Occurrence of enterococci in the environment and their value as an indicator of water quality

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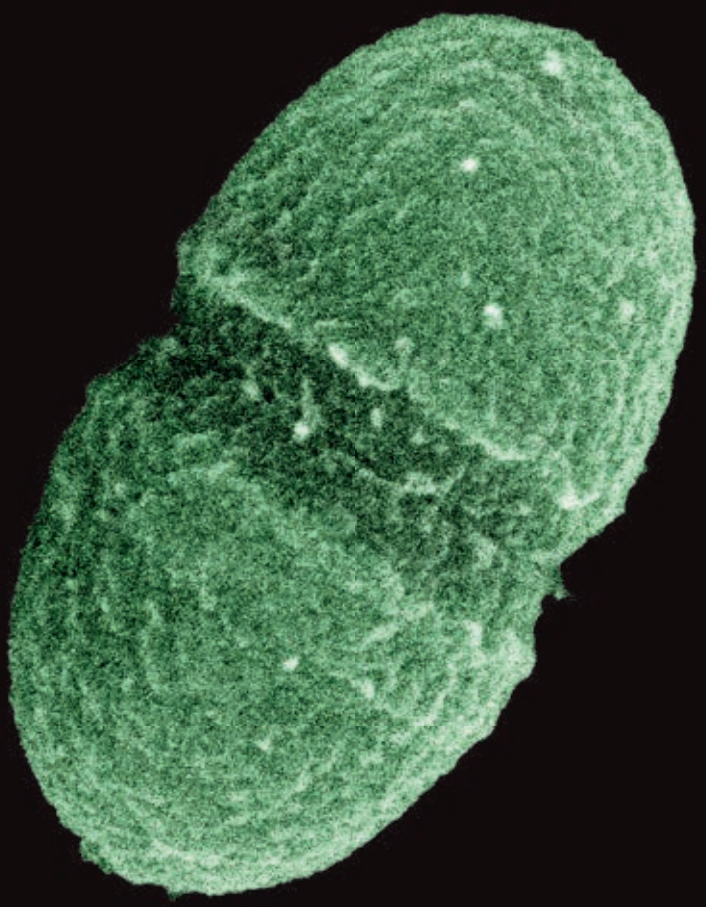

Maja Taučer-Kapteijn

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Occurrence of enterococci in the environment and their value as an indicator of water quality

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voorzitter van het College voor Promoties,
in het openbaar te verdedigen op
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door

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Moji mami in očetu

Aan Kees, Eva en David

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Chapter 1

Introduction

Health risk of enteric pathogens in water systems – the indicator concept

Enteric pathogens are excreted in the faeces of infected humans or animals and may contaminate water intended for human consumption (Figueras and Borrego, 2010). There are many different enteric microorganisms that are known to infect humans. These include bacteria such as *Salmonella*, *Campylobacter*, EHEC and *Shigella*. Also viruses infect humans such as enterovirus, norovirus, rotavirus, adenovirus, Hepatitis A and E virus. And parasitic protozoa such as *Cryptosporidium* and *Giardia* and parasitic worms (helminths). Worldwide approximately 842,000 people are estimated to die each year from diarrhoea as a result of consuming unsafe drinking-water, or through poor sanitation and/or insufficient hand hygiene (Anonymous, 2016). While the risk of outbreaks of waterborne diseases increases where standards of water, sanitation and hygiene are low, outbreaks are not limited to the underdeveloped world. In developed countries water treatment processes and regulations have greatly reduced the transmission of pathogens through public drinking water supplies, but water borne diseases still occur. A number of drinking water related outbreaks has occurred in Europe. For example, in Spain during the period of 1999–2006, 413 outbreaks were recorded that involved 23,642 cases (Martín Granado et al., 2008; Figueras and Borrego, 2010). These outbreaks occurred despite specific legislation designed to prevent them and the associated microbial control measures being carried out (Figueras & Borrego, 2010). Between 2000 and 2007, 13 of the total 14 European reporting countries (Belgium, Croatia, the Czech Republic, Estonia, Finland, Greece, Hungary, Italy, Lithuania, Norway, Slovakia, Spain, Sweden and the United Kingdom) reported a total of 354 outbreaks of disease arising from drinking water, resulting in over 47,617 cases of illness (Anonymous, 2009). Waterborne disease outbreaks still occur in the United States too (Cutler and Miller, 2005). The US Centre for Disease Control (CDC) reported 780 disease outbreaks associated with the consumption of contaminated drinking water from 1971 to 2006, resulting in 577,094 cases of illness (Craun et al., 2010). During 2011–2012, 32 drinking water associated outbreaks were reported to the US CDC Waterborne Disease and Outbreak Surveillance System, accounting for at least 431 cases of illness, 102 hospitalizations and 14 deaths (Beer et al., 2015). During the same period 90 recreational water associated outbreaks resulting in 1788 cases, 95 hospitalizations, and one death being reported to CDC (Hlavsa et al, 2015).

These data indicate that even in developed countries people may still get ill from drinking contaminated water. To prevent infections from water borne enteric pathogens, legislation in Europe, USA and other countries requires analysis of faecal indicator bacteria (FIB) to determine the microbiological quality of drinking and bathing water. Ideally, water should be analysed for the presence of enteric pathogens, but analysing all possible enteric pathogens is time-consuming and costly, and methods are not wide-spread. Since the common source of all of these pathogens is faecal pollution, FIB have been introduced to evaluate the microbiological quality of water. The detection of FIB plays an important role in the protection against infections caused by enteric pathogens via water. It allows for relatively quick and easy monitoring of microbiological water quality, indicating the level of contamination. It can also be used to determine the extent of elimination of (bacterial) pathogens during water treatment.

Indicator bacteria for faecal contamination of water

E. coli is globally most commonly used as microbial parameter for testing drinking water quality. Besides *Escherichia coli*, Enterobacteriaceae family (coliforms), *Enterococcus* spp. (enterococci) and *Clostridium perfringens* have been used for a long time as FIB, due to their high incidence in the faecal matter of humans and warm-blooded animals and due the availability of relatively fast and inexpensive methods to isolate and enumerate these organisms from water. Their presence in water indicates that faecal contamination has occurred and that pathogens may also be present, when the faecal source (host) was infected. Ashbolt et al. (2001) indicate that there is no universal indicator that covers all pathogens, but a spectrum of indicators for bacterial, viral and parasitic protozoans may cover most relevant pathogens of faecal origin. Different indicators may serve different purposes, from assessing the level of faecal contamination in source or bathing waters, determining the efficacy of water treatment processes for different types of pathogens, for assessing integrity of drinking water distribution networks, for tracking faecal sources etc. The validity of the use of indicators for these purposes is affected by differences in rates of removal or inactivation in water treatment processes, survival in water and other matrices (Ashbolt et al., 2001). Furthermore, enteric illness is not only caused by enteric bacteria, but may result from infection from pathogenic viruses, protozoa or parasitic worms (helminths). The viruses and protozoa have different environmental behaviour and survival characteristics than bacteria, which

means that faecal bacteria are not always an adequate indicator of their presence or absence (Medema et al., 2003a). The coliforms have been found to differ considerably from faecal pathogens in occurrence and resistance to stress (Desmarais et al., 2002; Harwood et al., 2005; Savichtcheva and Okabe, 2006). The occurrence of outbreaks of viral illnesses associated with drinking water meeting the coliform standards indicated that coliforms were an inadequate parameter to assess the virological quality of treated drinking water (Berg and Metcalf, 1978; Petrilli et al., 1974; Melnick and Gerba, 1982). Waterborne outbreaks of giardiasis and cryptosporidiosis, which have been reported in industrialised countries (Craun, 1990; MacKenzie et al., 1994; Craun et al., 1998; Craun et al., 2010) have also demonstrated the shortcoming of coliforms as an indicator for microbiologically safe drinking water. Groundwaters that are under the influence of surface waters or other contamination sources (i.e. surface run-off) can be contaminated with low levels of *Cryptosporidium* spp. and *Giardia* spp. (Hancock et al., 1997). They can also cause waterborne illness (Craun et al., 1998). Treatment of these waters with desinfectant using chlorine alone offers no protection against *Cryptosporidium* spp. and only limited protection against *Giardia* spp. (Medema, 1999). Given the shortcomings of the faecal indicator bacteria to ensure safety, there was a need of a more effective means for consistently ensuring the safety of a drinking-water supply. As a result theWorld Health Organisation (WHO) proposed Water Safety Plans (WSP's) in 2004. WSP's require a proactive risk assessment/risk management approach, based on understanding the hazards and hazardous events that may occur to a water supply system, and understanding of the efficacy of the control measures to adeqautely address these hazards. This covers all steps in the water supply from catchment to consumer, and ensures all controls are operating adequately by monitoring the control measures (WHO, 2016). Faecal indicator bacteria still do have a place in the WSP approach, in order to help in understanding hazards and controls, and to verify the adequacy of the control measures (WHO, 2016).

Enterococci as faecal indicators

This thesis focuses on enterococci in the environment, the occurrence and behaviour of different species of enterococci and their value as an indicator of faecal contamination. Studies evaluating enterococci as faecal indicators of surface water quality have shown that they have a strong correlation with swimming associated illness in both marine and fresh water (Kay et al., 1994; Wade et al., 2010; Bonilla et al., 2010; Heaney et al.,

2012). Enterococci tend to persist longer in the environment than coliforms and are less numerous than faecal coliforms and *E. coli* in human faeces, but are still sufficiently numerous to be detected after significant dilution (Stevens et al., 2003).

Enterococcus species isolated from enteric and non-enteric habitats

Enterococci are common inhabitants of the intestines of humans and animals. Faecal contamination of water bodies will lead to the presence of enterococci in these water bodies. There are several *Enterococcus* species. The use of different identification techniques provided information on the occurrence and the incidence of particular *Enterococcus* species in the excreta of different hosts. *E. faecalis* and *E. faecium* are considered to be the most abundant enterococci in human faeces (Devriese et al., 1994; Finegold et al., 1983; Noble, 1978; Patel et al., 1998). But a number of other species including *E. avium*, *E. hirae*, *E. durans*, *E. gallinarum*, *E. casseliflavus*, *E. mundtii*, *E. caccae* and *E. raffinosus* have also been isolated from human stools (Carvalho et al., 2006; Layton et al., 2010).

Devriese et al. (1987) reported *E. faecalis*, *E. faecium*, *E. hirae*, and *E. durans* as the most commonly isolated *Enterococcus* species from farm animals, however other species have been found occasionally or in particular age groups such as *E. cecorum* in older poultry (Aarestrup et al., 2002). *E. avium*, which was originally described as emanating from human faeces (Guthof, 1955) is common in chicken faeces (Nowlan and Deibel, 1967). In preruminant calves, the enterococcal flora mainly consists of *E. faecalis*, *E. faecium*, and *E. avium*, but later this flora is gradually replaced by *E. cecorum* (Devriese, et al., 1992a). Mostly *E. faecalis* and *E. faecium*, but also *E. hirae* and *E. cecorum* have been found from the intestines of swine (Devriese and Haesebrouck, 1991; Devriese et al., 1994). *E. faecalis* is also predominant in the intestinal flora of cats and dogs (Devriese et al., 1992b). However other species including *E. avium*, *E. raffinosus*, *E. durans*, *E. cecorum*, *E. gallinarum*, *E. canis* and *E. canintestini* (Devriese et al., 1992b; De Graef, et al., 2003; Naser, et al., 2005) have been found in cats and dogs.

In faecal samples of wild birds *E. faecium*, *E. durans*, and *E. gallinarum* have been detected (Silva, et al., 2011; Han, et al., 2011). Enterococci have been isolated also from wild boars, partridges and fish (*Liza ramada*) (Almeida, et al., 2011), red foxes (Radhouani,

et al., 2011) and wild rabbits (Silva, et al., 2010). The characterization of *Enterococcus* species was however not reported in these animals.
A variety of insects, including beetles, flies, bees, termites, and worms have been found to harbor enterococci (Martin and Mundt, 1972). A survey of *Drosophila*, Cox and Gilmore (2007) found *E. faecalis*, *E. faecium*, *E. gallinarum*, and *E. durans*, and localized them to the digestive tract. These findings were confirmed by other studies in which *Enterococcus casseliflavus*, *E. gallinarum*, *E. faecalis*, *E. faecium*, and *E. hirae* were isolated from insects (Macovei and Zurek 2006, Graham et al., 2009; Channaiah et al., 2010; Ahmad et al., 2011). The study on enterococci in human, other mammalian and avian faecal samples by Layton et al. (2010) suggested that no single species of *Enterococcus* is reliable as an exclusive indicator of human faecal pollution.

Alongside the excreta of humans and animals, enterococci have also been isolated from non-enteric environments. Some studies suggested that *Enterococcus casseliflavus* and *Enterococcus mundtii* may be abundant in environmental reservoirs (such as on plants) than other enterococcal species (Bahirathan et al., 1998; Ferguson et al., 2005; Wheeler et al., 2002). Some studies demonstrated high numbers of enterococci on flowering vegetation (Mundt, 1963), on forage crops (Müller et al., 2001), on *Cladophora* algae (Whitman et al., 2003), on beach wrack (Anderson et al., 1997; Grant, et al., 2001; Imamura et al., 2011), submerged aquatic vegetation (mostly *Hydrilla verticillata*) (Badgley et al., 2010a; Badgley et al., 2010b) and decaying vegetation on both fresh and marine beaches (Byappanahalli et al., 2003; Imamura et al., 2011). *Enterococcus* species have been recovered from sand from freshwater and marine beaches (*E. faecium*, *E. casseliflavus*, *E. durans*), and from marine sediments (*E. faecalis*, *E. faecium*, *E. hirae*, *E. casseliflavus*, and *E. mundtii*) (Ferguson et al., 2005).

Numerous recently characterized *Enterococcus* species such as *E. moraviensis*, *E. haemoperoxidus*, *E. rotai*, *E. ureilyticus*, *E. aquamarinus*, *E. rivorum*, *E. silesiacus*, *E. ureasiticus* and *E. quebecensis* have been isolated from water (Švec, et al., 2001; Švec, et al., 2005; Švec et al., 2006; Niemi, et al., 2012; Sistek, et al., 2012). Whether they are inhabitants of warm-blooded animals is still unknown.

Human health risk

All sources of *Enterococcus* in water, emanating from faeces of humans, livestock and wild warm-blooded animals, may contain pathogens that can infect humans. The risk of pathogen presence is the highest in human faeces. The subgroup named 'intestinal enterococci' (*E. faecium*, *E. faecalis*, *E. durans* and *E. hirae*), consisting of the most frequently isolated species from human and animal faeces, has been separated from other enterococci and suggested as being more specific for faecal pollution (Anonymous, 2011). Note however, that the method used for "intestinal enterococci," in the European Union drinking and bathing water quality standards (ISO 7899: 2000-2) is not only specific for just these four species; other species may also be isolated using this method (Byappanahalli et al., 2012a).

The human risk from water contaminated with wild animal faeces has been assumed to be lower than from human faeces, because the probability of human pathogens being present is highest in human faeces and in part because viruses, a common cause of illnesses from exposure to faeces, are highly host-specific. For example, human enteric viruses (such as noroviruses, hepatitis A and E viruses, rotaviruses and enteroviruses) in water originate predominantly from human faecal material (Medema et al., 2003b). *Shigella* spp. is also responsible for many waterborne disease cases and a large proportion of the deaths from waterborne disease (Traverso, 1996), and is almost exclusively of human faecal origin. Therefore, several studies proposed that the identification of human-specific enterococcal species or genotypes could aid in distinguishing between human faecal contamination and other environmental sources of the organisms. It has been suggested that *E. faecium* containing the enterococcal surface protein (*esp*) gene may be human-specific (Scott et al., 2005), but *esp*-containing *E. faecium* can also be found in a selected number of animal hosts (Layton et al., 2009; Whitman et al., 2007). The ratio between enterococci and *Escherichia coli*, which has been suggested to be used in order to indicate whether the contamination is from human or animal sources, is considered unreliable (Payment et al., 2003).

Human pathogens, such as *Campylobacter* spp., *Salmonella* spp. and *Cryptosporidium* spp., can be present in both human and animal wastes (Fenlon, 1981; Fayer et al., 1997). As reviewed by Medema et al. (2003b), waterfowl, calves, and rodents have been described as carriers of *Cryptosporidium* spp. and *Giardia* spp. *Cryptosporidium* spp. has additionally been found in sheep, swine and poultry. *Campylobacter* spp. has been isolated from

waterfowl and rodents, whereas *Salmonella* spp. has been isolated from cattle, pigs, sheep and waterfowl (Medema et al., 2003b). Genotyping of environmental isolates is important to determining the presence of genotypes that are pathogenic to humans, especially in more pristine environments (Medema, 1999; Medema et al., 2009). As reported by Chalmers (2012), most commonly associated species with human cryptosporidiosis and their hosts are *Cryptosporidium hominis* (humans), *C. parvum* (humans, ruminants) and *C. meleagridis* (homoiothermic animals: birds and mammals including humans). Less common, *C. canis* (dog), *C. cuniculus* (rabbits, humans), *C. felis* (cat), *C. ubiquitum* (various mammals) and *C. viatorum* (humans) are also associated with human cryptosporidiosis. The human-infectious potential of many wildlife-adapted *Cryptosporidium* is currently unknown and the UK outbreak caused by *C. cuniculus* should serve as a caution against assuming that these unusual species and genotypes are not significant (Chalmers et al., 2009; Robinson et al., 2011). Furthermore, one of the more common forms of pathogenic Enterohemorrhagic *E. coli* (EHEC) O157:H7 and other EHEC variants, are zoonotic pathogens associated with severe human illnesses. Ruminants such as cattle are considered as the dominant natural reservoir (Muniesa et al., 2006). Waterborne transmission of EHEC has been demonstrated in drinking waterborne outbreaks (Craun et al., 2010), where drinking water was contaminated with ruminant faeces (Hrudey et al., 2003; Olsen et al., 2016). Epidemiological data also indicate a relationship between adverse health effects and swimming in nonpoint source-affected waters (Haile et al., 1999).

