctolagus cuniculus</i>)	9	108
Dog (<i>Canis lupus familiaris</i>)	10	144
Goose (<i>Anser anser</i>)	20	231
Human	20	342
Sheep (<i>Ovis aries</i>)	11	126
Highland cattle (<i>Bos taurus</i>)	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 $D = \sum n(n-1)/N(N-1)$, 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 m²) 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

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

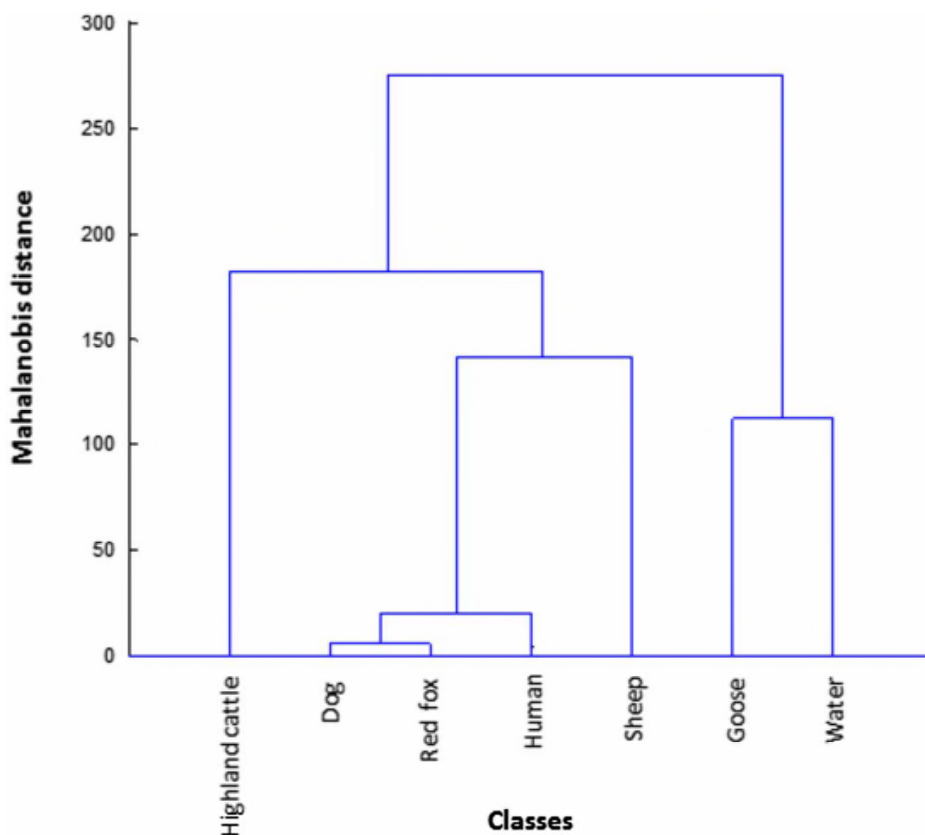


Figure 1 | Relationships between different classes (animal hosts and abstracted water) based upon their bacterial composition, using Mahalanobis distance (MATLAB).

