

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

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# Environmental growth of the faecal indicator *Enterococcus moraviensis*

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## ABSTRACT

Soil passage through sand dunes has previously been shown to remove enteric micro-organisms very effectively, and hence is used for the production of drinking water. However, enterococci have occasionally been isolated from abstracted water (after dune passage) in one of the dune infiltration areas in the Netherlands. *E. moraviensis* was the most frequently isolated species. Until now, no faecal sources of this species have been reported and the potential for growth under certain environmental conditions was reported for other *Enterococcus* species. The aim of this study was to determine the ability of *E. moraviensis* to grow in habitats present in the dune passage process (dune vegetation, sediment from abstraction wells, biofilm developed using abstracted water and soil). Different concentrations of boiled and filtered (0.45 µm) plant extracts obtained from dune vegetation supported growth (up to 6 log), with maximum concentrations after four to six days at 15 °C. Although *E. moraviensis* was shown to be able to attach to the biofilm, no growth was observed in biofilm or in sediment and soil. These observations confound the use of *E. moraviensis* as a faecal indicator.

## INTRODUCTION

Infiltration of pre-treated river water into sand dunes, with recovery after horizontal soil passage with a travel time of 60 days or more, is often used as one of the treatment steps in drinking water production in the Netherlands. For the Castricum dune infiltration area discussed in this study, effective removal of enteric micro-organisms has already been reported (Schijven *et al.* 1998). Recovered (abstracted) groundwater is the product of this process and is normally free of faecal indicator bacteria (FIB) and therefore considered free of enteric pathogens.

Enterococci are used as FIB, with 51 species currently recognized. They have been isolated from a variety of non-enteric habitats but are believed to have originated from faecal pollution by warm-blooded animals. They are not only natural members of the digestive microbiota in warm-blooded animals and humans (Flacklam *et al.* 2002; Layton *et al.* 2010), but may also be present in soil (Fujioka *et al.* 1999; Byappanahalli *et al.* 2012) and surface waters (Švec & Sedláček 1999), on insects and plants (Mundt 1963; Geldreich *et al.* 1964; Müller *et al.* 2001; Ott *et al.* 2001), in some foods – such as fermented products – and as probiotics (Aarestrup *et al.* 2002). The standard for enterococci in the EU Drinking Water Directive is 0 cfu/100 mL. In the Netherlands ISO 7899-2:2000 method is used for water quality monitoring, which implies that all *Enterococcus* species detected by this method serve equally to predict a potential health risk associated with drinking water. However, for some *Enterococcus* species, no faecal source has yet been reported (Lebreton *et al.*, 2014). *E. moraviensis* and *E. haemoperoxidus* are examples of such species, isolated from surface water without any evidence for faecal contamination (Švec *et al.*, 2001). Due to relatively high identification costs, information on the diversity of the *Enterococcus* species isolated from water is scarce. *E. moraviensis* has previously been isolated from water samples in the Netherlands, but could not be correctly identified using the Rapid ID 32 Strep biochemical identification test (Taučer-Kapteijn *et al.* 2013). With the introduction of new techniques, such as matrix-assisted laser desorption and ionization - time of flight mass spectrometry (MALDI-TOF MS) in water laboratories, means of identifying species have now become more readily available. Enterococci have occasionally been isolated from large samples (100 L) of water collected after infiltration and soil passage in the Castricum dune infiltration

area. This finding challenged the value of dune passage as very effective barrier against enteric micro-organisms. In 2011 and 2012, intensive monitoring of enterococci was conducted in this area to evaluate their potential breakthrough to the abstracted water. Enterococci were cultured from 67 (17.8 %) of 376 large samples (100 L), using the ISO method. From each of these 67 samples, between one and five isolates (183 isolates in total) were identified using a MALDI-TOF MS Biotyper. *E. moraviensis* was identified most often (n=74; 40.4%), followed by *E. faecalis* (n=62; 33.9%), *E. casseliflavus* (n=19; 10.4%), *E. haemoperoxidus* (n=8; 4.4%), *E. termitis* (n=6; 3.3%), *E. faecium* (n=5; 2.7%), *E. phoeniculicola* (n=5; 2.7%), *E. mundtii* (n=3; 1.6%) and *E. thailandicus* (n=1; 0.5%) (unpublished).