Persistence and growth in non-enteric environments

Although enterococci are believed to originate from the enteric environment, the occurrence of enterococci in non-enteric and apparently uncontaminated environments challenges this belief. When enterococci are released from the gastrointestinal tract of warm-blooded animals into secondary habitats, such as environmental waters aquatic vegetation or sediment, they are subjected to a series of biotic and abiotic stressors (sunlight, salinity, starvation, predation) that generally lead to a decline in the enterococci concentration over time (Byappanahalli et al., 2012a). However, many studies have clearly demonstrated the persistent nature and even the growth of some *Enterococcus* strains in extra-enteric habitats. Whitman and colleagues (2003) reported that the algal mats (*Cladophora*) collected along shorelines of southern and northern Lake Michigan in the Great Lakes contained a significant source of *E. coli*

and enterococci. In addition to these faecal bacteria, enteric pathogens such as *Shigella*, *Campylobacter*, and *Salmonella* were also isolated (Whitman et al., 2003). The findings of Whitman et al. (2003) have been confirmed by other studies of the Great Lakes (Verhougstraete et al., 2010). The high densities of enterococci in fresh *Cladophora* have been attributed to *in situ* growth (Byappanahalli et al., 2003). Enterococci grew over 100-fold in undiluted algal leachate at 35°C in 24 hours, suggesting that algal leachates (*Cladophora*) provide nutrients to sustain these bacteria (Byappanahalli et al., 2003). Although *Cladophora* is perennial in nature and it overwinters, leaving behind scattered basal stumps, there have been no reports of residual enterococci or other FIB surviving in these stumps under wintery conditions (Byappanahally et al., 2012a). In laboratory studies, Badgley et al. (2010a) described that enterococci survived longer and at much higher densities in mesocosms containing submerged aquatic vegetation (SAV) than in those without SAV. Furthermore, the recovery of a dominant *E. casseliflavus* strain indicated that this genotype was likely adapted to this vegetation (Badgley et al., 2010a). In other studies, enterococci have been isolated from plankton and macro-invertebrates (Maugeri et al., 2004; Signoretto et al., 2004). Signoretto et al. (2004) suggested that attachment to plankton contributes to the prolonged survival of *E. faecalis*. Some studies further suggest that populations of enterococci might be endogenous in sediments and soils and not exclusively of faecal origin (Byappanahally and Fujioka, 2004; Desmarais et al., 2002). Where enterococci have been isolated from fresh and marine water sediments (Ferguson et al., 2005; Obiri-Danso and Jones, 2000) and sand (Halliday and Gast, 2011; Yamahara et al., 2007), it has been reported that some sediments, soils (Byappanahalli and Fujioka, 2004; Mote et al., 2012) and beach sands (Yamahara et al., 2007) may also harbor enterococci. Rehydration has been observed to promote growth of enterococci in extra-enteric environments. Whitman et al. (2003) reported that enterococci survived in sun-dried algal mats stored at 4°C for over 6 months and displayed the ability to grow to high concentrations (10^8CFU/g) upon rehydration (Whitman et al., 2003). Similarly, Moriarty et al. (2012) observed the ability of enterococci to replicate in pooled simulated Canadian goose faeces in summer and winter. When initial enterococcal die-off was observed, between days 16 and 28, their concentration increased 10-fold which was associated with rainfall and rapid (re)hydration of faeces (Moriarty et al., 2012). Studies conducted in beach sand-filled columns found that transient growth of enterococci occurred after intermittent wetting of sand (Yamahara et al., 2009). Similar observations were made in soil. In one mesocosm study, densities of seeded *E. faecalis* remained nearly constant (6.0 log CFU/g dry soil) for 8 days when the moist soil (35% moisture, corresponding to a 60%

water-holding capacity) was allowed to desiccate (to the level of 12% moisture) under laboratory conditions at a temperature of 25°C. *E. coli* densities, on the other hand, declined drastically from 6.0 log CFU/g to 1 CFU/g in 4 days, but returned to the original levels upon rehydration (Byappanahalli and Fujioka, 2004).

Although enterococci are relatively common in some tropical soils (Byappanahalli et al., 2012b; Fujioka et al., 1999; Hardina and Fujioka, 1991), it has been argued that soil environments provide the necessary niche for populations of FIB to survive, adapt, and grow (Fujioka and Byappanahalli 2003; Ishii and Sadowsky, 2008; Winfield and Groisman, 2003). Studies of growth characteristics in these environments are rather limited (Byappanahalli et al., 2012a). An increasing concern is that enterococci and *E. coli* may not be reliable faecal indicators in all climatic zones; therefore additional alternative indicators (*C. perfringens*, coliphages) were proposed for these regions (Anonymous, 2010b). The paucity of available nutrients may limit the growth of enterococci in soil environments, however a likely habitat that provides conditions for growth is the plant rhizosphere, where microbial activity is known to be several fold higher than in the adjacent bulk soil (Sorensen, 1997; Byappanahalli et al., 2012a). The activity of protozoa and nematodes that graze on bacteria also appears to be more abundant in the rhizosphere (Anonymus, 2016a).

The replication of enterococci under natural conditions is likely to be limited because of desiccation, the paucity of, and competition for, available nutrients and other environmental stresses like UV sunlight, salinity, starvation, and predation (Byappanahalli et al., 2012a). There is a need to characterize the range of conditions (such as nutrients, moisture and temperature) under which these bacteria proliferate. Little is known about the interspecies diversity regarding the ability of enterococci to grow under certain extra-enteric conditions and the ability of pathogens to proliferate under the same conditions. Such information might be useful for the evaluation of different *Enterococcus* species as faecal indicators for water quality surveillance, particularly in more pristine water environments.

Characteristics of enterococci and detection methods

Taxonomy

Enterococci are facultatively anaerobic, Gram-positive bacteria, able to grow in the presence of 6.5% NaCl, 40% bile salt, at pH 9.6 and in temperatures ranging between 10 ºC and 45 ºC (Manero and Blanch, 1999). According to The List of Prokaryotic Names with Standing in Nomenclature (Anonymous, 2016b) there are currently 55 species described belonging to the *Enterococcus* genus. The taxonomy of the enterococci has changed considerably. The genus consisted of only 20 species at the end of the 20th century, however numerous new species have subsequently been described as the result of improvements in methods for differentiation (Euzéby, 2013).

Detection methods

In The Netherlands enterococci are usually isolated from water samples using membrane filtration in combination with Slanetz & Bartley agar (SBA) according to the standard method ISO 7899-2:2000 (Anonymous, 2000). This ISO method enables the detection of the species that have been reported as the predominant intestinal enterococci *E. faecalis*, *E. faecium*, *E. durans* and *E. hirae* (Anonymous, 2011), however many other enterococcal species can also be detected using this method. The use of the ISO method implies that all typical colonies on SBA that are confirmed to hydrolyze esculin in the presence of bile, by using bile esculin azide agar (BEAA), serve equally to predict a potential health risk associated with drinking water. However, it has been reported that genera *Pediococcus*, *Lactococcus*, *Aerococcus* and *Leuconostoc* may also occasionally exhibit positive reactions to Slanetz & Bartley agar tests when the colonies are confirmed by use of bile-esculin-agar. This may lead to false-positive results of the enterococci assay and unfavourable judgements about the quality of the water resulting in unnecessary restrictive consequences (Devriese et al., 1993; Leclerc et al., 1996; Pinto et al., 1999).

Quantitative Polymerase Chain Reaction (qPCR) assays that target the 23s rRNA operon have also been developed (Haugland et al., 2005; Ludwig and Schleifer, 2000) to detect *Enterococcus* spp. In the US a standard method for measuring enterococci in water has been developed by the Environmental Protection Agency (EPA). The EPA uses a quantitative polymerase chain reaction (QPCR) in conjunction with the TaqMan probe system (Anonymous, 2010a).

All these methods provide for the detection of a number of *Enterococcus* spp., which contains many species and which may have specific hosts or different environmental survival and growth characteristics. To better understand the differences in sources and the survival of different *Enterococcus* species in the environment, as well as to understand the suitability of different *Enterococcus* species as an indicator for human health risk, reliable identification techniques are needed.

Identification of *Enterococcus* species using MALDI-TOF MS

The identification of cultured microbial isolates in water laboratories in The Netherlands relied, until recently, on methods based on biochemical tests such as API (bioMerieux). The biochemical systems are limited in the sense that they are laborious, time-consuming and less reliable when it comes to environmental isolates. Due to relatively high identification costs, information on the diversity of the *Enterococcus* species isolated from water is scarce. 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, the identification of species-level has now become more readily available. This technique allows for the identification of microbial species within a few minutes by analyzing mass spectra of peptides and small proteins. Such a pattern was shown to be characteristic for microbial species (Mellman et al., 2008; Fenselau and Demirev, 2001; Holland et al., 1996; Krishnamurthy et. al, 1996). In this thesis, the reliability and the efficiency of MALDI-TOF MS identification for enterococci isolated from water is evaluated (Chapter 2).

Source tracking of enterococci using MALDI-TOF MS identification

Part of the research described in this thesis has been initiated due to the need to evaluate the occurrence and sources of enterococci in the Castricum dune infiltration area in The Netherlands. Infiltration of pre-treated river water into the dune area, with recovery after horizontal soil passage with a travel time of 60 days or more, has been used for over 60 years at several sites and has been considered to be the most important step for the removal of micro-organisms in drinking water production in The Netherlands. Recovered (abstracted) groundwater is the product of this process and is normally free of FIB, and therefore considered free of enteric pathogens (Schijven et al.,

1998; Hornstra, 2014). Low numbers of enterococci however have also been occasionally detected in large volume samples (100 L) in the recovered (abstracted) water. These observations occurred usually in the warmer period of the year. Understanding of the origin of these enterococci in abstracted water may lead to the identification of a specific animal host as a source of (faecal) pollution, or the environmental conditions that favor the survival or support the growth of these enterococci. Understanding the source is crucial for assessing associated health risk as well as determining actions necessary to solve the problem.

To study the sources of enterococci in abstracted water two approaches have been used:

1) MALDI-TOF MS has been applied for the intensive monitoring of enterococci species in abstracted water (large volume samples). The most abundant species isolated from the abstracted water was *E. moraviensis*. The combination of observations on the effective removal of enteric micro-organisms by passage through the dunes, the potential for enterococci species to grow in the environment under certain conditions, and the fact that no faecal source has been reported yet for *E. moraviensis*, led to the hypothesis that the occurrence of this species in abstracted water may be the result of growth in the environment rather than via a breakout of faecal contamination. The experiments aimed to answer the question whether biofilm, sediments from abstraction wells, as well as soil and plant extracts obtained from common dune vegetation may promote the growth of enterococci. If enterococci are able to multiply in one of these non-enteric habitats, it is important to know whether the ability to grow under such conditions is species specific. If *E. moraviensis* is better adapted to the environment and has higher ability to grow under non-enteric conditions than other *Enterococcus* species, this may clarify its frequent occurrence in abstracted water. In order to examine the likelihood of enterococcal growth in the environment, growth experiments were performed under the conditions of competititon with natural bacterial population (Chapter 3 and 4).

2) Parallel to the study mentioned above, it has been hypothesized that identification of the *Enterococcus* species from animal faeces using

MALDI-TOF MS can provide information on potential faecal sources of *E. moraviensis* in the Castricum dune infiltration area. Furthermore, it has been hypothesized that distribution of the different *Enterococcus* species found in abstracted water and the distribution of different *Enterococcus* species found in faecal samples may point towards the source of enterococci in abstracted water (Chapter 5).

The influence of protozoan predation on the numbers of enterococci and other FIB in the environment

When enterococci are released into the environment, they may be rendered inactive due to starvation, drought, UV (sunlight) etc., but also due to biological factors such as predation (Byappanahalli et al., 2012a). Grazing by protozoa, bacteriophage infection followed by virus-mediated lysis, and predation by some bacteria are among the biotic effects that control the abundance of prokaryotic organisms in the environment (Byappanahalli et al., 2012a). As reviewed by Byappanahalli et al. (2012a), predation by bacteria has been described for *Vibrio* spp., most notably *Vibrio parahaemolyticus*, where infection by predatory *Bdellovibrio* spp. plays a role in the population dynamics of these species (Mitchell, 1971; Sutton and Besant, 1994).

Bacteriophage infection affects a much wider range of bacteria, and viral infection was suggested to be a mechanism responsible for the elimination of up to 50% of autochthonous bacteria from aquatic habitats (Fuhrman and Noble, 1995; Proctor and Fuhrman, 1990; Thingstad, 2000). Bacteriophages that infect various *Enterococcus* spp. (hereafter termed enterophage) from different sources (i.e., raw sewage, cow manure, and environmental waters) were described (Bonilla et al., 2010; Morrison et al., 1997; Purnell et al., 2011; Santiago-Rodríguez et al., 2010). The relatively high concentrations of enterophage that specifically infect *E. casseliflavus*, *E. mundtii*, or *E. gallinarum* from cow faeces (10^4 to 10^5 PFU/100 ml) and *E. faecalis* or *E. faecium* (10^3 PFU/100 ml) from raw sewage (Purnell et al., 2011) indicate that, at least in these instances, lysis by enterophage can be a predatory factor on populations of enterococci.

Protozoa (e.g. cellular slime moulds) are present in soil and faecal matter and they also feed on bacteria (Raper, 1984). This may lead to reduction of FIB numbers in faecal matter and in soil. Protozoan predation in aquatic ecosystems is well studied

(Pernthaler, 2005). Some studies estimate that protozoan grazing is responsible for up to 90% of the overall mortality of both autochthonous and allochthonous microorganisms from freshwater and marine environments (Anderson et al., 1986; Menon et al., 2003). A direct correlation between rates of predation and temperature was found in a variety of environments, with higher rates of grazing and an increase in protozoan concentrations at higher temperatures (An et al., 2002; Anderson et al., 1983; Barcina et al., 1991; Sherr et al., 1988). The predilections of protozoa for the morphology of prey and the physiological state were observed for different types of protozoa (Beardsley et al., 2003; Gonzalez et al., 1990a; Matz et al., 2002; Simek et al., 1994; Verity, 1991). For example, lower rates of grazing were observed for Gram-positive organisms (including *E. faecalis*) than for *E. coli* (Davies et al., 1995; Gonzalez et al., 1990b; Iriberri et al., 1994a; Iriberri et al., 1994b; Nilsson, 1987). Hartke et al. (Hartke et al., 2002) showed a more active grazing of zooflagellate protozoa on *E. faecalis* cells harvested from the exponential growth phase than on glucose-starved cells, while nanoflagellates did not appear to exhibit a preference (Hartke et al., 2002).

In terrestrial systems, amoebae are considered important predators of bacteria and many of these amoebae belong to slime moulds. However, the role of protozoa in soil and faeces on the concentration of FIB has not yet been explored. Chapter 6 presents the results of the experiments to determine whether cellular slime moulds (dictyostelids) can be isolated from soil and dung obtained from infiltration area and if they are able to consume enterococci and other FIB. In order to estimate the potential predation pressure caused by these organisms in terrestrial ecosystems, the growth rate of cellular slime moulds was examined using *Pseudomonas fluorescens* as a nutritional source.

The significance of antibiotic-resistant enterococci in the aquatic environment

Enterococci from the gastro-intestinal tract of healthy humans are generally not virulent. Some *Enterococcus* strains have nevertheless emerged as leading causes of hospital-acquired infections (Tendolkar et al., 2003). Ampicillin and vancomycin are important antibiotics in the treatment of those infections. In infections with ampicillin resistant enterococci (ARE), vancomycin can still be used, but this is increasingly leading to the development of *Enterococcus* strains that are also not susceptible to vancomycin anymore. These strains are known as vancomycin resistant enterococci

(VRE). Therapeutic options for VRE infections are therefore becoming limited. *E. faecalis* accounts for most of the enterococci infections of humans, usually representing 80 to 90 % of clinical isolates. *E. faecium* is detected less frequently (5% to 15 %) but is of higher significance because of a high incidence of resistance to multiple antibacterial agents, including vancomycin (Cetinkaya et al., 2000). In The Netherlands, VRE (*E. faecium*) is one of the organisms on the watch list of especially resistant micro-organisms.

Aquatic environments could play a role in the transmission of antibiotic-resistant enterococci. The transfer of resistant bacteria from environmental compartments to humans may occur through contaminated food (Perreten et al., 1997), manure (if used as a fertilizer) and contaminated surface water used for irrigation or as recreational water. Wastewater and sludge from municipal sewage water treatment plants have been reported as favourable environments, consisting of variable mixtures of bacteria, nutrients and antimicrobial agents, for both survival and gene transfer (Lindberg et al., 2004), spreading resistant bacteria in both aquatic and terrestrial environment (Iversen et al., 2004). An additional concern is the possible presence of resistant enterococci in surface water used as a source for the production of drinking water. The presence of a reservoir of VRE in the environment could pose a threat for the transmission of vancomycin resistant bacteria to humans, either of enterococcal strains harbouring vancomycin-resistance genes, or other bacterial species via the horizontal spread of the genetic elements.

This thesis

Questions raised from drinking water practices led to the initiation of a number of investigations in order to find answers for practical problems. The *Enterococcus* genus consists of a relatively large number of species, possibly with different ecological demands. The identification of enterococci on the species level may help to identify sources of contamination (microbial source tracking). Since enterococci occur in human and husbandry wastewater, dispersion of antibiotic-resistant strains cannot be ruled out.

These questions form the frame of this thesis; individual research questions and general approach are summarized here.

1. The MALDI-TOF MS technique opens the possibility to identify enterococci isolates. In our study, a large number of water-isolated strains were identified using MALDI-TOF and these identifications were compared with biochemical and molecular identification techniques (Chapter 2).

2. An important trigger for these studies was the dominant occurrence of *E. moraviensis* in many of the large volume samples. As there was no primary indication of faecal contamination, a number of experiments were carried out to test possible environmental growth of this species. Growth on abstracted water, biofilms in the wells, sediments form the wells, humus and on plant extract was tested. Growth on plant extracts was observed in these tests (Chapter 3).

3. The observation of growth of *E. moraviensis* on plant extract led to a comparison to other *Enterococcus* species (*E. casseliflavus, E. hirae, E. faecalis* and *E. faecium*). Can these species grow on plant extracts? Are they capable of growing in competition with the natural microbial flora? Two types of tests were carried out. First, tests were done with plant extracts where most of the natural microbial community was removed by membrane filtration (0.22 µm), in order to test the growth potential of these species. In the second set up, the natural microbial community was not removed in order to test whether the competition with the natural microbial community would reduce the growth, or even lead to the disappearance of enterococci (Chapter 4).

4. It was necessary to evaluate the possible sources of faecal contamination in the vicinity of the abstraction wells. Can *E. moraviensis* (and *E. haemoperoxidus*) be found in animal faeces? If so, is it possible to link the species distribution found in the water samples with that of certain animals (or man) to each other? The faeces samples from humans and several animals living in the dune area were investigated for the presence and species diversity of enterococci. The diversity pattern observed in each species was compared (via hierarchical clustering) with the species distribution of species in the water samples (Chapter 5) to obtain indications about the water contamination source.

5. Reduction of bacterial numbers in soil and faeces in the vicinity of abstraction wells has not yet been studied. Drought, UV (sun light), high temperatures, etc., inactivate bacteria but also predation by protozoa is a factor to consider. Cellular slime moulds (dictyostelids) are quite common terrestrial amoebae. It has been investigated whether these organisms can be isolated from soil and dung from dune infiltration area and whether these consume faecal indicator bacteria (Chapter 6).

6. The presence of ampicillin- (ARE) and vancomycin (VRE)- resistant enterococci in treated wastewater and in surface water used for the production of drinking water was evaluated, using a modified version of the Slanetz & Bartley medium, enriched with 16 mg L^{-1} ampicillin or 16 mg L^{-1} vancomycin (Chapter 7).

7. In Chapter 8 the results are discussed and general conclusions are drawn on the value of enterococci as a faecal indicator. This includes studying the sources, environmental occurrence and fate of enterococci, and the significance of ARE and VRE in the water environment. The findings of every individual chapter are placed in a broader perspective for each subject, including the implications for drinking-water practices and recommendations for further research.

References

Aarestrup FM, Butaye P, Witte W. 2002. Non-human reservoirs of enterococci. In Gilmore MS, Clewell DB, Courvalin P, Dunny G M, Murray BE, Rice LB (Eds.), The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance, pp. 55-100. Washington, District of Columbia: ASM Press.

Ahmad A, Ghosh A, Schal C, Zurek L. 2011. Insects in confined swine operations carry a large antibiotic resistant and potentially virulent enterococcal community. *BMC Microbiol*. 11: 23.

Almeida T, Brandão A, Muñoz-Atienza E, Gonçalves A, Torres C, Igrejas G, Hernández PE, Herranz C, Cintas LM, Poeta P. 2011. Identification of bacteriocin genes in enterococci isolated from game animals and saltwater fish. *J Food Protect.* 74(8):1252–1260.

An YJ, Kampbell DH, Breidenbach GP. 2002. Escherichia coli and total coliforms in water and sediments at lake marinas. *Environ. Pollut*. 120:771–778.

Anderson IC, Rhodes MW, Kator HI. 1983. Seasonal variation in survival of *Escherichia coli* exposed in situ in membrane diffusion chambers containing filtered and nonfiltered estuarine water. *Appl. Environ. Microbiol*. 45:1877–1883.

Anderson A, Larsson U, Hagstrom A. 1986. Size selective grazing by a microflagellate on pelagic bacteria. *Mar. Ecol. Prog. Ser.* 33:51–57.

Anderson SA, Turner SJ, Lewis GD. 1997. Enterococci in the New Zealand environment: implications for water quality monitoring. *Water Sci. Technol*. 35(11-12):325–331.

Anonymous. 2000. ISO 7899-2:2000. Water quality -- Detection and enumeration of intestinal enterococci -- Part 2: Membrane filtration method. Geneva, Switzerland.

Anonymous. 2009.WHO Europe. European Environment and Health Information System. Outbreaks of water borne diseases. Fact sheet 1.1, Code: RPG1_WatSan_E1.

Anonymous. 2010a. US Environmental Protection Agency. *Method A: Enterococci in Water by TaqMan® Quantitative Polymerase Chain Reaction (qPCR) Assay.* Washington, D. C.: United States Environmental Protection Agency.

Anonymous, 2010b. Environmental Protection Agency 2010. Assessment of the Extra-Enteric Behavior of Fecal Indicator Organisms in Ambient Waters. (https://www.epa.gov/sites/production/files/2015-11/documents/asssessment-fecal-indicator-ambient-waters.pdf)

Anonymous. 2011. WHO. Guidelines for drinking-water quality - 4th ed.

Anonymous. 2016. WHO. Drinking-water. Fact sheet. (http://www.who.int/mediacentre/factsheets/fs391/en/)

Anonymous. 2016a. The Soil Food Web. US Department for Agriculture. Natural Resources Conservation Service Soils.

Anonymous. 2016b. LPSN (List of Prokaryotic names with Standing in Nomenclature). http://www.bacterio.cict.fr/e/enterococcus.html.

Ashbolt NJ, Grabow WOK, Snozzi M. 2001. Indicators of microbial water quality. In: Fewtrell L, Bartram J. (Ed.).WHO, Water Quality: Guidelines, Standards and Health. IWA Publishing, London, UK.

Badgley BD, Thomas FI, Harwood VJ. 2010a. The effects of submerged aquatic vegetation on the persistence of environmental populations of Enterococcus spp. (1271-1281, Ed.) *Environ. Microbiol*. 12(5).

Badgley BD, Nayak BS, Harwood VJ. 2010b. The importance of sediment and submerged aquatic vegetation as potential habitats for persistent strains of enterococci in a subtropical watershed. *Water Res*. 44(20):5857–5866.

Bahirathan M, Puente L, Seyfried P. 1998. Use of yellow pigmented enterococci as a specific indicator of human and non-human sources of fecal pollution. *Can J Microbiol*. 44(11):1066–1071.

Barcina I, Gonzalez JM, Iriberri J, Egea L. 1991. Role of protozoa in the regulation of enteric bacteria populations in seawater. *Mar. Microb. Food Webs.* 5:179–188.

Beardsley C, Pernthaler J, Wosniok W, Amann R. 2003. Are readily culturable bacteria in coastal North Sea waters suppressed by selective grazing mortality? *Appl. Environ. Microbiol.* 69:2624–2630.

Beer KD, Gargano JW, Roberts VA, Hill VR, Garrison LE, Kutty PK, Hilborn ED, Wade TJ, Fullerton KE, Yoder JS. 2015. Surveillance for Waterborne Disease Outbreaks Associated with Drinking Water — United States, 2011–2012. Morbidity and Mortality Weekly Report. 64(31):842-848.

Berg GT and Metcalf T. 1978. Indicators of viruses in waters. In: Indicators of Viruses in Water and Food. Ann Arbor Science Publishers.

Bonilla N, Santiago T, Marcos P, Urdaneta M, Domingo JS, Toranzos GA. 2010. Enterophages, agroupofphages infecting *Enterococcus faecalis*, and their potential as alternate indicators of human faecal contamination. *Water Sci. Technol*. 61(2):293–300.

Byappanahalli MN, Shively DA, Nevers MB, Sadowsky MJ, Whitman RL. 2003. Growth and survival of *Escherichia coli* and enterococci populations in the macro-alga *Cladophora* (Chlorophyta). FEMS Microbiology Ecology. 46(2):203–211.

Byappanahalli MN, Nevers MB, Korajkic A, Staley ZR, Harwood VJ. 2012a. Enterococci in the environment. *Microbiol. Molec. Biol. Rev*. 76(4):685–706.

Byappanahalli MN, Roll BM, Fujioka RS. 2012b. Evidence for occurrence, persistence, and growth of Escherichia coli and enterococci in Hawaii's soil environments. *Microbes Environ*. 27:164–170.

Byappanahalli M, Fujioka R. 2004. Indigenous soil bacteria and low moisture may limit but allow faecal bacteria to multiply and become a minor population in tropical soils. *Water Sci. Technol*. 50(1):27–32.

Carvalho MG, Shewmaker PL, Steigerwalt AG, Morey RE, Sampson AJ, Joyce K, Barrett TJ, Teixeira LM, Facklam RR. 2006. *Enterococcus caccae sp. nov.* isolated from human stools. International Journal of Systematic and Evolutionary Microbiology. 56(7):1505–1508.

Cetinkaya Y, Falk P, Mayhall CG. 2000. Vancomycin-Resistant Enterococci. *Clin. Microbiol. Rev.* 13(4), 686–707.

Chalmers RM, Robinson G, Elwin K, Hadfield SJ, Xiao L, Ryan U, Modha D, Mallaghan C, 2009. *Cryptosporidium* sp. rabbit genotype, a newly identified human pathogen. *Emerg. Infect. Dis.* 15, 829e830.

Chalmers RM. 2012. Waterborne outbreaks of cryptosporidiosis. *Ann Ist super sAnIta* . 48 (4), 429-446.

Channaiah LH, Subramanyam B, McKinney LJ, Zurek L. 2010. Stored-product insects carry antibiotic-resistant and potentially virulent enterococci. *FEMS Microbiol. Ecol*. 74:464-471.

Cox CR and Gilmore MS. 2007. Native microbial colonization of *Drosophila melanogaster* and its use as a model of *Enterococcus faecalis* pathogenesis. *Infect. Immun*. 75(4):1565–1576.

Craun GF. 1990. Waterborne giardiasis. In: Meyer EA (ed). Human Parasitic Diseases, Vol. 3, Giardiasis. Elsevier Science Publ., Amsterdam, The Netherlands, pp. 267-293.

Craun GF, Hubbs SA, Frost F, Calderon RL, Via SH. 1998. Waterborne outbreaks of cryptosporidiosis. *J. Am. Water Works Assoc.* 90:81-91.

Craun GF, Brunkard JM, Yoder J S, Roberts VA, Carpenter J, Wade T, Calderon RL, Roberts JM, Beach M J, Roy SL. 2010. Causes of Outbreaks Associated with Drinking Water in the United States from 1971 to 2006. *Clin. Microbial. Rev.* 23(3): 507–528.

Cutler D and Miller G. 2005. The role of public health improvements in health advances: the twentieth-century United States. *Demography*. 42:1–22.

Davies CM, Long JA, Donald M, Ashbolt NJ. 1995. Survival of fecal microorganisms in marine and freshwater sediments. *Appl. Environ. Microbiol*. 61:1888–1896.

De Graef EM, Devriese LA, Vancanneyt M, Baele M, Collins MD, Lefebvre K, Swings J, Haesebrouck F. 2003. Description of *Enterococcus canis sp. nov.* from dogs and reclassification of *Enterococcus porcinus* Teixeira *et al*. 2001 as a junior synonym of *Enterococcus villorum* Vancanneyt *et al*. 2001. *Int. J. Syst. Evol. Microbiol*. 53(4):1069–1074.

Desmarais TR, Solo-Gabriele HM, Palmer CJ. 2002. Influence of soil on fecal indicator organisms in a tidally influenced subtropical environment. *Appl. Environ. Microbiol*. 68 (3):1165-72.

Devriese LA, Van De Kerckhove A, Kilpper-Bälz R, Schleifer KH. 1987. Characterization and Identification of *Enterococcus* Species Isolated from the Intestines of Animals. *Int. J. Syst. Evol. Microbiol*. 37: 257-259.

Devriese LA, Haesebrouck F. 1991. *Enterococcus hirae* in different animal species. *Veterin. Rec*. 129:391–392.

Devriese LA, Laurier L, De Herdt P, Haesebrouck F. 1992a. Enterococcal and streptococcal species isolated from faeces of calves, young cattle and dairy cows. *J. Appl. Bacteriol*. 72(1):29–31.

Devriese LA, Cruz Colque JI, De Herdt P, Haesebrouck F. 1992b Identification and composition of the tonsillar and anal enterococcal and streptococcal flora of dogs and cats. *J. Appl. Bacteriol*. 73(5):421–425.

Devriese LA, Pot B, Collins MD. 1993. Phenotypic identification of the genus *Enterococcus* and differentiation of phylogenetically distinct enterococcal species and species group. *J. Appl. Bacteriol*. 75, 399–408.

Devriese LA, Pot B, Van Damme L, Kersters K, Haesebrouck F. 1994. Identification of *Enterococcus* species isolated from foods of animal origin. *Int. J. Food Microbiol*. 26(2):187–197.

Euzéby JP. 2013. List of Prokaryotic names with Standing in Nomenclature. (Parte AC, Editor) Retrieved November 20, 2016: http://www .bacterio.cict .fr/e/enterococcus.html.

Fayer R, Speer CA, Dubey JP. 1997. The general biology of *Cryptosporidium*. In: *Cryptosporidium* and Cryptosporidiosis. Fayer R (Ed.) CRC Press, Boca Raton, USA. pp. 1-42.

Fenlon DR. 1981. Seagulls (*Larus* spp.) as vectors of salmonellae: an investigation into the range of serotypes and numbers of salmonellae in gull faeces. *J. Hygiene* (London). 86(2), 195-202.

Fenselau C and Demirev PA. 2001. Characterization of intact microorganisms by MALDI mass spectrometry. *Mass Spectr. Rev.* 20(4), 157-171.

Ferguson DM, Moore DF, Getrich MA, Zhowandai MH. 2005. Enumeration and speciation of enterococci found in marine and intertidal sediments and coastal water in southern California. *J. Appl. Microbiol*. 99(3):598–608.

Figueras MJ and Borrego JJ. 2010. New Perspectives in Monitoring Drinking Water Microbial Quality. *Int. J. Environ. Res. Publ. Health*. 7(12):4179-4202.

Finegold SM, Sutter VL, Mathisen GE. 1983. Normal indigenous intestinal flora. In Hentges DJ. Human intestinal microflora in health and disease (pp. 3-29). Waltham: Academic Press.

Fuhrman JA and Noble RT. 1995. Viruses and protists cause similar bacterial mortality in coastal seawater. *Limnol. Oceanogr*. 40:1236–1242.

Fujioka RSB, Byappanahalli MN, (ed). 2003. Proceedings and report: tropical water quality indicator workshop SR-2004–01. University of Hawaii Water Resources Research Center, Honolulu, HI: http://www.wrrc.hawaii.edu/tropindworkshop.html.

Fujioka R, Sian-Denton C, Borja M, Castro J, Morphew K. 1999. Soil: the environmental source of Escherichia coli and enterococci in Guam's streams. *J. Appl. Microbiol*. 85(Suppl 1): 83S–89S.

Gonzalez JM, Sherr EB, Sherr BF. 1990a. Size-selective grazing on bacteria by natural assemblages of estuarine flagellates and ciliates. *Appl. Environ. Microbiol*. 56: 583–589.

Gonzalez JM, Iriberri J, Egea L, Barcina I. 1990b. Differential rates of digestion of bacteria by freshwater and marine phagotrophic protozoa. *Appl. Environ. Microbiol*. 56: 1851–1857.

Graham JP, Price LB, Evans SL, Graczyk TK, Silbergeld EK. 2009. Antibiotic resistant enterococci and staphylococci isolated from flies collected near confined poultry feeding operations. *Sci. Total Environ*. 407:2701- 2710.

Grant SB, Sanders BF, Boehm AB, Redman JA, Kim JH, Mrse RD, Chu AK, Gouldin M, McGee CD, Gardiner NA, Jones BH, Svejkovsky J, Leipzig GV, Brown A. 2001. Generation of enterococci bacteria in a costal saltwater marsh and its impact on surf zone water quality. *Environ. Sci. Technol*. 15(35):2407–2416.

Guthof O. 1955. A new serologic group of alphahemolytic Streptococci (serologic group Q). *Zentralbl Bakteriol Orig*. 164:60–3.

Haile RW, Witte JS, Gold M, Cressey R, McGee C, Millikan RC, Glasser A, Harawa N, Ervin C, Harmon,P, Harper J, Dermand J, Alamillo J, Barrett K, Nides M, Wang G. 1999. The health effects of swimming in ocean water contaminated by storm drain runoff. *Epidemiol*.10:355-363.

Halliday E and Gast RJ. 2011. Bacteria in beach sands: an emerging challenge in protecting coastal water quality and bather health. *Environ. Sci. Technol*. 45(2):370–379.

Han D, Unno T, Jang J, Lim K, Lee SN, Ko G, Sadowsky MJ, Hur HG. 2011. The occurrence of virulence traits among high-level aminoglycosides resistant *Enterococcus* isolates obtained from feces of humans, animals, and birds in South Korea. *Int. J. Food Microbiol*. 144(3):387–392.

Hardina CM and Fujioka RS. 1991. Soil: the environmental source of *Escherichia coli* and enterococci in Hawaii's streams. *Environ. Toxicol. Water Qual*. 6:185–195.

Hartke A, Lemarinier S, Pichereau V, Auffray Y. 2002. Survival of *Enterococcus faecalis* in seawater microcosms is limited in the presence of bacterivorous zooflagellates. *Curr. Microbiol*. 44: 329–335.

Harwood VJ, Levine AD, Scott TM, Chivukula V, Lukasik J, Farrah SR, Rose JB. 2005. Validity of the indicator organism paradigm for pathogen reduction in reclaimed water and public health protection. *Appl. Env. Microbiol*. 71: 3163-3170.

Haugland RA, Siefring SC, Wymer LJ, Brenner KP, Dufour AP. 2005. Comparison of *Enterococcus* measurements in freshwater at two recreational beaches by quantitative polymerase chain reaction and membrane filter culture analysis. *Water Res*. 39(4):559–568.

Heaney CD Sams E, Dufour AP, Brenner KP, Haugland RA, Chern E, Wing S, Marshall S, Love DC, Serre M, Noble R, Wade TJ. 2012. Fecal indicators in sand, sand contact, and risk of enteric illness among beachgoers. *Epidemiol*. 23:95–106.

Hlavsa MC, Roberts VA, Kahler AM, Hilborn ED, Mecher TR, Beach MJ, Wade TJ, Yoder JS. 2015. Outbreaks of Illness Associated with Recreational Water — United States, 2011–2012. Morbidity and Mortality Weekly Report. 64(24):668-672.

Holland RD, Wilkes JG, Rafii F, Sutherland JB, Persons CC, Voorhees KJ, Lay JOJ. 1996. Rapid identification of intact whole bacteria based on spectral patterns using matrix- assisted laser desorption/ionization with time-of- flight mass spectrometry. *Rap. Commun. Mass Spectr.* 10(10), 1227-1232.

Hornstra L. 2014. Modelling water movement and transport of bacteriophage MS2 through an unsaturated sandy soil. BTO rapport: BTO 2014.219(s).

Hrudey SE, Payment P, Huck PM, Gillham RW, Hrudey EJ, 2003. A fatal waterborne disease epidemic in Walkerton, Ontario: comparison with other waterborne outbreaks in the developed world. *Water Sci. and Technol*. 47(3): 7-14.

Imamura GJ, Thompson RS, Boehm AB, Jay JA. 2011.Wrack promotes the persistence of fecal indicator bacteria in marine sands and seawater. *FEMS Microbiol. Ecol*. 77(1):40–9.

Iriberri J, Azua I, Labiruaiturburu A, Artolozaga I, Barcina I. 1994a. Differential elimination of enteric bacteria by protists in a fresh-water system. *J. Appl. Bacteriol*. 77: 476–483.

Iriberri J, Ayo B, Artolozaga I, Barcina I, Gea LE. 1994b. Grazing on allochthonous vs autochthonous bacteria in river water. *Lett. Appl. Microbiol*. 18:12–14.

Ishii S and Sadowsky MJ. 2008. *Escherichia coli* in the environment: implications for water quality and human health. *Microbes Environ*. 23:101–108.

Iversen A, Kühn I, Rahman M, Franklin A, Burman LG, Olsson-Liljequist B, Torell E, Möllby R. 2004. Evidence for transmission between humans and the environment of a nosocomial strain of Enterococcus faecium. *Environ. Microbiol*. 6 (1):55–61.

Kay D, Fleisher JM, Salmon RL, Jones F, Wyer MD, Godfree AF, Zelenauch-Jacquotte Z, Shore R. 1994. Predicting Likelihood of Gastroenteritis from Sea Bathing: Results from Randomised Exposure. *Lancet* 344(8927):905-909.

Krishnamurthy T, Ross PL, Rajamani U. 1996. Detection of pathogenic and non-pathogenic bacteria by matrix- assisted laser desorption/ionization with time-of-flight mass spectrometry. *Rap. Commun. Mass Spectr.*10(8):883-888.

Layton BA, Walters SP, Boehm AB. 2009. Distribution and diversity of the enterococcal surface protein (*esp*) gene in animal hosts and the Pacific coast environment. *J. Appl. Microbiol*. 106(5):1521–1531.

Layton BA, Walters SP, Lam LH, Boehm AB. 2010. *Enterococcus* species distribution among human and animal hosts using multiplex PCR. *J. Appl. Microbiol*. 109(2):539–574.

Leclerc H, Devriese LA, Mossel DAA. 1996. Taxonomical changes in intestinal (faecal) enterococci and streptococci: consequences on their use as indicators of faecal contamination in drinking water. *J. Appl. Bacteriol*. 81:459–466.

Lindberg R, Jarnheimer P, Olsen B, Johansson M, Tysklind M, 2004. Determination of antibiotic substances in hospital sewage water using solid phase extraction and liquid chromatography/mass spectrometry and group analogue internal standards. *Chemosph*. 57:1479–1488.

Ludwig W and Schleifer KH. 2000. How quantitative is quantitative PCR with respect to cell counts? *Syst. Appl. Microbiol*. 23(4):556–562.

MacKenzie WR, Hoxie NJ, Proctor ME, Gradus MS, Blair KA, Peterson DE, Kazmierczak JJ, Addiss DG, Fox KR, Rose JB and Davis JP. 1994. A massive outbreak in Milwaukee of Cryptosporidium infection transmitted through the public water supply. *N. Engl. J. Med*. 331(3):161-167.

Macovei L and Zurek L. 2006. Ecology of Antibiotic Resistance Genes: Characterization of Enterococci from Houseflies Collected in Food Settings. *Appl. Environ. Microbiol*. 72(6):4028-4035.

Manero A, and Blanch AR. 1999. Identification of *Enterococcus* spp. with a Biochemical Key. *Appl. Environ. Microbiol*., 65(10):4425 4430.

Martín Granado A, Varela Martínez M, Torres Frías A, Ordóñez Banegas P, Martínez Sánchez EV, Hernández Domínguez M, Hernández Pezzi G, Tello Anchuela O. 2008. Vigilancia epidemiológica de brotes de transmisión hídrica en España. 1999–2006. *Bol. Epidemiol. Semanal*.16:25–28.