Information on environmental adaptations of enterococci and their growth on vegetation is scarce. Indication of environmental growth of enterococci on plant material has been observed in studies in *Cladophora* algae, where sun-dried algae stored for 6 months supported growth of enterococci to a density of  $10^7$  cfu g<sup>-1</sup> (Whitman *et al.* 2003). Further, Byappanahalli *et al.* (2003) demonstrated that *Cladophora* leachate readily supported in vitro multiplication of *Escherichia coli* and enterococci, suggesting that leachates contain necessary growth-promoting substances. The combination of observations on the effective removal of enteric micro-organisms by passage through the sand dunes, the potential for enterococci species to grow in the environment under certain conditions, the fact that the most abundant species in the abstracted water are *E. moraviensis* and *E. haemoperoxidus* and the fact that no faecal source has been reported yet for these species led to the hypothesis that the occurrence of these enterococci in abstracted water is the result of growth in the environment rather than breakthrough of faecal contamination.

In the dune filtration area, decaying vegetation may provide nutrients for the growth of microbes. The amount of nutrients percolating into the ground depends upon the growth cycle of plants and the season. During rainy periods, the nutrients will infiltrate into the soil and become available to bacterial community. If FIB are able to grow in the soil, this may reduce their usefulness as faecal indicators. The objective of this study is to determine if *E. moraviensis* is able to grow under ambient conditions that may occur in the percolating water and in the aquifer storage and recharge system. Specific habitats studied were extracts from plants growing in this area, sediments obtained from abstraction wells and biofilms in those wells. If *E. moraviensis* is able to grow in one of these environments, occasional release from them could explain its occurrence in large (100 L) samples taken from the abstracted water.

## METHODS

### Bacterial strain

*E. moraviensis* strain used in this study was isolated from abstracted water from the Castricum dune infiltration area (the Netherlands) using the standard ISO 7899-2:2000 method and identified using MALDI-TOF MS (Biotyper, Bruker). An inoculum flask containing  $10^4$  cfu mL<sup>-1</sup> of *E. moraviensis* in stationary phase was prepared according to van der Kooij (2001). For the identification of isolated strains, MALDI-TOF MS was used in accordance with the manufacturer's instructions. In each target plate run we used *E. coli* Bacterial Test Standard (BTS) provided by manufacturer to control the quality of the measurement. The measurements with BTS reliability of 2.3 were used in this study.

### Enumeration method

Colonies were enumerated using membrane filtration and Slanetz & Bartley Agar according to the ISO 7899-2:2000 standard method. In order to monitor the growth, 1 mL was filtered using 0.45 µm cellulose nitrate filter (Sartorius Stedim) and incubated on Slanetz and Bartley Agar (SBA) for 48 hours at 37 °C each time. SBA agar quality control was performed using ATTC 27270 *E. faecium* strain. Laboratory quality control in general was carried out in conformity with the quality requirements of ISO 17025.

## **AOC-free glassware**

Assimilable organic carbon-free (AOC-free) 1000 mL Erlenmeyer flasks, beakers and pipettes (Duran) were cleaned according to the AOC Manual (van der Kooij 2001).

## **Plant material**

In May 2013, a total of 800 g of plant material composed of leaf parts of *Ammophila arenaria* (80 %), *Rubus fruticosus* (10 %), *Plantago lanceolata* (5 %), and *Rumex acetosa* (5%) were collected in the vicinity of abstraction wells (Q 400) in the Castricum dune infiltration area (The Netherlands). Plant material was transported by sterile plastic bag to the laboratory, where collected plant leaves were aseptically cut into pieces of c. 3 cm length using sterile scissors and mixed to obtain a homogenous plant mixture, which was then stored at -20 °C for further use in preparation of plant extract agar and boiled plant extract. In August 2013, a total of 600 g of plant material composed of leaf parts of the most common dune plant (*Ammophila arenaria*) were collected at the same location and prepared in the same way and were used in experiments with filtered extract.