Martin JD and Mundt JO. 1972. Enterococci in insects. *J. Appl. Microbiol*. 24(4):575–580.

Matz C, Boenigk J, Arndt H, Jurgens K. 2002. Role of bacterial phenotypic traits in selective feeding of the heterotrophic nanoflagellate Spumella sp. *Aquat. Microb. Ecol*. 27:137–148.

Maugeri TL, Carbone M, Fera MT, Irrera GP, Gugliandolo C. 2004. Distribution of potentially pathogenic bacteria as free living and plankton associated in a marine coastal zone. *J. Appl. Microbiol*. 5:354–361.

Medema GJ. 1999. PhD Thesis: *Cryptosporidium* and *Giardia*: New Challenges to the Water Industry. University of Utrecht, Utrecht, The Netherlands.

Medema GJ, Payment P, Dufour A, Robertson W, Waite M, Hunter P, Kirby R, Andersson Y. 2003a. Safe Drinking Water: An Ongoing Challenge. In: Assessing Microbial Safety of Drinking Water Improving Approaches and Methods. Published on behalf of the World Health Organization and the Organisation for Economic Co-operation and Development by IWA Publishing, Alliance House,12 Caxton Street, London SW1H 0QS, UK.

Medema GJ, Shaw S, Waite M, Snozzi M, Morreau A, Grabow W. 2003b. Catchment characterization and source water quality. In: Assessing Microbial Safety of Drinking Water Improving Approaches and Methods. Published on behalf of the World Health Organization and the Organisation for Economic Co-operation and Development by IWA Publishing, Alliance House,12 Caxton Street, London SW1H 0QS, UK.

Medema G, Teunis P, Blokker M, Deere D, Davison A, Charles P, Loret JF. 2009. World Health Organization. Risk Assessment of *Cryptosporidium* in Drinking-water.

Mellman A, Cloud J, Maier T, Keckevoet U, Ramminger I, Iwen P, Dunn J, Hall G, Wilson D, LaSala P, Kostrzewa M, Harmsen D. 2008. Evaluation of Matrix-Assisted Laser Desorption Ionization-Time-of-Flight Mass Spectrometry in Comparison to 16S rRNA Gene Sequencing for Species Identification of Nonfermenting Bacteria. *J. Clin.l Microbiol*. 46(6):1946-1954.

Melnick JL and Gerba CP. 1982. Viruses in surface and drinking waters. *Environ. Int*. 7:3-7.

Menon P, Billen G, Servais P. 2003. Mortality rates of autochthonous and fecal bacteria in natural aquatic ecosystems. *Water Res*. 37:4151– 4158.

Mitchell R. 1971. Role of predators in the reversal of imbalances in microbial ecosystems. *Nature* 230:257–258.

Moriarty EM, Weaver L, Sinton LW, Gilpin B. 2012. Survival of *Escherichia coli*, Enterococci and *Campylobacter jejuni* in Canada Goose Faeces on Pasture. *Zoon. Publ. Health*. 59 (7):490–497.

Morrison D, Woodford N, Cookson B. 1997. Enterococci as emerging pathogens of humans, p 89S–99S. In Andrew PW, Mitchell TJ (ed), The biology of streptococci and enterococci. Blackwell Science, Oxford, United Kingdom.

Mote BL, Turner JW, Lipp EK. 2012. Persistence and growth of the fecal indicator bacteria enterococci in detritus and natural estuarine plankton communities. *Appl. Environ. Microbiol*. 78(8):2569–2577.

Mundt JO. 1963. Occurrence of enterococci on plants in a wild environment. *Appl. Microbiol*. 11:141–144.

Muniesa M, Jofre J, García-Aljaro C, Blanch A.R. 2006. Occurrence of *Escherichia coli* O157:H7 and other enterohemorrhagic *Escherichia coli* in the environment. *Environ. Sci. Technol*. 40:7141-7149.

Müller T, Ulrich A, Ott EM, Müller M. 2001. Identification of plant-associated enterococci. *J. Appl. Microbiol*. 91(2):268–278.

Naser SM, Vancanneyt M, De Graef E, Devriese LA, Snauwaert C, Lefebrve K, Hoste B, Švec P, Decostere A, Haesebrouck F, Swings J. 2005. *Enterococcus canintestini sp. nov.*, from faecal samples of healthy dogs. *Int. J. Syst. Evol. Microbiol*. 55(5):2177–2182.

Niemi RM, Ollinkangas T, Paulin L, Švec P, Vandamme P, Karkman A, Kosina M, Lindström K. 2012. *Enterococcus rivorum sp. nov.* from water of pristine brooks. *Int. J. Syst. Evol. Microbiol*. 62(9):2169–2173.

Nilsson JR. 1987. Structural aspects of digestion of *Escherichia coli* in *Tetrahymena*. *J. Protozool*. 34:1–6.

Noble CJ. 1978. Carriage of group D streptococci in the human bowel. *J. Clin. Pathol*. 31(12):1182–1186.

Nowlan SS and Deibel RH. 1967. Group Q streptococci. I. Ecology, serology, physiology, and relationship to established enterococci. *J. Bacteriol*. 94(2):291–296.

Obiri-Danso K and Jones K. 2000. Intertidal sediments as reservoirs for hippurate negative campylobacters, salmonellae and faecal indicators in three EU recognized bathing waters in north west England. *Water Res*. 34:519–527.

Olsen SJ, Miller G, Breuer T, Kennedy M, Higgins C, Walford J, McKee G, Fox K, Bibb W, Mead P. 2002. A Waterborne Outbreak of *Escherichia coli* O157:H7 Infections and Hemolytic Uremic Syndrome: Implications for Rural Water Systems. *Emerging Infectious Diseases*. 8(4):370-375.

Patel R, Piper KE, Rouse MS, Steckelberg JM, Uhl JR, Kohner P, Hopkins MK, Cockerill FR 3rd, Kline BC. 1998. Determination of 16S rRNA sequences of enterococci and application to species identification of nonmotile *Enterococcus gallinarum* isolates. *J. Clin. Microbiol*. 36(11):3399–3407.

Payment P, Waite M, Dufour A. 2003. Introducing parameters for the assessment of drinking water quality. In: Assessing Microbial Safety of Drinking Water Improving Approaches and Methods. Published on behalf of the World Health Organization and the Organisation for Economic Co-operation and Development by IWA Publishing, Alliance House,12 Caxton Street, London SW1H 0QS, UK.

Pernthaler J. 2005. Predation on prokaryotes in the water column and its ecological implications. *Nature reviews*. (3):537-546.

Perreten V, Schwarz F, Cresta L, Boeglin M, Dasen G, Teuber M, 1997. Antibiotic resistance spread in food. *Nature* 389:801–802.

Petrilli FL, Crovari P, DeFlora S. Vannucci A. 1974. The virological monitoring of water. I. Drinking water. *Boll. Ist. Seiroter,* Milan 53:434- 442.

Pinto B, Pierotti R, Canale G, Reali D.1999. Characterization of "faecal streptococci" as indicators of faecal pollution and distribution in the environment. *Lett. Appl. Microbiol*. 29:258–263.

Proctor LM, Fuhrman JA. 1990. Viral mortality of marine-bacteria and cyanobacteria. *Nature* 343:60 – 62.

Purnell SE, Ebdon JE, Taylor HD. 2011. Bacteriophage lysis of *Enterococcus* host strains: a tool for microbial source tracking? *Environ. Sci. Technol*. 45:10699 –10705.

Radhouani H, Igrejas G, Carvalho C, Pinto L, Gonçalves A, Lopez M, Sargo R, Cardoso L, Martinho A, Rego V, Rodrigues R, Torres C, Poeta P. 2011. Clonal lineages, antibiotic resistance and virulence factors in vancomycin-resistant enterococci isolated from fecal samples of red foxes (*Vulpes vulpes*). *J. Wildlife Diseases*. 47(3):769–773.

Raper KB. 1984. The Dictyostelids. Princeton University press. I-x; 1 – 453.

Robinson G, Chalmers RM, Stapleton C, Palmer SR, Watkins J, Francis C, Kay D. 2011. A whole water catchment approach to investigating the origin and distribution of *Cryptosporidium* species. *J. Appl. Microbiol*. 111:717e730.

Santiago-Rodríguez TM, Dávila C, González J, Bonilla N, Marcos P, Urdaneta M, Cadete M, Monteiro S, Santos R, Domingo JS, Toranzos GA. 2010. Characterization of *Enterococcus faecalis*-infecting phages (enterophages) as markers of human fecal pollution in recreational waters. *Water Res*. 44:4716 – 4725.

Savichtcheva O, Okabe S. 2006. Alternative indicators of fecal pollution: Relation with pathogens and conventional indicators, current methodologies for direct pathogen monitoring and future application perspectives. *Water Res*. 40: 2463- 2476.

Schijven JF, Hoogenboezem W, Nobel PJ, Medema GJ, Stakelbeek A. 1998. Reduction of FRNA-bacteriophages and faecal indicator bacteria by dune infiltration and estimation of sticking efficiencies. *Water Sci. Technol*. 38 (12):127-131.

Scott TM, Jenkins TM, Lukasik J, Rose JB. 2005. Potential use of a host associated molecular marker in Enterococcus faecium as an index of human fecal pollution. *Environ. Sci. Technol*. 39(1):283–287.

Sherr BF, Sherr EB, Rassoulzadegan F. 1988. Rates of digestion of bacteria by marine phagotrophic protozoa: temperature dependence. *Appl. Environ. Microbiol*. 54:1091–109

Signoretto C, Burlacchini G, Lleò MM, Pruzzo C, Zampini M, Pane L, Franzini G, Canepari P. 2004. Adhesion of *Enterococcus faecalis* in the nonculturable state to plankton is the main mechanism responsible for persistence of this bacterium in both lake and seawater. *Appl. Environ. Microbiol*. 70(11):6892–6896.

Silva N, Igrejas G, Figueireido N, Gonçalves A, Radhouani H, Rodrigues J, et al. 2010. Molecular characterization of antimicrobial resistance in enterococci and *Escherichia coli* isolates from European wild rabbit (*Oryctolagus cuniculus*). *Sci. Tot. Environ*. 408(20):4871–4876.

Silva N, Igrejas G, Rodrigues T, Gonçalves A, Felgar AC, Pacheco R, Gonçalves D, Cunha R, Poeta P. 2011. Molecular characterization of vancomycin-resistant enterococci and extended-spectrum -lactamase-containing *Escherichia coli* isolates in wild birds from the Azores Archipelago. *Avian Pathol*. 40(5):473–479.

Simek K, Vrba J, Hartman P. 1994. Size-selective feeding by *Cyclidium* sp. on bacterioplankton and various sizes of cultured bacteria. *FEMS Microbiol. Ecol*. 14:157–167.

Sistek V, Maheux AF, Boissinot M, Bernard KA, Cantin P, Cleenwerck I, De Vos P, Bergeron MG. 2012. *Enterococcus ureasiticus sp. nov.* and *Enterococcus quebecensis sp. nov.* isolated from water. *Int. J. Syst. Evol. Microbiol*. 62(6):1314–1320.

Sorensen J. 1997. The rhizosphere as a habitat for soil microorganisms. In van Elsas JD, Trevors JT, Wellington EMH, editors. (ed), Modern soil microbiology. Marcel Dekker, Inc, New York, NY.

Stevens M, Ashbolt N, Cunliffe D. 2003. Recommendation to change the use of coliforms as microbial indicators of drinking water quality. Australian Government National Health and Medical Research Council.

Sutton DC, Besant PJ. 1994. Ecology and characteristics of bdellovibrios from 3 tropical marine habitats. *Mar. Biol*. 119:313–320.

Švec P, Devriese LA, Sedlacek I, Baele M, Vancanneyt M, Haesebrouck F, Swings J, Doskar J. 2001. *Enterococcus haemoperoxidus sp. nov.* and *Enterococcus moraviensis sp. nov.* isolated from water. *Int. J. Syst. Evol. Microbiol*. 51:1567–1574.

Švec P, Vancanneyt M, Devriese LA, Naser SM, Snauwaert C, Lefebvre K, Hoste B, Swings J. 2005. *Enterococcus aquamarinus sp. nov.* isolated from sea water. *Int. J. Syst. Evol. Microbiol*. 55:2183–2187.

Švec P, Vancanneyt M, Sedlácek I, Naser SM, Snauwaert C, Lefebvre K, Hoste B, Swings J. 2006. *Enterococcus silesiacus sp. nov.* and *Enterococcus termitis sp. nov. Int. J. Syst. Evol. Microbiol*. 56(3):577–581.

Tendolkar PM, Baghdayan AS, Shankar N. 2003. Pathogenic enterococci: new developments in the 21st century. *Cell. Mol. Life Sci*. 60(12):2622–2636.

Thingstad TF. 2000. Elements of a theory for the mechanisms controlling abundance, diversity, and biogeochemical role of lytic bacterial viruses in aquatic systems. *Limnol. Oceanogr*. 45:1320 –1328.

Traverso HP. 1996. Water and health in Latin America and the Caribbean: infectious waterborne diseases. *In*: Water Quality in Latin America. Balancing the Microbial and Chemical Risks in Drinking Water Disinfection. Craun, G.F. (Ed.) ILSI Press, Washington DC, USA, pp. 45-54.

Verhougstraete MP, Byappanahalli MN, Whitman RL, Rose JB. 2010. *Cladophora* in the Great Lakes: impacts on beach water quality and human health. *Water Sci. Technol*. 62(1): 68–76.

Verity PG. 1991. Feeding in planktonic protozoans—evidence for nonrandom acquisition of prey. *J. Protozool*. 38:69–76.

Wade TJ, Sams E, Brenner KP, Haugland R, Chern E, Beach M, Wymer L, Rankin CC, Love D, Li Q, Noble R, Dufour AP. 2010. Rapidly measured indicators of recreational water quality and swimming-associated illness at marine beaches: a prospective cohort study. *Environ. Health*. 9(1):66.

Winfield MD, Groisman EA. 2003. Role of non-host environments in the lifestyles of *Salmonella* and *Escherichia coli*. *Appl. Environ. Microbiol*. 69:3687–3694.

Wheeler AL, Hartel PG, Godfrey DG, Hill JL, Segars WI. 2002. Potential of *Enterococcus faecalis* as a human fecal indicator for microbial source tracking. *J Environ Qual*. 31(4):1286–1293.

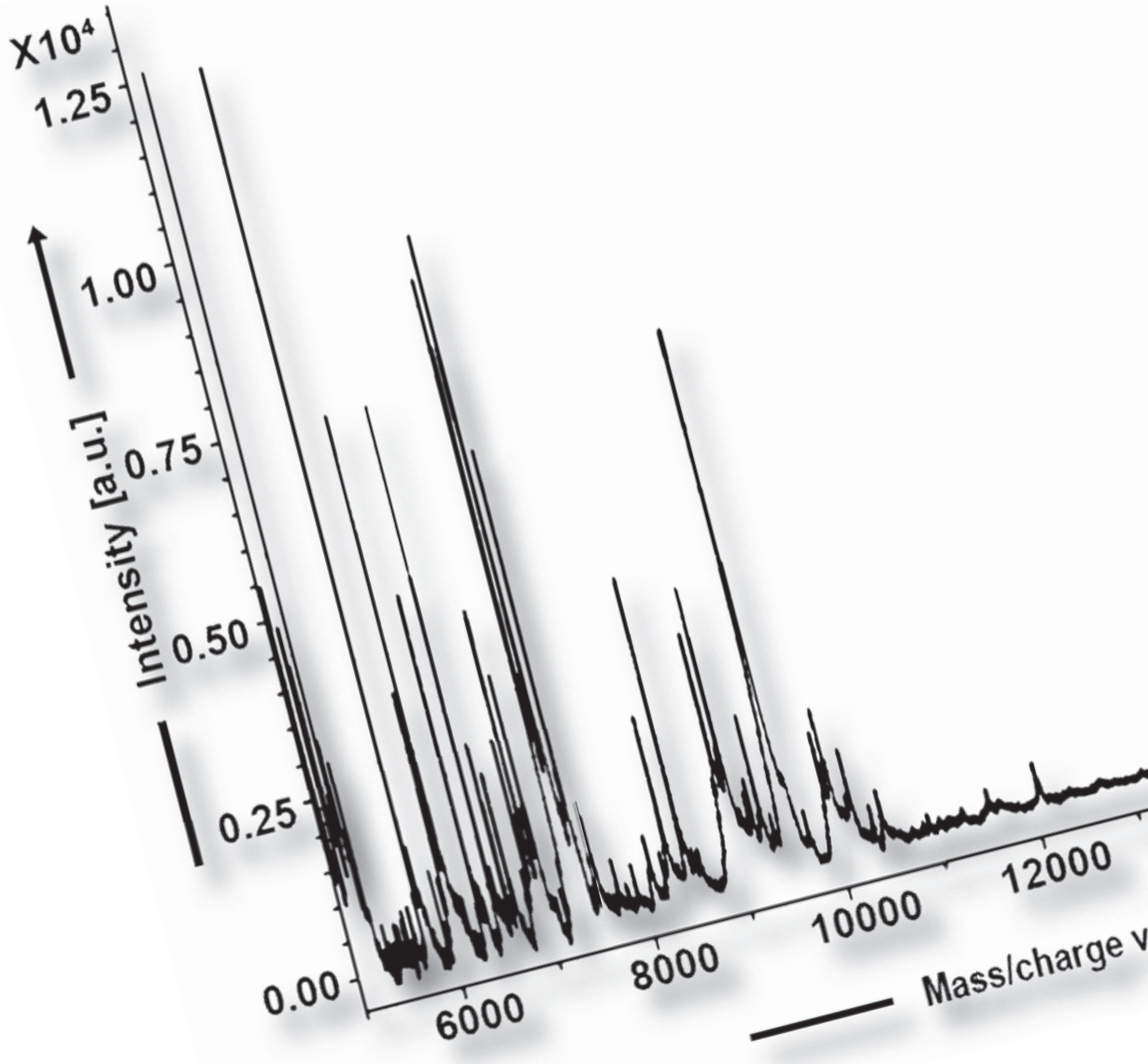
Whitman RL, Shively DA, Pawlik H, Nevers MB, Byappanahalli MN. 2003. Occurrence of *Escherichia coli* and enterococci in *Cladophora* (Chlorophyta) in nearshore water and beach sand of Lake Michigan. *Appl. Environ. Microbiol*. 69(8):4717–4719.

Whitman RL, Przybyla-Kelly K, Shively DA, Byappanahalli MN. 2007. Incidence of the enterococcal surface protein (*esp*) gene in human and animal fecal sources. *Environ. Sci. Technol*. 41(17):6090–6095.

World Health Organisation (WHO). 2016. Quantitative microbial risk assessment: application for water safety management.

Yamahara KM, Layton BA, Santoro AE, Boehm AB. 2007. Beach sands along the California coast are diffuse sources of fecal bacteria to coastal waters. *Environ. Sci. Technol*. 41(13):4515–4521.

Yamahara KM, Walters SP, Boehm AB. 2009. Growth of enterococci in unaltered, unseeded sands subjected to tidal wetting. *Appl. Environ. Microbiol*. 75:1517–1524.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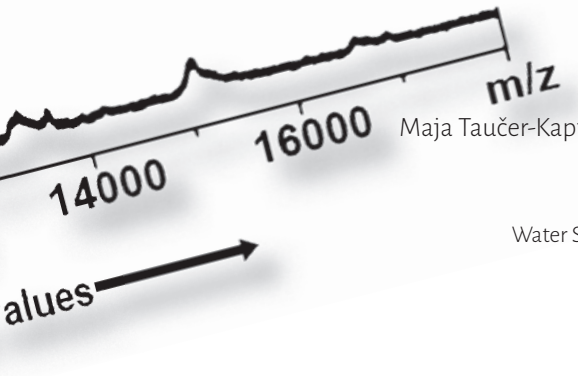
Chapter 2

Comparison between Rapid ID 32 Strep System, Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry and 16S rRNA gene sequence analysis for the species identification of *Enterococcus spp.* isolated from water.



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ABSTRACT

Matrix-Assisted Laser Desorption Ionisation-Time of Flight Mass Spectrometry (MALDI-TOF MS) has increasingly been used for rapid and reliable identification of clinically relevant micro-organisms. To establish the applicability of this technique in (drinking) water quality analysis, the MALDI-TOF MS identification results for *Enterococcus spp.* isolated from various water environments were compared with those obtained using the commercial Rapid 32 ID Strep system. One hundred and one bacterial isolates were isolated from various types of water and determined as enterococci on the basis of their growth on Slanetz-Bartley agar in typical colonies. The isolates were identified by MALDI-TOF MS and the commercial biochemical test Rapid 32 ID Strep. Isolates yielding in discrepant identifications were genotyped using 16S rRNA gene sequence analysis. For 86 isolates (85%), the results of Rapid ID 32 Strep were identical to those obtained with MALDI-TOF MS. Six isolates were impossible to be classified by means of the Rapid 32 ID Strep test. And for eight out of a total of nine discrepant results (89%), the 16S analyses confirmed the MALDI-TOF MS identification. MALDI-TOF MS produced highly reproducible results. These results indicated that the use of two different culture media had no effect on the identification. In addition, no significant differences ($p=0.32$; $n=20$) were evident between the scores obtained from a 20-fold measurement of the same isolate. The results of this study showed that MALDI-TOF MS identification (Bruker) is a reliable method for identifying *E. faecium*, *E. faecalis*, *E. durans*, *E. hirae* and *E. casseliflavus* isolated from water samples. *E. mundtii* and *E. moraviensis* were not included in the Rapid 32 ID Strep database and could therefore not be identified using that test set. However, MALDI-TOF MS and 16S identified all six isolates as members of these species.

INTRODUCTION

Enterococci are widely used in drinking water microbiology as indicators for fecal contamination and additionally as indicators for the removal of bacteria by water treatment processes. Their natural habitats are the human and animal intestinal tracts, but they are also found in environmental habitats (Gelsomino et al., 2002). Outside of the host organism, enterococci are able to survive for longer periods under a wide range of temperatures, pH conditions and salinity levels, and they are resistant to the bactericidal effects of detergents (Shepard and Gilmore, 2002). Some species, including *E. faecium* and *E. faecalis*, appear to be true fecal indicators. For others, such as *E. gallinarum* and *E. casseliflavus*, it is suggested that grasslands are important habitats (LeClerc *et al.*, 1996). Identification of enterococci species level may provide information on the nature of fecal contamination events and the source of fecal contamination. When the commensal relationship with the host is disrupted, enterococci, particularly *E. faecalis*, may become opportunistic pathogens and cause invasive diseases (Jett et al., 1994). In the laboratory, enterococci are detected by culture methods that use their ability to grow in the presence of azide. Confirmation of presumptive enterococci is performed by testing their ability to hydrolyze aesculin in the presence of bile within 2 h. However, this method gives no information on species composition. Thus far, identification of cultured microbial isolates in water laboratories relies on methods based on biochemical tests such as Api (bioMérieux). Species identification by means of commercial systems is performed when the results obtained through the use of routine procedures do not provide sufficient information on, for instance, the potential source of contamination or the fecal nature of the contamination. It is often necessary to obtain additional information on the species in question, to collect information on the probability of a true fecal or potential environmental source, or for source tracking studies. The biochemical systems are limited in the sense that they are laborious and less reliable when it comes to environmental isolates. In the last decade, the molecular technique real-time PCR (Polymerase Chain Reaction) has become an important, routinely used detection technique for some of the relevant species for water research (e.g. *E. coli*, *Legionella*). Although PCR is accurate, its use for direct detection of (indicator) bacteria is still limited. Reliable quantification at low levels in environmental samples is still difficult, hampering the introduction of PCR in water laboratories. Another drawback of direct PCR determination is the fact that PCR results do not discriminate between viable and nonviable bacteria. PCR is therefore more often used for colony confirmation, whereas species identification and source tracking still requires

sequence analysis. Matrix Assisted Laser Desorption Ionisation -Time of Flight Mass Spectrometry (MALDI-TOF MS) allows the identification of microbial species within a matter of minutes. This is done by analysing mass spectra of peptides and small proteins. Such a pattern was shown to be characteristic for microbial species (Mellman et al., 2008; Fenselau and Demirev, 2001; Holland et al., 1996; Krishnamurthy et. al, 1996). The general principle of MS is to produce, separate, and detect gas-phase ions. The sample is embedded in the crystalline structure of small organic compounds (matrix) and deposited on a conductive sample support. The crystals are irradiated with a laser beam. The laser energy causes structural decomposition of the irradiated crystal and generates a particle cloud from which ions are extracted by an electric field. This results in the disintegration of the crystal. Following acceleration through the electric field, the ions drift through a field-free path before reaching the detector. Ion masses (mass-to-charge ratios *[m/z]*) are calculated by measuring their Time Of Flight (TOF), which is longer for larger molecules than for smaller ones. In recent years, MALDI-TOF MS has been increasingly used to identify clinically relevant bacteria. The advantages of MALDI-TOF MS are short preparation time, rapid measurement, automatic spectrum analysis and no use of consumables. Furthermore, it is possible to add new mass spectra to the database. However, this technique also has two disadvantages: the necessity to culture the micro-organisms before identification can be completed, and the relatively expensive equipment to do so. In this study, the application of MALDI-TOF MS for water research using *Enterococcus spp*. as a model was examined. Earlier, Benagli et al. (2011) have proven MALDI-TOF MS to be a fast, reliable and cost-effective technique for the identification of clinically relevant *E. faecalis* and *E. faecium*. We applied the same criteria to the environmental isolates *E. faecium*, *E. faecalis E. hirae*, *E. durans* and *E. casseliflavus*.

METHODS

Bacterial isolates. All isolates (n=101) were obtained from water samples, collected over a period of 1 year. The origins of the isolates were: surface water, water at different stages of drinking water treatment and drinking water samples from drinking water distribution systems, mainly after repair works. All isolates were cultured using membrane filtration on solid selective Slanetz and Bartley agar (SBA) for 44 h at 37 ˚C. To confirm typical colonies, the membrane, with all the colonies, was transferred onto bile-aesculin-azid agar (BEAA) for 2 h at 44 ˚C (according to NEN-EN-ISO 7899-2:2000). Only confirmed enterococci colonies, which were able to hydrolyse aesculin

and induced a brown to black colour in the confirmation test were used in this study. Fresh pure cultures on SBA were made. Furthermore, we took a colony from the SBA plate to inoculate Columbia Sheep-Blood agar (CSB). Isolates on CSB were cultured for 24 h at 37 ˚C. Fresh colonies (within 1 hour after incubation) on SBA and CSB agar were used for MALDI-TOF MS. The influence of using less fresh colony material was not studied. For the biochemical Rapid ID 32 Strep test (bioMérieux) only colonies on CSB agar were used, as prescribed by the manufacturer.

Biochemical identification. For biochemical identification, the Rapid ID 32 Strep tests (bioMérieux) were performed according to manufacturer's instructions. The identification results were obtained by entering the test results into the Analytical Profile Index WEB (https://apiweb.biomerieux.com/) using the identification software. A reliability percentage of ≥ 95% was used for species identification.

MALDI-TOF MS analysis. A colony of a freshly cultured isolate on SBA was thinly smeared onto a steel target plate (Bruker Daltonik) using a toothpick. Each colony was overlaid with 1 ml of matrix solution (saturated solution of µ-cyano-4-hydroxycinnamic acid in 50% acetonitrile, and 2.5% trifluoracetic acid) and dried at room temperature. Measurements were performed using a Microflex mass spectrometer (Bruker Daltonik, Bremen, Germany) using FlexControl software (version 2.0). Spectra were recorded in the positive linear mode (laser frequency, 60 Hz; ion source 1 voltage, 20.0 kV; ion source 2 voltage, 16.7 kV; lens voltage, 7.0 kV; mass range, 2,000 to 20,000 Da). For each spectrum 240 shots in 40-shot steps from different positions of the target spot (automatic mode) were collected and analysed. The BioTyper database contains spectra of approximately 34 *Enterococcus* species and is regularly updated by Bruker Daltonik. The software compared the obtained spectrum with those present in the database. During the matching process the obtained spectra were scored on the basis of three matching criteria: 1) the position of peaks in the measured spectrum and how well they matched the position of peaks in the spectrum from the database, 2) overall match between the measured spectrum and the spectrum in the database and 3) the height of the peaks in both spectra. For each criterion a maximum score of 10 could be obtained. Identical matches were given a score of 1000 (10x10x10). The final results of the mass spectra matching process were expressed in scores ranging from 0 to max 3 (^{10}log1000). For each isolate, the highest score of a match against a spectrum in the database was used for identification. For reliable species identification, only scores of ≥2.0 were used, which has been established as a criterion in the validation of MALDI-

TOF MS (Moliner, 2010; Risch et al., 2010). In each target plate run we used *Escherichia coli* Bacterial Test Standard (BTS) provided by manufacturer to control the quality of the measurement. According to the manufacturer's instructions only the measurements with BTS reliability of ≥2.3 were used in this study.

16S rRNA analyses. In case of discrepant results between Rapid ID 32 Strep and MALDI-TOF MS identifications, additional identification was performed by 16S rRNA whole gene sequencing analyses after PCR using 8F: AGAGTTTGATCATGGCTCAG and 1392R: ACGGGCGGTGTGTACA primers. A sequence was attributed to a certain species when similarity (>99%) was obtained using BLAST (Basic Local Alignment Search Tool) software.

Reproducibility test. Three isolates per species (n=15) (*E. faecium, E. faecalis, E. durans, E. hirae* and *E. casseliflavus),* originating from different water samples were used for the MALDI-TOF MS reproducibility test and Rapid 32 ID Strep identification. From each isolate, a pure culture was made on SBA and identified by MALDI-TOF MS. Subsequently, we took a colony from SBA and inoculated the Columbia Sheep-Blood (CSB) agar to obtain the colonies which were identified using Rapid 32 ID Strep. We repeated the same procedure after a week with the same isolates, using fresh pure cultures.

Influence of culture media on MALDI-TOF results. Since the manufacturer of Rapid 32 ID Strep prescribes the use of Columbia Sheep-Blood agar (CSB), which is not routinely used in the water laboratory, we examined the influence of this culture medium on MALDI-TOF MS identification. From almost all isolates included in this study (n=93) pure cultures were made on SBA. These pure cultures were subsequently identified by MALDI-TOF and further used to inoculate Columbia sheep-blood agar (bioMérieux) for 24 hours at 37 ˚C. The colonies were then analysed by MALDI-TOF. During the study we verified whether or not the amount of colony material influenced the reliability. We repeated the identification of a randomly chosen *E. faecium* in 20-fold. Ten times the colony was smeared on the target plate in a thin layer and another 10 times it was smeared in a thick layer. As only one isolate was used for the 20-fold test, these results are not representative for all isolates used in this study. However, the results may provide information on the sensitivity of the analysis and the way the analysis was conducted. The robustness of the analysis is important if the method is going to be used routinely.

RESULTS AND DISCUSSION

We obtained 101 isolates of positive enterococci on BEAA agar from water samples and identified them using Rapid ID 32 Strep system and MALDI-TOF MS. For 86 isolates (85%), the results of Rapid ID 32 Strep were identical to those obtained with MALDI-TOF MS. 16S rRNA analysis was performed on nine isolates with discrepancies in identification between the two methods used to compare the corresponding identification by means of MALDI-TOF and Rapid ID 32 Strep (see Table 2.1). Five strains that showed identical identification with MALDI-TOF and Rapid ID32 were subjected to 16S rRNA analysis to confirm the identification. Discrepant results were observed in isolates identified as *E. gallinarum* by Rapid ID 32 Strep, where all six isolates were identified and confirmed as *E. faecium* using MALDI-TOF MS and 16S analysis (shown in Table 1). One out of a total of 14 isolates identified by Rapid ID 32 Strep as *E. faecium* was identified by MALDI-TOF MS as *E. faecalis* and one out of a total of 22 isolates identified by Rapid ID 32 Strep as *E. hirae* was identified as *E. durans*. In both cases, the 16S rRNA gene analysis confirmed the MALDI-TOF MS identification. For *E. faecalis* and *E. durans* the agreement between Rapid 32 ID and MALDI-TOF MS results was 100%. A noteworthy result was observed for *E. casseliflavus*. Using MALDI-TOF MS one of the *E. casseliflavus* isolates was identified as *E. cecorum*. When the analysis was repeated, it was identified as *E. casseliflavus*. The 16S rRNA gene analysis identified *E. casseliflavus*.

Table 2.1. Comparison between Rapid ID 32 Strep, MALDI-TOF MS and 16S rRNA identification for *Enterococcus spp*.

	No. of isolates	Rapid ID 32 Strep	MALDI-TOF MS	16S rRNA
Agreement	13	*E. faecium*	*E. faecium*	*E. faecium* [a]
	26	*E. faecalis*	*E. faecalis*	*E. faecalis* [a]
	21	*E. hirae*	*E. hirae*	*E. hirae* [a]
	11	*E. durans*	*E. durans*	*E. durans* [a]
	15	*E. casseliflavus*	*E. casseliflavus*	*E. casseliflavus* [a]
	3	no reliable identification [b]	*E. mundtii*	*E. mundtii*
	3	no reliable identification [b]	*E. moraviensis*	*E. moraviensis*
Disagreement	1	*E. faecium*	*E. faecalis*	*E. faecalis*
	6	*E. gallinarum*	*E. faecium*	*E. faecium*
	1	*E. hirae*	*E. durans*	*E. durans*
	1	*E. casseliflavus*	*E. cecorum*/*E. casseliflavus* [c]	*E. casseliflavus*
Total	(n=101)			

[a] Not all, but one randomly chosen isolate was identified by 16S rRNA.

[b] Species not included in database of Rapid 32 ID test.

[c] By repeating the measurement twice, different identifications were obtained: *E. cecorum* and *E. casseliflavus*. Both times the reliability score was ≥ 2.0.

Once the correct (defined as identical identification by at least two methods) identity was determined, the list of species included in this study was made. The number of isolates per species is listed in Table 2.2.

Table 2.2. Isolates (n=101) of *Enterococcus spp.* obtained from water samples.

Enterococcus spp.	(n=101)
E. faecium	19
E. faecalis	27
E. hirae	21
E. durans	12
E. casseliflavus	16
E. mundtii	3
E. moraviensis	3

Using the MALDI-TOF MS database, we were able to identify 34 *Enterococcus* species, while we only identifyed nine using the Rapid 32 ID Strep system. Some *Enterococcus* species, which are not included in the list of species that can be identified by Rapid ID 32 Strep, such as *E. mundtii* and *E. moraviensis*, were identified during our study using MALDI-TOF MS. Three isolates of these two species were compared to the 16S sequencing results. For all six isolates the 16S confirmed the identifications obtained by MALDI-TOF MS. In this case the Rapid 32 ID Strep identifications were considered unreliable. The MALDI-TOF MS appeared to be very time efficient; 2 minutes were needed to prepare the isolate for the measurement and another 2 minutes were needed to identify it. The preparation of one isolate using Rapid ID Strep required 5 minutes to prepare the test and after the 4.5-h incubation an additional 10 minutes were needed to read and interpret the result.

Reproducibility test. With regard to both methods (Rapid 32 ID Strep and MALDI-TOF MS respectively), no discrepancy has been observed between identification of all 15 isolates obtained on a single day and the duplicate test repetition performed on another day. Using Rapid 32 ID Strep, on both days one of the *E. hirae* isolates was identified with a low reliability percentage (77.3%), but confirmed by MALDI-TOF MS and 16S rRNA gene sequence analysis as *E. hirae*.

Use of another culture medium. To examine if the reliability of MALDI-TOF MS identification depended on the use of another cultivation medium, the results obtained using CSB agar were compared with those obtained using SBA agar. There

was no difference found in the identification between the two culture media. Using the Wilcoxon signed-rank test, no significant difference was found either between the reliability score obtained using SBA and using CSB agar; p=0.304 (n=93). However, when the same test was carried out per species, a significant difference was found for *E. hirae* (p=0.021). The reliability score obtained for *E. hirae* with CSB agar was higher than with SBA, but we have not been able to explain the cause of this variation. As the observed difference did not influence the identification, only the reliability of the identification, this result may be less significant.

Reliability. The identification score of the 10-fold measurement of thinly smeared *E. faecium* varied between 2.399 and 2.540, and when the same isolate was smeared thickly, the score varied between 1.998 and 2.457. Using the Wilcoxon signed-rank test, we found a significant difference (p=0.01, n=10) between the reliability scores obtained from 10 thickly and 10 thinly smeared colonies. In all cases *E. faecium* was identified.

The MALDI-TOF MS and Rapid 32 ID Strep identification methods were compared for the identification of *Enterococcus* species isolated from surface water, process and drinking water samples. 16S rRNA gene sequencing was used to verify the correct identification. The 16S rRNA gene sequencing has shown to be a reliable and universal technique for species identification in clinical microbiology (Clarridge, 2004; Vargha et al., 2006). The results of this study show that MALDI-TOF MS can be used to efficiently identify enterococci (*E. faecium, E. faecalis, E. durans, E. hirae, E. casseliflavus)* isolated from water samples. For *E. mundtii* (n=3) and *E. moraviensis* (n=3) we did not have enough isolates to show the reliability of MALDI-TOF MS, although the 16S rRNA gene sequencing confirmed the MALDI-TOF MS identification for six isolates in 100% of the cases. To explain the discrepancy between two MALDI-TOF MS identification results of *E. casseliflavus*, additional research is needed. Discrepancy between MALDI-TOF MS and Rapid 32 ID Strep was observed in six isolates of *E. faecium* identified by Rapid 32 ID as *E. gallinarum*. When the Rapid 32 ID test for these isolates was repeated, different identification results were obtained: one *E. gallinarum* was identified this time as *E. faecium*. We also noticed that one of the biochemical tests (raffinose) has an important influence on the biochemical discrimination between these two species. A positive raffinose test indicated *E. gallinarum*, where a negative test indicated *E. faecium*. For all six isolates, 16S rRNA gene sequencing confirmed *E. faecium* as the identity. Variation of scores obtained in 20 consecutive runs for one randomly chosen isolate showed good repeatability. However, the preparation of the sample influences the reliability score

and should therefore be taken into consideration when implementing MALDI-TOF MS in routine testing. We observed lower scores when running a thick layer of colony material. It was shown, that the use of two different culture media, Slanetz & Bartley and Columbia Sheep-Blood agar, did not influence the MALDI-TOF MS identification. Several studies have reported on the use of MALDI-TOF MS for specific micro-organisms and yeast obtained from reference collections and clinical isolates (van Veen et al., 2010; Seng et al., 2009). These studies concluded that MALDI-TOF MS can replace conventional systems for identification of bacteria in a conventional clinical laboratory. Until now, only a few studies included isolates obtained from environmental samples. A recent study by Moliner et al. (2010) using a large collection of clinical and environmental *Legionella* strains, demonstrated that MALDI-TOF is a reliable tool for the rapid identification of *Legionella* isolates at the species level. However, for the most common pathogenic species, *L. pneumophila*, it could not discriminate among serogroups, using the present database. Identifying enterococci on species level requires a new approach for enterococci analysis within a water laboratory and interpretation of the results. The advantage of rapid identification is the possibility of a rapid response in the case of a true positive result. Rapid identification also avoids a response based on a "false positive" test result. As published previously (Health Protection Agency, 2007), some strains of *Aerococcus viridans* are also bile-aesculin positive and can therefore be confused with *Enterococcus spp.* in water testing. *A. viridans* has been observed in many non-fecal environments (Facklam and Elliott ,1995), it does not possess Lancefield group D antigen and cannot be used to indicate fecal pollution. Beside rapid identification there are also other possible applications of MALDI-TOF MS, which are more suitable for research purposes than for the routine use. For example, trained personnel could extend the database, add mass spectra to the database and exchange data with other users. This is especially relevant for environmental isolates. However, good quality assurance on newly entered spectra is of great importance. The Microflex database currently consists of 34 *Enterococcus* clinically relevant species. Although this number may seem relatively small, the identification with MALDI-TOF is specific enough to discriminate between these clinical and our environmental strains and hence between true fecal species and potential environmental species. As suggested earlier (Giebel et al., 2008), MALDI-TOF MS–based fingerprinting of environmental isolates of fecal indicators as shown in this study for environmental isolates of enterococci, has the potential to become a tool for bacterial source tracking (BST). To establish the value of such a tool, a large number of environmental isolates need to be analysed.