## **Plant extract agar**

Exactly 200 g of cut plant mixture was suspended in 1L of abstracted water and boiled for 2 minutes. After cooling down to room temperature (21 °C), the plant material mixture remained in the water for 12 h. Subsequently the plant leaves were filtered from the extract using sterile metal sieve (300 µm). The extract was boiled again for 10 min, and during this process 15 g L<sup>-1</sup> of technical agar (Oxoid) was added under sterile conditions. Finally, the plant extract agar was autoclaved at 121 °C for 15 min and poured into 10 sterile Petri dishes. When the solid plant extract agar cooled down to room temperature, approximately 50 cfu of *E. moraviensis* was inoculated onto the agar and spread out using sterile inoculation spreader (Sarstedt). As control, 5 plates with only technical agar (without addition of the plant extract) (Oxoid) were prepared following the manufacturer's instructions. The same amount of *E. moraviensis* was inoculated and spread over the technical agar. The cultures were kept at 15 °C for 14 days in a sterile plastic bag to prevent dehydration. Colonies grown on the plant extract agar were identified using MALDI-TOF MS.

## **Boiled plant extract**

Exactly 200 g of plant mixture and 1L of abstracted water were placed in an AOC-free beaker and boiled for 2 minutes. After cooling down to room temperature the plant material mixture remained in the water for 12 h. Thereafter the extract was decanted and sterilized for 15 min at 121 °C. In total 16 AOC-free 1L Erlenmeyer flasks were filled in duplicates with 60 mL plant extract in concentrations of 200 g L<sup>-1</sup>, 20 g L<sup>-1</sup>, 2 g L<sup>-1</sup> and 0.2 g L<sup>-1</sup>. Pasteurized abstracted water (30 minutes at 65 °C) was used to prepare plant extract dilutions and to prepare two control flasks (AOC-free 1L Erlenmeyer flasks filled with 60 mL pasteurized abstracted water). *E. moraviensis* (stationary phase) was inoculated into each flask to obtain a concentration of approximately 100 cfu mL<sup>-1</sup>. Flasks were finally incubated at 15 °C and the growth of *E. moraviensis* was tested every second day, using the SBA enumeration method as mentioned above.

## **Filtered plant extract**

Exactly 50 g of the plant material mentioned above was suspended in 1L of abstracted water for 5 hours at room temperature. Subsequently, the extract was decanted and filtered using sterile 0.45 µm filter (Millipore) to remove plant parts and bacteria. AOC-free Erlenmeyer flasks, containing plant extract (60mL) in concentrations of 50, 5 and 0.5 g L<sup>-1</sup> were prepared in duplicates. Filtered abstracted water (Millipore 0.45 µm) was used as a control experiment and (60 mL in duplicate) to prepare plant extract dilutions. *E. moraviensis* was inoculated into the flasks to obtain solutions of

approximately 100 cfu mL<sup>-1</sup>. The solutions were incubated at 15 °C and the concentration of *E. moraviensis* was measured daily using the SBA enumeration method.

### **Sediments in recharge mains**

Because sediments accumulate relatively fast in the pipes carrying abstracted water after dune filtration, we investigated whether or not these sediments may support the growth of *E. moraviensis*. Sediment (500 g) was taken from the bottom of an abstraction well in the Castricum infiltration area using a sterile 1.5 L dip flask. Sediment consisted of abstracted water (30%), sand, 'flocculated iron' and iron bacteria. Three equal series (in total 36 flasks) of sediment suspensions were prepared. Preparation of a series was as follows: while stirring the sediment subsamples (15, 45, 225, 450 or 900 mg) were distributed (in duplicates) into a total of 10 AOC-free 1L flasks. To each flask, aliquots (600 mL) of abstracted water were added. Two control flasks were filled with only abstracted water. All flasks were first pasteurized for 30 min at 65 °C and subsequently, *E. moraviensis* was added to each flask to obtain a concentration of c. 20 cfu mL<sup>-1</sup>. The first series was incubated (static) at 15 °C, the second at 20 °C and the third at 25 °C. Growth was examined every three days by shaking the suspensions for a moment and then analysing 1 mL for enterococci using SBA enumeration method.

### **Biofilm**

To examine whether *E. moraviensis* is able to attach to the biofilm and grow there, two experiments using biofilm monitors (BFMs) were conducted. A BFM is a device consisting of vertical glass columns containing glass cylinders stacked on top of each other. Water flowing downwards through the columns and cylinders is collected periodically from the system and investigated for total counts of the micro-organism of interest, as described earlier (Van der Kooij & Veenendaal 1992). The first experiment was conducted to test the attachment of *E. moraviensis* to the biofilm. At one of the abstracted water collection points, two biofilm monitors (BFM1 and BFM2) were installed for 42 days to allow biofilm to develop. The temperature of the abstracted water varied between 8 °C and 18 °C and the flow through the BFMs was 180 L h<sup>-1</sup>. After 42 days, BFM1 was closed at the bottom, filled with abstracted water (200 mL) and inoculated with 1 mL of a suspension of *E. moraviensis* (10<sup>4</sup> cfu mL<sup>-1</sup>) in stationary phase. Next, the suspension in BFM1 was shaken gently for 30 minutes. BFM1 was then flushed for one hour at a flow rate of 160 L h<sup>-1</sup>. Finally, the BFM1 column with the glass rings was disconnected from the biofilm monitor and taken to the laboratory at 4 °C. The biofilm material and attached *E. moraviensis* were collected from all 35 rings using low frequency ultrasonication (Branson 5200 ultrasonication bath), and subsequently enumerated using membrane filtration. All colonies were confirmed to be *E. moraviensis* using MALDI-TOF MS (Biotyper). BFM2 was not inoculated, but used as a control for possible background contamination with enterococci. BFM2 was treated in the same way as BFM1.

In the second experiment, we studied whether or not *E. moraviensis* is able to multiply in biofilm. Cleaned and sterilized, BFM1 and BFM2 were placed at the same collection point as in the first experiment. The temperature of the abstracted water varied between 9 °C and 18 °C. The flow rate was 160 L h<sup>-1</sup>. After 42 days, the column of BFM1 was disconnected, filled with 200 ml of abstracted water and inoculated with 1 mL of a suspension of *E. moraviensis* (2\*10<sup>4</sup> cfu mL<sup>-1</sup>) in stationary phase. The column was shaken slowly for 30 minutes, placed back in the monitor and flushed for one hour at a flow rate of 160 L h<sup>-1</sup>. To confirm the attachment to the biofilm, 13 rings were taken out of the column, put into sterilized tap water and transported to the laboratory for the enumeration of enterococci. The column with the remaining rings was placed back in BFM1. Biofilm on these rings was used as a growth medium for *E. moraviensis* for the 4 weeks. Therefore, BFM1 was switched on again with the flow rate of 160 L h<sup>-1</sup> and, to evaluate the growth in the biofilm, between four and 16 rings were taken weekly from BFM1 to enumerate the enterococci. On the same days, the same number of rings in BFM2 was tested for enterococci (SBA).

## Soil

A soil profile (30×30×30) was excavated at a 2 meter distance from abstraction well PCR11 in the dune infiltration area and transported to the laboratory, where a dark soil layer at a depth of 5 cm was extracted using sterile scoop. Small pieces of plant roots, Nematoda (13.8 per ml soil), a few Rotifera and Annelida were observed in this layer and a single Tardigrade. The composition of the extracted soil layer used in this experiment was analysed: 15.7% water, 84.3% dry residue (dr), 4.1% humus, 0.9% CaCO<sub>3</sub>, phosphate = 220 mg P/kg dr, Kjeldahl nitrogen = 2,000 mg N/kg dr, pH = 7.3. According to NEN 5104 this soil layer was classified as moderate silty sand.