CONCLUSIONS

It has been shown that MALDI-TOF MS can be used for rapid, efficient and reliable identification of *E. faecium*, *E. faecalis*, *E. durans*, *E. hirae* and *E. casseliflavus* isolated from water samples. To establish the identification reliability of two other species, *E. mundtii* and *E. moraviensis*, more isolates needed to be tested. We obtained high reproducibility of the identification and obtained the same result when using two different culture media. To further advance the implementation of this identification technique in (drinking) water laboratories requires the validation of MALDI-TOF MS for other micro-organisms isolated from water.

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REFERENCES

Benagli C., Rossi V., Dolina M., Tonolla M. and Petrini O. (2011). Matrix- Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry for the Identification of Clinically Relevant Bacteria. *PloS One 2011*, 6(1): e164224.

Clarridge J.E. (2004). Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical Microbiology Reviews*, 17(4), 840-862.

Facklam R., and Elliott J. A. (1995). Identification, classification, and clinical relevance of catalase-negative, gram-positive cocci, excluding the Streptococci and Enterococci. *Clinical Microbiology Reviews*, 8(4), 479-495.

Fenselau C., and Demirev P. A. (2001). Characterization of intact microorganisms by MALDI mass spectrometry. *Mass Spectrometry Reviews*, 20(4), 157-171.

Gelsomino R., Vancanneyt M., Cogan T. M., Condon S. and Swings J. (2002). Source of Enterococci in a Farmhouse Raw-Milk Cheese. *Applied and Environmental Microbiology*, 68(7) 3560–3565.

Giebel R.A., Fredenberg W., Sandrin T.R. (2008). Characterization of environmental isolates of Enterococcus spp. by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Water Research*, 42(4-5), 931- 40.

Health Protection Agency (2007). Identification of *Streptococcus species*, *Enterococcus species* and morphologically similar organisms. National Standard Method BSOP ID 4 Issue 2.1http://www.hpa-standardmethods.org.uk/pdf sops.asp.

Holland R. D., Wilkes J. G., Rafii F., Sutherland J. B., Persons C. C., Voorhees K. J., and Lay J. O. J. (1996). Rapid identification of intact whole bacteria based on spectral patterns using matrix- assisted laser desorption/ ionization with time-of- flight mass spectrometry. *Rapid Communications in Mass Spectrometry*, 10(10), 1227-1232.

Jett B. D., Huycke M. M. and Gilmore M. S. (1994).Virulence of Enterococci. *Clinical Microbiology Reviews* 7(4), 462-478.

Krishnamurthy T., Ross P. L., and Rajamani U. (1996). Detection of pathogenic and non-pathogenic bacteria by matrix- assisted laser desorption/ionization with time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry*, 10(8), 883-888.

Leclerc, H., Devriese, L.A. and Mossel, D.A.A. (1996) Taxonomical changes in intestinal (faecal) enterococci and streptococci: consequences on their use as indicators of faecal contamination in drinking water. *Journal of Applied Bacteriology* 81, 459–466.

Mellman, A., Cloud J., Maier T., Keckevoet U., Ramminger I., Iwen P., Dunn J., Hall G., Wilson D., LaSala P., Kostrzewa M. and Harmsen D. (2008). Evaluation of Matrix-Assisted Laser Desorption Ionization-Time-of-Flight Mass Spectrometry in Comparison to 16S rRNA Gene Sequencing for Species Identification of Nonfermenting Bacteria. *Journal of Clinical Microbiology,* 46(6), 1946-1954.

Moliner C., Ginevra C., Jarraud S., Flaudrops C., Bedotto M., Couderc C., Etienne J. and Fournier P. E. (2010). Rapid identification of *Legionella* species by mass spectrometry. *Journal of Medical Microbiology*, 59, 273-284.

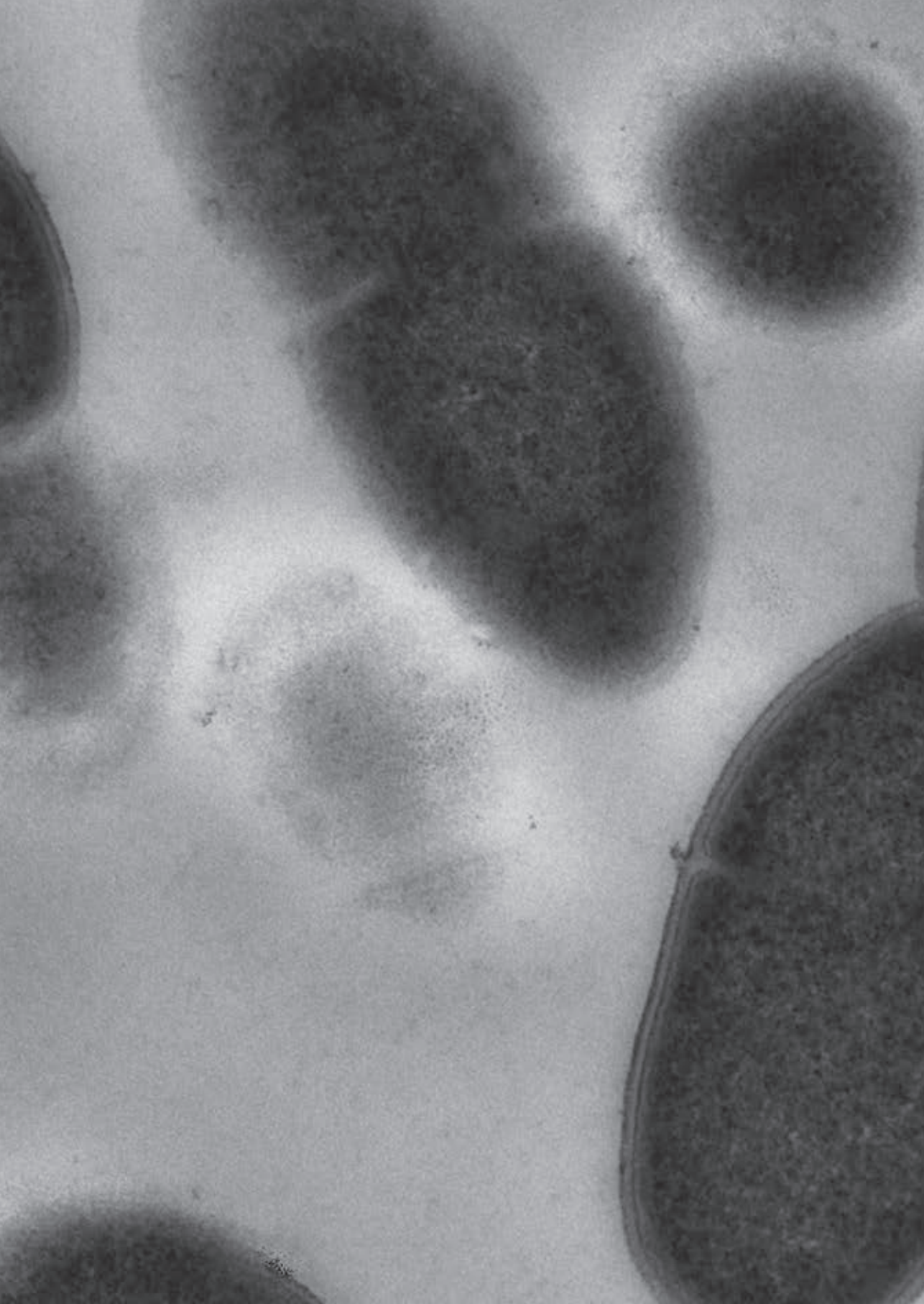
Risch M., Radjenovic D., Han J. N., Wydler M., Nydegger U. and Risch L. (2010). Comparison of MALDI_TOF with conventional identification of clinically relevant bacteria. *Swiss Medical Weekly*, 140:w13095.

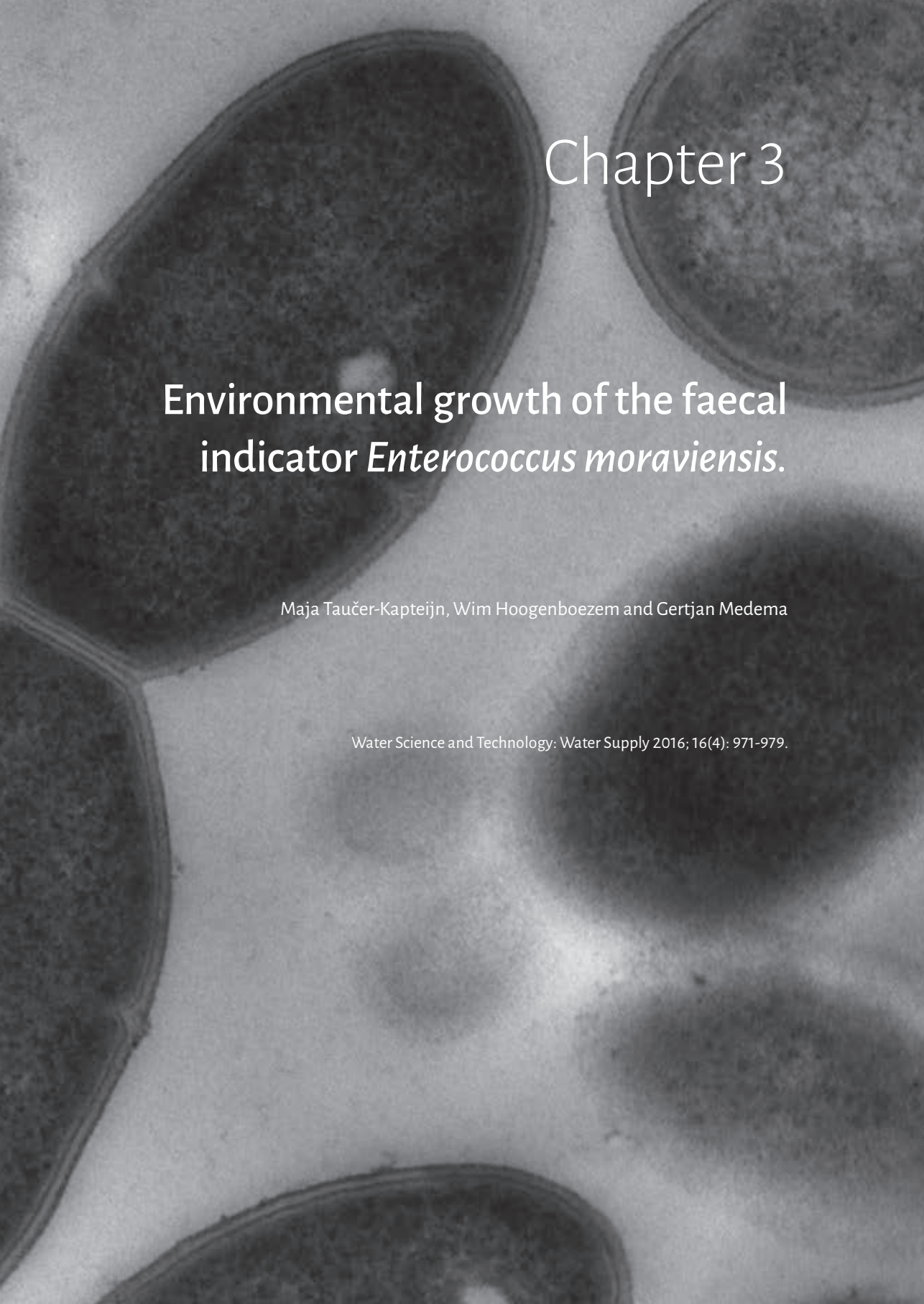
Seng P., Drancourt M., Gouriet F., La S.B., Fournier P.E., Rolain J.M. and Raoult D. (2009). Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Clinical Infectious Diseases*, 49(4), 543-551.

Shepard B. D. and Gilmore M. S. (2002). Antibiotic-Resistant Enterococci: The Mechanisms and Dynamics of Drug Introduction and Resistance. *Microbes and Infection Institut Pasteur,* 4(2), 215-224.

Vargha M., Takats Z., Konopka A., and Nakatsu C. H. (2006). Optimization of MALDI-TOF MS for strain level differentiation of *Arthrobacter* isolates. *Journal of Microbiological Methods*, 66(3), 399-409.

van Veen S. Q., Claas E. C. J., and Kuijper Ed J. (2010). High-Throughput Identification of Bacteria and Yeast by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry in Conventional Medical Microbiology Laboratories. *Journal of Clinical Microbiology*, 48(3), 900-907.





Chapter 3

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^{-1} (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^{-1} 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 leave 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^{-1} 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 de 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^{-1}, 20 g L^{-1}, 2 g L^{-1} and 0.2 g L^{-1}. 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^{-1}. 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^{-1} 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^{-1}. 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^{-1}. 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^{-1}. 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* (10^4 cfu mL^{-1}) 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^{-1}. 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^{-1}. 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* ($2*10^4$ cfu mL^{-1}) 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^{-1}. To confirm the attachment to the bilfilm, 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^{-1} 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% CaCO3, 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^{-1} 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^{-1}) 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*, 10^2 *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.

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

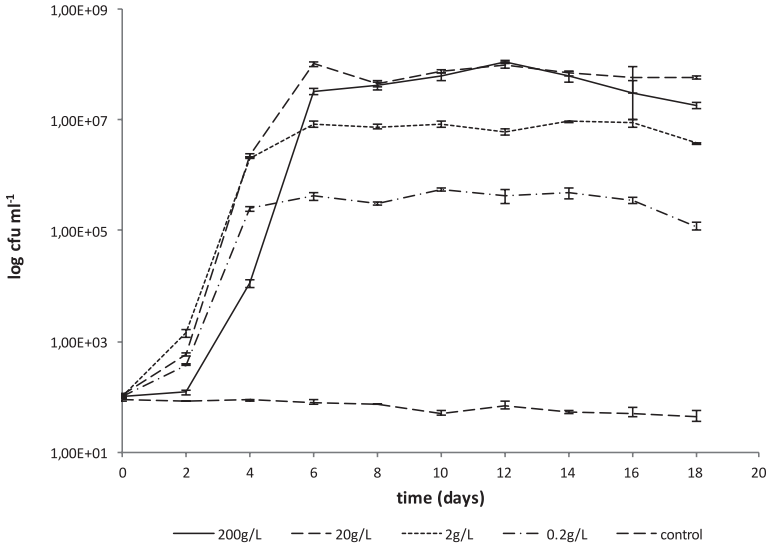

Figure 3.1. Growth of *E. moraviensis* at 15 ˚C on sterilized plant extract. Extract concentrations: (—) 200 g L^{-1}, (---) 20 g L^{-1}, (---) 2 g L^{-1}, (- . -) 0.2 g L^{-1} and (- -) control. Bars indicate standard deviations of duplicate measurements.

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^{-1}, 5 g L^{-1} and 0.5 g L^{-1}), rapid growth occurred and a maximum was reached within 3 days. Yields were $5.3*10^3$ cfu g^{-1}, $8.3*10^3$ cfu g^{-1} and $2.1*10^4$ cfu g^{-1}, 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.

Dune vegetation provides nutrients suitable to support the growth of *E. moraviensis* at 15 ˚C. Boiled and filtered plant extracts showed different yields (Δyields = 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).

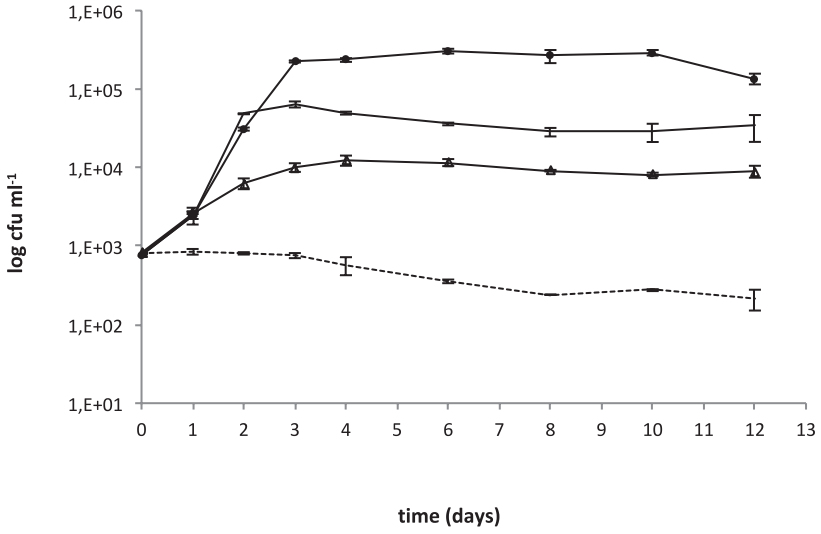

Figure 3.2. Growth of *E. moraviensis* on filtered (0.45 µm) plant extract at 15 ˚C. Extract concentrations: (•) 50 g L^{-1}, (–) 5 g L^{-1}, (Δ) 0.5 g L^{-1} and (---) control. Bars indicate standard deviations of duplicate measurements.

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^{-1} at 15 ˚C, -0.22 d^{-1} at 20 ˚C and -0.50 d^{-1} at 25 ˚C (Figure 3.3).

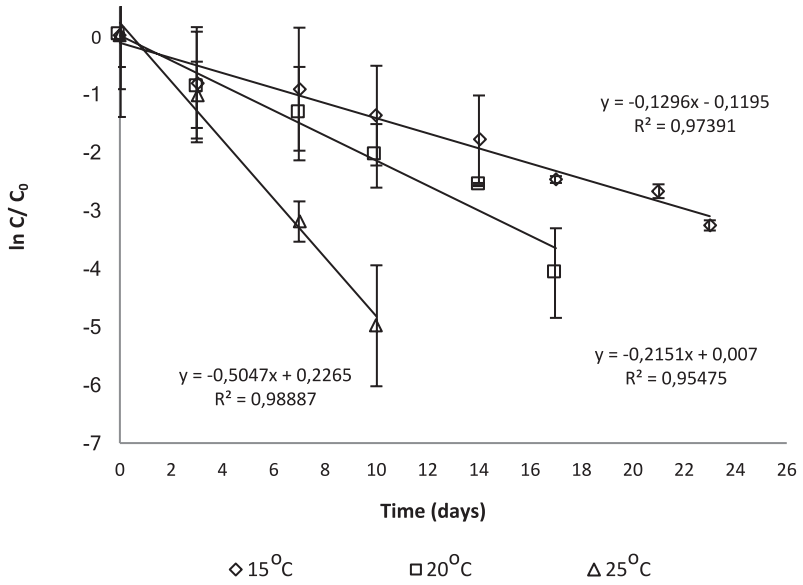

Figure 3.3. Die-off rate of *E. moraviensis* at 15 ˚C (◇), 20 ˚C (□) and 25 ˚C (Δ) in sediment. Bars indicate standard deviations of duplicate measurements.