Each of the three AOC-free Erlenmeyer flasks was filled with 600 mL of abstracted water and 50 g of extracted soil. The flasks were pasteurized for 30 minutes at 65 °C. When the suspensions had cooled to room temperature, *E. moraviensis* was inoculated up to a concentration of 100 cfu mL<sup>-1</sup> and flasks were incubated at 15 °C. In order to follow the growth, every 2 days the flasks were shaken for a moment and 1 mL samples then analysed for enterococci using the SBA enumeration method.

## RESULTS AND DISCUSSION

### Plant extract agar

Agar enriched with sterilized plant extract (200 g L<sup>-1</sup>) promoted the growth of *E. moraviensis* at 15 °C. Within 10 days incubation at 15 °C, with a rate of recovery of approximately 90%, on average 45 small, round, whitish colonies had formed on all 10 plant extract agar plates and were all identified as *E. moraviensis* using a MALDI Biotyper. No growth was observed at all on the five control agar plates. This result demonstrates the ability of *E. moraviensis* to grow on plant material collected in the Castricum dune filtration area at relatively low temperature (15 °C).

### Boiled plant extract

To investigate the dose effect of plant extract on the growth of *E. moraviensis*, 10<sup>2</sup> *E. moraviensis* cells were added to different concentrations of the extract. As shown in Figure 3.1, in the first 4 to 6 days a rapid increase in *E. moraviensis* was observed at all concentrations, with only a short lag phase. No growth was observed in control samples, but the concentrations remained constant for at least 18 days. The growth curves show similar slopes, but reach different levels depending upon the extract concentration. Lower concentrations reach distinctly lower maximum levels. After reaching the maximum level, the concentration remained more or less constant for at least 12 days.

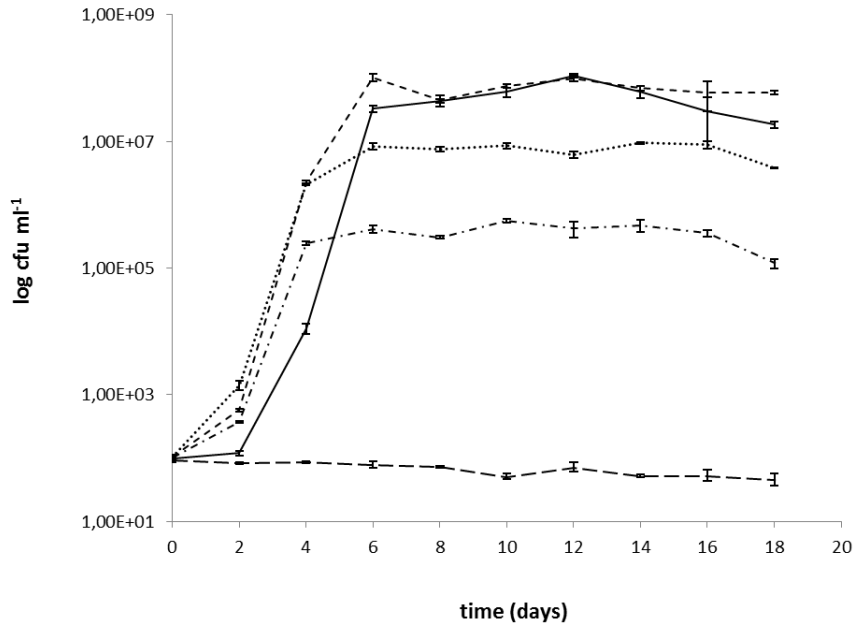


Figure 3.1. Growth of *E. moraviensis* at 15 °C on sterilized plant extract. Extract concentrations: (—) 200 g L<sup>-1</sup>, (---) 20 g L<sup>-1</sup>, (···) 2 g L<sup>-1</sup>, (- · -) 0.2 g L<sup>-1</sup> and (- - -) control. Bars indicate standard deviations of duplicate measurements.

Yields were  $2.2 \cdot 10^5$  cfu g<sup>-1</sup>,  $3.2 \cdot 10^6$  cfu g<sup>-1</sup>,  $3.4 \cdot 10^6$  cfu g<sup>-1</sup> and  $1.8 \cdot 10^6$  cfu g<sup>-1</sup>, respectively. It is also noteworthy that the yield at the highest concentration (200 g L<sup>-1</sup>) was lower than at 20 g L<sup>-1</sup>, which probably indicates a growth inhibition at the highest extract concentration.