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^{-1}; 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^{-1} varied between 21 and 60. On average, 34 cfu ring^{-1} (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^{-1} (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 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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REFERENCES

Aarestrup F., Butaye P. & Witte W. 2002 Nonhuman reservoirs of enterococci. In *The enterococci*, M. S. Gilmore (ed.), DC:ASM Press, Washington, pp. 55-99.

Badgley B. D., Thomas F. I. M. & Harwood V. J. 2010 The effects of submerged aquatic vegetation on the persistence of environmental populations of *Enterococcus* spp. *Environmental Microbiology*, 12 (5),1271-1281.

Byappanahalli M. N., Shively D. A., Nevers M. B., Sadowsky M. J. & Whitman R. L. 2003 Growth and survival of *Escherichia coli* and enterococci populations in the macro-alga *Cladophora* (Chlorophyta). *FEMS Microbiology Ecology*, 46 (2), 203-211.

Byappanahalli M. N., Roll B. M. & Fujioka R. S. 2012 Evidence for occurrence, persistence and growth of *Escherichia coli* and enterococci in Hawaii's soil environments. *Microbes and Environments*, 27 (2), 164-170.

Euzéby, J. P. 2009 Enterococcus. Dictionnaire de Bactériologie Vétérianire. In Microbiological Examination Methods of Food and Water: A Laboratory Manual, N. da Silva, M. H. Taniwaki, V. C. A. Junqueira, N. F. A. Silveira, M. S. do Nascimento & R. A. R. Gomes (eds.) CRC Press, Boca Raton, FL, p.140.

Flacklam R. R., Carvalho M. & Teixeira L. 2002 History, taxonomy, biochemical characteristics and antibiotic susceptibility testing of enterococci. In: *The enterococci*, M. S. Gilmore (ed.), DC: ASM Press, Washington, pp. 1-54.

Fujioka R., Sian-Denton C., Borja M., Castro J. & Morphew K. 1999 Soil: the environmental source of *Escherichia coli* and enterococci in Guam's streams. *Journal of Applied Microbiology*, 85 (S1), 83S-89S.

Geldreich E. E., Kenner B. A. & Kabler P. W. 1964 Occurrence of Coliforms, Fecal Coliforms, and Streptococci on Vegetation and Insects. *Applied Microbiology*, 12 (1), 63-69.

ISO (7899-2:2000).Water quality. Detection and enumeration of intestinal enterococci. Part 2: Membrane filtration method.

Layton B. A., Walters S. P., Lam L. H. & Boehm A. B. 2010 *Enterococcus* species distribution among human and animal hosts using multiplex PCR. *Journal of Applied Microbiology*, 109 (2), 539-547.

Lebreton F., Willems R. J. L., & Gilmore M. S. 2014. *Enterococcus* Diversity, Origins in Nature, and Gut Colonization in Enterococci: From Commensals to Leading Causes of Drug Resistant Infection, M. S. Gilmore, D. B. Clewell, Y. Ike & and N. Shankar (ed.). Boston: Massachusetts Eye and Ear Infirmary.

Müller T., Ulrich A., Ott E. M. & Müller M. 2001 Identification of plant-associated enterococci. *Journal of Applied Microbiology*, 91 (2), 268-78.

Mundt J. O., Coggins J. H. Jr & Johnson L. F. 1962 Growth of *Streptococcus faecalis* var. *liquefaciens* on plants. *Applied Microbiology* 10 (6), 552-555.

Mundt J. O. 1963 Occurrence of enterococci on plants in wild environment. *Applied Microbiology* 11 (2), 141-144.

Noble R. T., Lee I. M. & Schiff K. C. 2004 Inactivation of indicator micro-organisms from various sources of faecal contamination in seawater and freshwater. *Journal of Applied Microbiology*, 96 (3), 464–472.

Ott E. M., Müller T., Müller M., Franz C. M., Ulrich A., Gabel M. & Seyfarth W. 2001 Population dynamics and antagonistic potential of enterococci colonizing the phyllosphere of grasses. *Journal of Applied Microbiology* 91 (1), 54-66.

Schijven J. F., Hoogenboezem W., Nobel P. J., Medema G. J. & Stakelbeek A. 1998 Reduction of FRNA-bacteriophages and faecal indicator bacteria by dune infiltration and estimation of sticking efficiencies. *Water Science and Technology*, 38 (12), 127-131.

Sinton L. W., Davies-Colley R. J. & Bell R. G. 1994 Inactivation of enterococci and fecal coliforms from sewage and meatworks effluents in seawater chambers. *Applied and Environmental Microbiology*, 60 (6), 2040–2048.

Sinton L. W., Hall C. H., Lynch P. A. & Davies-Colley, R. J. 2002 Sunlight inactivation of fecal indicator bacteria and bacteriophages from waste stabilization pond effluent in fresh and saline waters. *Applied and Environmental Microbiology*, 68 (3), 1122–1131.

Švec P. & Sedláček I. 1999 Occurrence of *Enterococcus* spp. in waters. *Folia Microbiologica*, 44 (1), 3-10.

Švec, P. & Devriese, L. A. 2009 Genus I Enterococcus (ex Thiercelin and Jouhaud 1903) Schleifer and Kilpper-Bälz 1984. In: Bergey's Manual of Systematic Bacteriology, P. De Vos, G. M. Garrity, D. Jones, N. R. Krieg, W. Ludwig, F. A. Rainey, K. Schleifer & W. B. Whitman (eds), 2nd edn, Volume 3. Springer, New York, pp. 594–607.

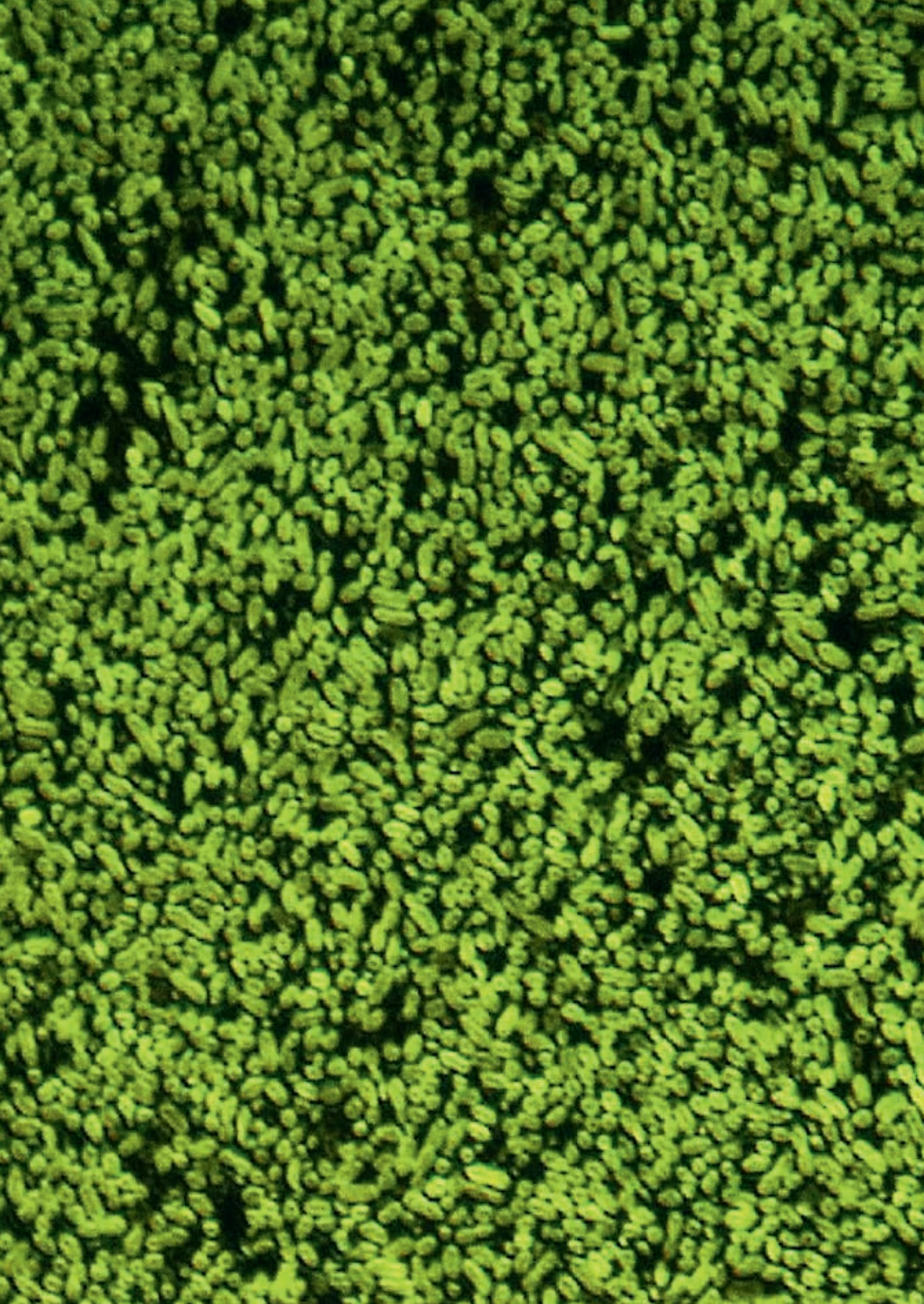
Švec P., Devriese L. A., Sedláček I., Baele M., Vancanneyt M., Haesebrouck F., Swings J. & Doškar J. 2001 *Enterococcus haemoperoxidus* sp. nov. and *Enterococcus moraviensis* sp. nov., new species isolated from water. *International Journal of Systematic and Evolutionary Microbiology*, 51, 1567-1574.

Taučer-Kapteijn M., Medema G. & Hoogenboezem W. 2013 Comparison between Rapid ID 32 Strep System, Matrix Assisted Laser Desorption Ionisation–Time of Flight Mass Spectrometry and 16S rRNA gene sequence analysis for the species identification of *Enterococcus* spp. isolated from water. *Water Science & Technology: Water Supply*, 13 (5), 1383-1389.

van der Kooij D. & Veenendaal H. R. 1992 Assessment of the biofilm formation characteristics of drinking water. Proceedings Water Quality Technology Conference. American Water Works Association, Denver, pp. 1099-1110.

van der Kooij D. (2001). AOC Manual. BTO Report. 212 (S). Kiwa Water Research.

Whitman R. L., Shively D. A., Pawlik H., Nevers M. B. & Byappanahalli M. N. 2003 Occurrence of Escherichia coli and enterococci in Cladophora (Chlorophyta) in nearshore water and beach sand of Lake Michigan. *Applied and Environmental Microbiology*, 69 (8), 4714–4719.



Chapter 4

Possible environmental growth of faecal indicators (*Enterococcus moraviensis, E. casseliflavus, E. hirae, E. faecalis* and *E. faecium*) on plant extract.

Maja Taučer-Kapteijn, Wim Hoogenboezem and Gertjan Medema

This chapter has been submitted for publication.

ABSTRACT

This study compared the extent of growth for *E. moraviensis*, *E. casseliflavus*, *E. hirae*, *E. faecalis* and *E. faecium* on extracts of marram grass (*Ammophila arenaria*), a common plant in vegetation of the Castricum dune infiltration area (The Netherlands). Nutrient concentrations of 50 g L^{-1}, 5 g L^{-1} and 0.5 g L^{-1} and a temperature of 15 ˚C were used in two different approaches. The first approach examined the growth of enterococci when the major part of the indigenous microbial population was removed. In the second approach, enterococci had to compete with indigenous flora. In the first approach, plant extract promoted nutrient concentration dependent growth of all five *Enterococcus* species. Culturable *Enterococcus* cells were detectable for several months in the plant extract. When the indigenous microbial community was not removed from the plant extract, different *Enterococcus* species differed in their ability to multiply. At the highest nutrient concentration (50 g L^{-1}), an initial increase (from 7.2×10^{1} to 4.3×10^{2}) in the numbers of CFU mL^{-1} was observed for *E. moraviensis*; an increase was not observed for the other species studied. These findings can have implications for the suitability of *E. moraviensis* as an indicator of water quality, especially in suspensions rich in plant material. Although the ability to grow on plant extract has been shown for five *Enterococcus* species, the results of this study indicate that predation and competition for the available nutrients hinder the replication and persistence of enterococci in the environment.

INTRODUCTION

Faecal indicator bacteria (FIB) have been widely used as water quality parameters, indicating the potential presence of enteric pathogens. The genus *Enterococcus* has been recognized as a reliable FIB, due to the following important characteristics: i) intestines of human and other warm-blooded animals are a natural habitat of *Enterococcus* spp., ii) enterococci tend to persist longer in the environment than other FIB like the coliforms (Stevens et al., 2003), and iii) previous epidemiological studies have demonstrated a correlation between the concentration of enterococci in surface waters and an increase in swimmer-associated gastroenteritis (Kay et al. 1994; He & Jiang, 2005). Presently it is assumed that when enterococci are shed from the gastrointestinal tract of animal hosts, they will decay in secondary environmental habitats due to ecological stressors, such as nutrient deprivation, predation or ultraviolet radiation. More recently, however, the occurrence, persistence, and population dynamics of enterococci in extra-enteric environmental habitats have received increasing attention (Byappanahalli et al., 2012). Recent characterization of enterococci in extra-enteric habitats has revealed the presence of apparently stable, potentially naturalized, environmental populations (Badgley et al., 2010a). Such populations may confound the use of enterococci as FIB, especially when they are present in high abundance in the absence of faecal contamination and human pathogens (Staley et al., 2014). It has been suggested that water quality studies might benefit from focusing on a subset of *Enterococcus* spp. that are consistently associated with sources of faecal pollution including domestic sewage, which poses significant human health risks, rather than utilizing the entire genus. *E. faecium* and *E. faecalis* have been suggested as good faecal species, as they have been identified consistently as the dominant *Enterococcus* spp. in human faeces (Chenoweth & Schaberg, 1990; Gelsomino et al., 2003; Harwood et al., 2004) and in sewage (Manero et al., 2002; Harwood et al., 2004). It has also been suggested that some of the *Enterococcus* species (intestinal enterococci: *E. faecalis, E. faecium, E. hirae* and *E. durans*) might be more reliable indicators of faecal contamination than other members of *Enterococcus* spp., which can be found in faeces but is also associated with vegetation (*E. casseliflavus* and *E. mundtii*) (Ulrich & Müller, 1998; Salminen et al., 2004). The technique that clearly differentiates faecal from naturally adapted strains of enterococci is not yet available. More information on sources, survival and the ability of different species to grow in extra-enteric environments is desirable. 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 (Švec et al., 2001; Švec et al. 2005; Carvalho et al., 2006; Sukontasing et al., 2007; Cotta et al., 2013; Frolková et al., 2013). The information on host associated *Enterococcus* species may assist in finding the contamination source and help in the selection of effective preventive measures. Lack of information on faecal sources and the ecology of newly described species, makes the evaluation of their reliability as FIB difficult.

Detection of enterococci during drinking water production process requires rapid detection and localization of faecal contamination in order to control the possible presence of pathogens in the drinking water that is produced. If faecal sources are not readily identifiable and *Enterococcus* species other than the intestinal enterococci are detected, uncertainty arises about the integrity of the water treatment system versus the validity of the indicator and the existence of extra-enteric sources. Faecal sources are not established for all *Enterococcus* species and little is known about the survival of enterococci under certain environmental conditions on the species level. This study was prompted by the occasional detection of enterococci in 100 L samples of water abstracted from a shallow aquifer in a natural dune infiltration area for drinking water production (Castricum, The Netherlands). *Enterococcus moraviensis* was the species most frequently identified in these samples. Removal of micro-organisms during soil passage in dune infiltration areas is often used as one of the treatment steps and main barriers for enteric pathogens in drinking water production in The Netherlands (Taučer-Kapteijn et al., 2013). Recovered (abstracted) groundwater is normally free of FIB and, together with bacteriophages, they are very effectively removed by the soil passage process (Schijven et al., 1998). The abstracted water is therefore considered safe with regard to enteric pathogens, but occasional presence of enterococci challenges this assumption. In our previous study (Taučer-Kapteijn et al., 2016), growth of *E. moraviensis* on filtered plant extract prepared from certain dune plants was observed, where the majority of the indigenous microbial community was removed. The objective of this study was to determine if, and to what extent, other *Enterococcus* species (*E. casseliflavus*, *E. hirae*, *E. faecalis* and *E. faecium*) are also able to grow under the same environmental conditions. In addition, the same experiment, however using unfiltered plant extract, was performed to evaluate the ability of these species to grow at 15 °C when in competition with natural microbial community.

MATERIALS AND METHODS

Bacterial strains and identification method

The *E. moraviensis, E. casseliflavus, E. faecalis, E. faecium, E. hirae* strains used in this study were 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 3.1, Bruker). The identification of isolates was performed in accordance with the manufacturer's instructions. In each MALDI-TOF MS identification run, we used the *Escherichia coli* Bacterial Test Standard (BTS) provided by the manufacturer in order to ensure the quality of the measurement.

Five inoculum flasks, each containing 10^4 cfu mL^{-1} of *E. moraviensis, E. casseliflavus, E. faecalis, E. faecium* or *E. hirae* in stationary phase were prepared according to the specifications stipulated by Van der Kooij (2001).

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 April 2014, a total of 900 g of plant material composed of leaf parts of the most common dune plant *Ammophila arenaria* were collected in the vicinity of abstraction wells (Q 400) in the Castricum dune infiltration area (The Netherlands). Plant material was transported in sterile plastic bags to the laboratory, where collected plant leaves were aseptically cut into pieces of c. 3 cm length using sterile scissors and then mixed to obtain a homogeneous plant mixture, which was stored at -20 ˚C for further use in the preparation of plant extract.

Microcosms with filtered plant-extract

Exactly 400 g of plant material mentioned above was suspended in 8 L of abstracted water for five hours at room temperature. Subsequently the extract was decanted and filtered using a sterile 0.22 µm filter (Millipore) to remove plant parts and indigenous bacteria. Plant extract was divided into AOC-free Erlenmeyer flasks, to obtain plant extract (600 mL) in concentrations of 50 g L^{-1}, 5 g L^{-1} and 0.5 g L^{-1} for each *Enterococcus* species in duplicate. Filtered abstracted water from Castricum dune infiltration area (The Netherlands) (Millipore 0.22 µm) was used as a control experiment (600 mL

in duplicate) and to prepare plant extract dilutions. *E. moraviensis*, *E. casseliflavus*, *E. faecalis*, *E. faecium* or *E. hirae* were inoculated into separate flasks to obtain an initial concentration of approximately 100 cfu mL^{-1}. In the solutions, which were kept in the dark at 15 ˚C, the concentration of enterococci was measured over time using the enumeration method described below. In order to follow the growth of bacteria that could not be removed by filtration, dilutions of 50 g L^{-1}, 5 g L^{-1}, 0.5 g L^{-1} and a control (filtered abstracted water without plant extract) were prepared in duplicates without the addition of enterococci. Total plate counts were determined over time using Plate Count Agar (PCA, Oxoid) according to NEN-EN-ISO 6222:1999.