### Filtered plant extract

The most common grass species in the dunes, *Ammophila arenaria*, was used to prepare plant extract and applied as a growth medium for *E. moraviensis* at 15 °C. To avoid degradation of larger organic molecules into smaller molecules during sterilization, we applied filtration of prepared plant extract through 0.45 µm pore size membrane filters to test a more natural formation of the extra nutrients available from plant materials. At all concentrations of filtered plant extract (50 g L<sup>-1</sup>, 5 g L<sup>-1</sup> and 0.5 g L<sup>-1</sup>), rapid growth occurred and a maximum was reached within 3 days. Yields were  $5.3 \cdot 10^3$  cfu g<sup>-1</sup>,  $8.3 \cdot 10^3$  cfu g<sup>-1</sup> and  $2.1 \cdot 10^4$  cfu g<sup>-1</sup>, respectively (Figure 3.2). No growth was observed in either of the two control samples. Decrease in concentrations was observed within 6 days of incubation.

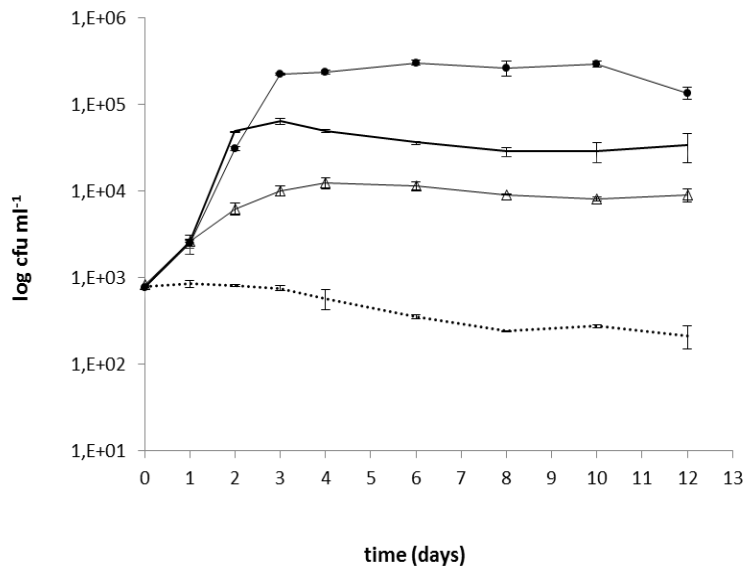


Figure 3.2. Growth of *E. moraviensis* on filtered (0.45 µm) plant extract at 15 °C. Extract concentrations: (●) 50 g L<sup>-1</sup>, (■) 5 g L<sup>-1</sup>, (Δ) 0.5 g L<sup>-1</sup> and (□) control. Bars indicate standard deviations of duplicate measurements.

Dune vegetation provides nutrients suitable to support the growth of *E. moraviensis* at 15 °C. Boiled and filtered plant extracts showed different yields ( $\Delta$ yields = c. 2 log/g), presumably due to more readily degradable molecules forming during boiling, although the difference in yields might also be the result of using two different batches of plant material. These findings are in line with earlier observations on environmental adaptations in enterococci. Mundt and co-workers (1962) demonstrated the ability of *E. faecalis* to grow on germinating seeds and plants; in studies using freshwater algae *Cladophora*, Whitman *et al.* (2003) demonstrated the survival of enterococci for more than 6 months in sun-dried *Cladophora* mats, with ready growth upon rehydration; and Byappanahalli *et al.* (2003) have demonstrated enterococcal growth, which was directly related to the concentration of algal leachate. Furthermore, it has been suggested that submerged aquatic vegetation indirectly facilitates persistence of *E. casseliflavus* in aquatic habitats (Badgley 2010).

### Sediments in recharged mains

No growth was observed in any of the five aliquots (15, 45, 225, 450 and 900 mg) of sediment at any of the three incubation temperatures (15 °C, 20 °C and 25 °C). The amount of sediment had no effect upon the mortality rate, so the average die-off for all sediment concentrations was calculated for each temperature. Higher temperatures reduced the period of bacterial survival. The exponential decay constants were -0.13 d<sup>-1</sup> at 15 °C, -0.22 d<sup>-1</sup> at 20 °C and -0.50 d<sup>-1</sup> at 25 °C (Figure 3.3).



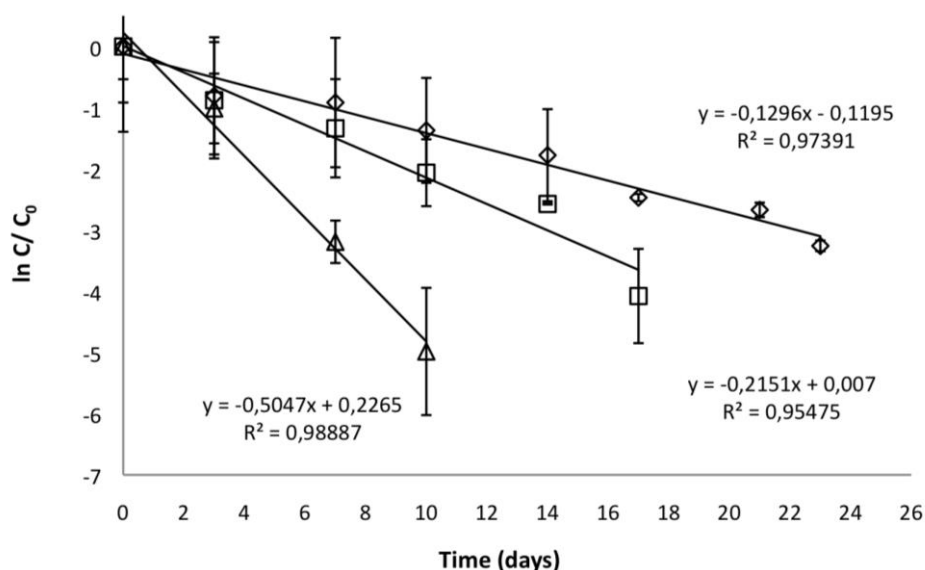


Figure 3.3. Die-off rate of *E. moraviensis* at 15 °C (◇), 20 °C (□) and 25 °C (Δ) in sediment. Bars indicate standard deviations of duplicate measurements.

Inactivation of *E. moraviensis* in sediments, described by exponential inactivation kinetics, with higher inactivation rates at higher temperatures, is in line with the temperature-dependent decay of enterococci already reported (Sinton *et al.* 1994, 2002; Noble *et al.* 2004). This is probably due to the increase in the rate of biochemical reactions at higher temperatures.

## Biofilm

First, the ability of *E. moraviensis* to attach to biofilm was studied. The average number of *E. moraviensis* bacteria attached to the rings in BFM1 was 16 cfu ring<sup>-1</sup>; std=6.4. In total, 3% of inoculated enterococci were found to attach to the biofilm within a relatively short time (30 min). In the control, BFM2, no enterococci were found.

In the second experiment, BFM1 with biofilm was inoculated and used to determine the growth of *E. moraviensis*. For the initial measurement, 13 out of the total of 43 rings were analysed (Table 3.1). The number of cfu ring<sup>-1</sup> varied between 21 and 60. On average, 34 cfu ring<sup>-1</sup> (std=11.8; n=13) were obtained. Comparing these results with the first experiment, the attachment of *E. moraviensis* was confirmed and it seems that adhesion capacity is related to the concentration applied. Within a week, no growth was observed, but the number of *E. moraviensis* had decreased to an average of 5 cfu ring<sup>-1</sup> (std=4.5; n=4) and after 3 weeks *E. moraviensis* was not detected in biofilm formed on the rings. So die-off of *E. moraviensis* was observed in the biofilm, rather than any growth.

Table 3.1. Die-off of *E. moraviensis* in biofilm. Values are the average number of cfu per ring from a number (n) of rings.

No. of rings	day 0	week 1	week 2	week 3	week 4
n=13	34 (std=11.8)				
n=4		5 (std=4.5)			
n=6			0.2 (std=0.4)		
n=4				0.5 (std=0.6)	
n=16					0.0 (std=0)

On the sampling days, the same number of rings from BFM2 were tested for the presence of enterococci to control for background contamination. Interestingly, 2 cfu of *E. moraviensis* were observed in one of four rings in BFM2 in the second week, which means that the abstracted water used to feed the BFMs during the experiment still incidentally contained this species. No *E. moraviensis* was found in BFM2 in the subsequent weeks, indicating that these micro-organisms were transient occupants of the biofilm, not able to multiply on biofilm. Even though the attachment of *E. moraviensis* to biofilms was demonstrated, this species was not able to colonize the biofilm and its numbers in the biofilm declined once the flow of water was restored. This indicates that *E. moraviensis* is unable to compete with the other bacteria in the biofilm under these conditions.

## Soil

To answer the question of whether the dark soil layer at the depth of 5 cm obtained in the dune infiltration area can serve as a nutrition source for *E. moraviensis*, we followed the growth of this species at 15 °C in a pasteurized suspensions of 50 g of soil and 600 mL of abstracted water. In none of the triplicates was growth observed. The exponential decay constant was  $-0.61 \text{ d}^{-1}$ . Numbers remained relatively stable up to 5 days, after which they started to decrease. This can be explained by the fact that due to the large percentage of sand and the small amount of organic matter the type of soil used in this study was less humus-rich soil.

Besides *E. casseliflavus*, *E. mundtii*, *E. sulfureus*, and *E. haemoperoxidus*, *E. moraviensis* belongs to the most common *Enterococcus* species in the environment (plants, soil and water) (Euzéby 2009). According to the information summarized by Švec & Devrieze (2009), the enterococci are a temporary part of the microflora of plants, probably disseminated by insects. The soil is not naturally inhabited by enterococci but can be contaminated from animals, plants, wind or rain. In waters the presence of enterococci is considered an indication of faecal contamination. The fact that *E. moraviensis* has not yet been associated with animal material, but has been commonly found in the environment and has been shown to grow in the presence of plant extracts, confound the use of this species as a faecal indicator. The reveal the environmental significance of *E. moraviensis*, it is important to investigate whether its faecal origin can be found. A sanitary survey of the area where *E. moraviensis* was observed and extended screening for *Enterococcus* species in faecal samples and in plants is needed to answer this question. Applying the results of this study to the practical situation in the Castricum dune infiltration area, where the surface is generally covered with vegetation, it is possible that leaf litter becomes soaked with rainwater and consequently that nutrient-enriched water percolates into the ground, which may induce the growth of *E. moraviensis* in topsoil in this area. This study has demonstrated that *E. moraviensis* is able to grow on plant material on or in the top layer of the soil, but does not grow in the abstraction wells (sediments and biofilm) or in soil in the conditions tested. Therefore, the presence of *E. moraviensis* in the abstracted water may be the result of its growth on material from decaying plants rather than faecal contamination. Assuming this occurs primarily in the topsoil, the presence of *E. moraviensis* in abstracted water suggests a contamination pathway from the topsoil through the soil to the abstraction wells.

## CONCLUSION

Exponential growth of *E. moraviensis* was observed in different concentrations of plant extracts obtained from dune vegetation. It has also been demonstrated that *E. moraviensis* was not able to grow in the sediments or biofilm obtained from abstraction wells or on soil from the Castricum dune infiltration area used in this study. The fact that *E. moraviensis* has not yet been isolated from faecal matter and that it is able to grow on plant material makes this species less suitable as a faecal indicator. The experiments in this study were conducted using filtrated media, in the absence of the natural microbiome. To estimate the capacity of enterococci to grow under natural circumstances in the presence of competing micro-organisms, additional experiments in the presence of the natural

microbiome are required. The occurrence of *E. moraviensis* in abstracted water may not necessarily indicate faecal pollution, but it does appear to indicate that bacteria from the unsaturated soil layer are able to reach the groundwater.

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