Microcosms with unfiltered plant-extract

With the exception of the filtration step, a batch of unfiltered plant extract was prepared following the same procedure as filtered plant extract. To enumerate enterococci that may naturally be present on the plant material, 100 mL of the batch was analysed using the SBA enumeration method (see below). The batch was then divided into portions of 60 mL in concentrations of 50 g L^{-1}, 5 g L^{-1} and 0.5 g L^{-1}. Unfiltered abstracted water was used as a control experiment and to prepare plant extract dilutions. Subsequently, *E. moraviensis*, *E. casseliflavus*, *E. faecalis*, *E. faecium* or *E. hirae* were inoculated into separate flasks to obtain solutions with an initial concentration of approximately 100 cfu mL^{-1}. These were then kept in the dark at 15 ˚C. Enterococci and the total plate counts were monitored according to the standard enumeration methods over time.

Enumeration methods

ISO 7899-2:2000

A volume of 1 mL was taken from the flask and used to prepare dilutions to 10^7 using sterile tap water. Subsequently, samples were filtered using 0.45 µm cellulose nitrate filter (Sartorius Stedim) and incubated on Slanetz and Bartley Agar (SBA, Oxoid) for 48 h at 37 ˚C. A confirmations test was performed by the incubation for 2 h at 44 ˚C using Bile-esculin-azide agar (BEAA). Typical colonies that showed a dark brown to black colour in the medium directly surrounding the presumptive colony were counted as enterococci. The number of enterococci per sample was calculated by multiplying the average number of typical colonies per BEAA plate by the reciprocal of the dilution used. To identify *Enterococcus* species grown on the BEAA medium, the MALDI-TOF MS identification method was applied according to the manufacturer's instructions. Quality control was performed using the ATCC 27270 *E. faecium* strain.

NEN-EN-ISO 6222:1999

Each time 1 mL was sampled from the flask and used to prepare a dilution series from 0 to 10^7 using sterile tap water. A volume of 15 ml of tempered (45 °C) Plate Count Agar (PCA, Oxoid) was then added to the sterile petri dishes containing 0.1 mL of the sample. Plates were gently swirled for a few seconds, then allowed to cool and solidify and finally incubated for 72 ± 4 h at 22 °C. Colonies were counted on plates containing 30 - 300 colonies. The bacterial count per sample was calculated by multiplying the average number of colonies per plate by the reciprocal of the dilution used.

Microscopy

The sediment from the filtered and unfiltered suspension of randomly selected samples of the *E. hirae* (50 g L^{-1}) microcosm was inspected for the presence of protozoa. 2 µL of the sediment was used for microscopic investigation. Microscope slides were inspected using phase-contrast microscope (Olympus BHS, Japan) with the magnification of 400×.

Predation test

To test for the occurrence of predation by eukaryotes, four turbid bacterial suspensions of 4 ml were prepared dispersing colonies of *Pseudomonas fluorescens* in sterile tap water. 1 ml of filtered and unfiltered plant extract suspension was added to the *Pseudomonas fluorescens* suspensions. Tubes were prepared in duplicate and were incubated at 15 ºC. After 3 days of incubation the turbidity of the suspensions was inspected visually and microscopically for the presence of protozoa.

RESULTS

Filtered plant extract of *Ammophila arenaria* (a common plant in dune vegetation) promoted growth of all *Enterococcus* species (*E. moraviensis*, *E. casseliflavus*, *E. hirae*, *E. faecalis* and *E. faecium*) used in this study. Growth maxima (10^5-10^7 CFU mL^{-1} at 50 g L^{-1} and 10^3—10^5 CFU mL^{-1} at 5 g L^{-1}) were reached between three and seven days and were dependent on the concentration of the nutrients. Growth at low nutrient level 0.5 g L^{-1} was observed only in *E. moraviensis* and *E. casseliflavus* (1.5 log and 2 log respectively), with a maximum density after seven and ten days respectively. This finding indicates differences among *Enterococcus* species to grow on plant extract, based on the minimum nutrient concentration required for growth at 15 ºC. For all plant extract concentrations,

the highest yields were obtained for *E. casseliflavus* and *E. moraviensis*. The lowest yields were obtained for *E. faecium*, indicating limited ability of this species to grow under the conditions examined in this study. After the growth phase, the numbers of enterococci in the control flasks changed slightly over time (Figure 4.1). Because the standard deviations did not overlap, differences in the performance of various species are considered to be meaningful.

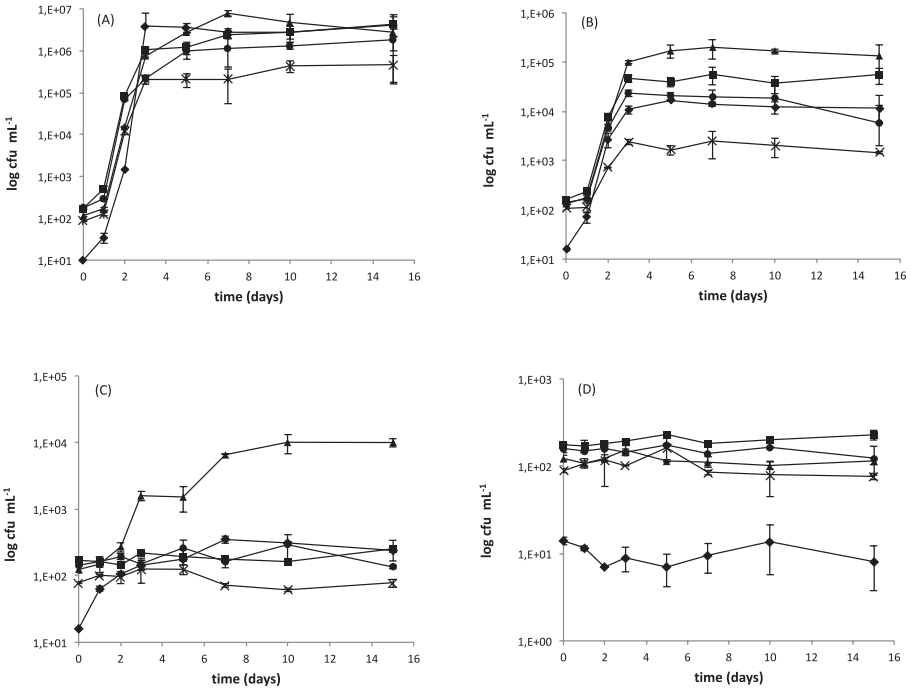


Figure 4.1. Growth of *E. moraviensis* (♦), *E. casseliflavus* (▲), *E. hirae* (■), *E. faecalis* (●) and *E. faecium* (×) at plant extract concentrations of 50 g L^{-1} (A), 5 g L^{-1} (B), 0.5 g L^{-1} (C) and 0 g L^{-1} (controls) (D) at 15 °C. Bars indicate standard deviations of duplicate measurements.

Monitoring total plate counts (PCA) without addition of enterococci showed that the autochthonous community had not been completely removed by 0.22 µm filtration, although this was not noticed in the first series of tests (day 0), as all counts were <1 in all flasks. Although it is not clear how much of this natural microbial heterotrophic community had remained in the extract, its growth reached maxima of 10^8 cfu mL^{-1}, 10^7 cfu mL^{-1}, and 10^6 cfu mL^{-1}, at 50 g L^{-1} and 5 g L^{-1} and 0.5 g L^{-1} respectively within five days (Figure 4.2).

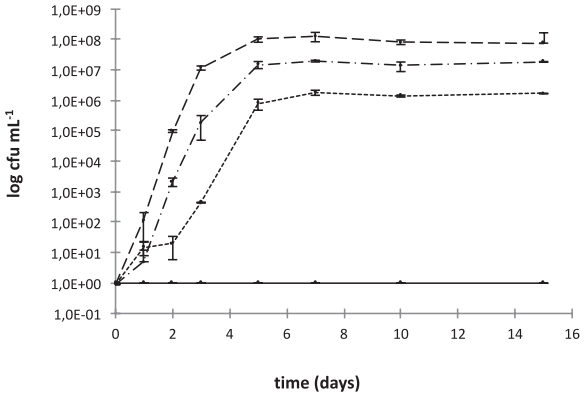


Figure 4.2. Growth of natural microbial population on filtered (0.22 µm) plant extract at 15 °C was monitored using PCA medium. Plant extract concentrations were: (– –) 50 g L^{-1}, (–•–) 5 g L^{-1}, (- - -) 0.5 g L^{-1} and (—) control. Bars indicate standard deviations of duplicate measurements.

Comparison of growth levels of PCA bacteria (Figure 4.2) with growth levels of enterococci in the first experiment (Figure 4.1) indicates that enterococci generally took advantage of the available nutrients in the first 48 hours, however differences per species were observed. No growth has been observed in the filtered abstracted water (control) indicating the competing flora depended heavily on the nutrients from the plant extracts.

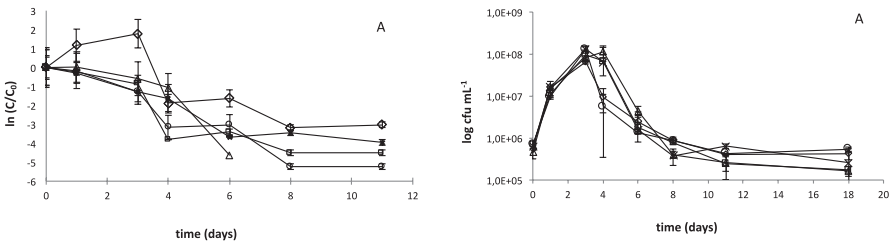
After growth maxima were reached, the enterococci were detectable by culture method for a longer period (52 - 283 days) (Table 4.1). However, their persistence differed per species. *E. moraviensis* and *E. faecalis* persisted for less than 121 days, while *E. casseliflavus*, *E. hirae* and *E. faecium* persisted for a longer period of time. For *E. moraviensis*, *E. casseliflavus* and *E. hirae* the survival rate was proportional to the dose of nutrients.

Table 4.1. Persistence of *Enterococcus* species at different concentrations of filtered plant-extract in days (x).

	50 g L^{-1}	5 g L^{-1}	0.5 g L^{-1}
E. moraviensis	73 < x < 121	73 < x < 121	52 < x < 73
E. casseliflavus	121 < x < 273	73 < x < 121	73 < x < 121
E. hirae	283 < x < 353	121 < x < 283	121 < x < 273
E. faecalis	73 < x < 121	73 < x < 121	73 < x < 121
E. faecium	273 < x < 283	121 < x < 273	121 < x < 273

The experiment in which the natural microbial community was not removed from the plant extract demonstrates the differences between the *Enterococcus* species regarding their ability to multiply in competition with the indigenous community. Furthermore, the presence of predators (protozoa) has been shown and may have caused a reduction of concentrations of enterococci in this experiment. At the highest concentration of nutrients (50 g L^{-1}), *E. moraviensis* was the only species with initial increase (up to 4.3×10^2) in the numbers of CFU mL^{-1} (Figure 4.3). This initial increase, which lasted only for three days, was followed by rapid decrease in numbers of *E. moraviensis*, which coincides in time with the decrease of the total number of bacteria in these flasks, caused by the limitation of the nutrients. At the lower concentration of nutrients (5 g L^{-1} and 0.5 g L^{-1}), only decay of all *Enterococcus* species was observed. Average survival time of enterococci at 50 g L^{-1} and 5 g L^{-1} and 0.5 g L^{-1} was 11 days, 9 days and 12 days respectively. Enterococci held in the suspension without the addition of plant extract (control) were culturable for at least 18 days.

Average start concentrations of the total numbers of bacteria per mL^{-1} in 50 g L^{-1} and 5 g L^{-1} and 0.5 g L^{-1} solutions were 6.0×10^5 CFU mL^{-1}, 6.7×10^4 CFU mL^{-1} and 7.0×10^3 CFU mL^{-1} corresponding to the three dilutions of the plant extract, meaning that the addition of enterococci (approximately 100 CFU mL^{-1}) represented only a small fraction of the total number of bacteria. Rapid growth at these nutrient concentrations reached average maxima of 1.1×10^8, 3.4×10^7 and 2.9×10^6 respectively within three to four days. This did not allow time for extensive multiplication of enterococci under conditions of competition with other bacteria which are better adapted to grow on plant material. Different yields were obtained in the control flasks (Figure 4.3), which remain unexplained since the same batch of abstracted water has been used to dilute the extract and similar densities of enterococci were present in the inoculum flasks used.



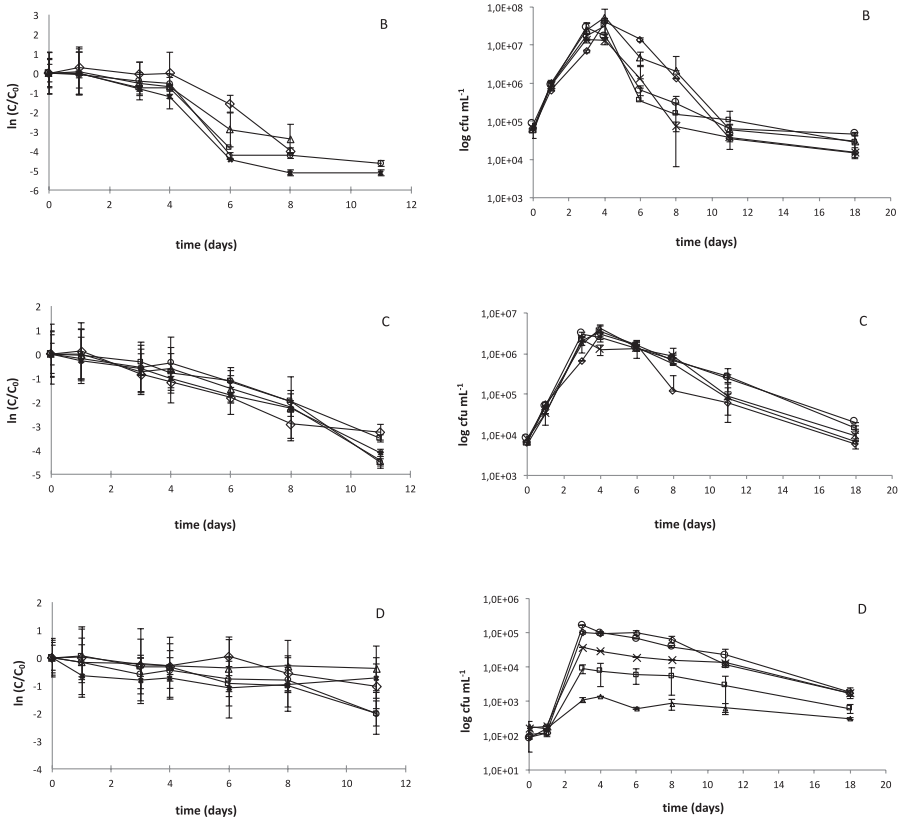


Figure 4.3. Left: Decay of *Enterococcus* species under conditions of competition with indigenous microbial population monitored using SBA medium: *E. moraviensis* (◇), *E. casseliflavus* (□), *E. hirae* (△), *E. faecalis* (○) and *E. faecium* (×). Right: Total numbers of bacteria monitored using PCA medium. Signs in the right column indicate the suspension containing particular *Enterococcus* species. Letters refer to the plant extract concentration of 50 g L^{-1} (A), to 5 g L^{-1} (B), 0.5 g L^{-1} (C) and to the control measurement (D). Bars indicate standard deviations of duplicate measurements.

Using the same dose of plant extract, similar decay constants were obtained for different *Enterococcus* species (Table 4.2). Using phase contrast microscopy, high numbers of protozoa (magnification of 400×) were observed in the unfiltered samples, which was not the case in the filtered samples. Three types of amoebae belonging to genera *Vexillifera*, *Mayorella and Limax*-type were observed. Moreover, several species of heterotrophic flagellates were also present in de sediment of the experimental flask. The predation test confirmed differences in the predation capacity between the filtered and unfiltered extract. Within three days of incubation, the turbid suspensions with unfiltered plant-extract turned into a clear suspension, containing large numbers of heterotrophic flagellates, while the suspensions containing filtered extract remained

turbid and no heterotrophic flagellates were observed. These results indicate that differences in the decay rates can be attributed to the activity of protozoa.

Table 4.2. The exponential decay constants per day for *Enterococcus* spp. in filtered and unfiltered plant-extract at 15 °C.

	Filtered				*Unfiltered*			
	50 g L^{-1}	5 g L^{-1}	0.5 g L^{-1}	Control	50 g L^{-1}	5 g L^{-1}	0.5 g L^{-1}	Control
E. moraviensis	-0.07	-0.05	-0.03	-0.01	-0.34*	-0.48	-0.33	-0.08
E. casseliflavus	-0.06	-0.05	-0.04	-0.02	-0.46	-0.59	-0.31	-0.18
E. hirae	-0.03	-0.04	-0.03	-0.02	-0.71	-0.47	-0.39	-0.03
E. faecalis	-0.07	-0.05	-0.02	-0.03	-0.53	-0.51	-0.38	-0.16
E. faecium	-0.05	-0.03	-0.05	-0.02	-0.4	-0.57	-0.36	-0.05

** calculated from day 3 when decay started.*

DISCUSSION

This study compared the extent of growth of different *Enterococcus* species: *E. moraviensis*, *E. casseliflavus*, *E. hirae*, *E. faecalis* and *E. faecium* on the plant extract of *Ammophila arenaria*, a common type of dune vegetation in the Castricum infiltration area (The Netherlands). Using two different approaches, we found that while the major part of the indigenous microbial population and protozoa were removed from the extract by filtration, the nutrients from the plant extract promoted a rapid and significant growth of all five *Enterococcus* species at 15 °C. Increased filtered extract concentrations resulted in increased maximal concentrations. Except for *E. moraviensis* (50 g L^{-1}), no growth was observed in unfiltered extract. The experiment using filtered extract showed the persistence of enterococci for several months. Growth maxima as well as the time to die-off and reach of nonculturability have been shown to be species–specific. Growth of *E. moraviensis* and also *E. casseliflavus* at lower concentrations of the extract (5 g L^{-1} and 0.5 g L^{-1}) have shown greater ability to grow on plant material than *E. hirae*, *E. faecalis* and *E. faecium* when the indigenous microbial population is limited. Compared to *E. moraviensis* and *E. casseliflavus*, reduced ability to grow at different doses of nutrients (50 g L^{-1} and 5 g L^{-1} and 0.5 g L^{-1}) was found in *E. faecium*. These results are in accordance with earlier studies in which enterococci have been shown to persist for weeks in environmental habitats that are likely to have high nutrient contents including soil, sediment, and sand (Byappanahalli & Fujioka, 2004; Byappanahalli et al., 2006;

Davies et al., 1995; Hartz et al., 2008; Lee et al., 2006; Mallmann & Litsky, 1951; Staley et al., 2014).

The competition with other bacteria which are better adapted to grow on plant material and predation by protozoa, appeared to be limiting factors for growth of all of the *Enterococcus* species used in this study. This is in line with earlier studies where competition for nutrients with indigenous microorganisms in environments such as soil, sediment, sand, and vegetation has limited the success of enterococci in secondary habitats (Staley et al., 2014). The results of this study indicate that the ability of enterococci to grow on plant material may be species-specific. At the nutrient concentration level of 50 g L^{-1}, *E. moraviensis* showed an initial increase in CFU mL^{-1} in the presence of the indigenous microbial community, until the moment that the nutrient became limited (after 3 days). This was not observed in other species used in this study. These findings may limit the suitability of *E. moraviensis* as an indicator of water quality, especially in environments rich in decaying plant material.

Since *E. moraviensis* has been the most frequently detected *Enterococcus* species in abstracted water in the Castricum dune filtration area (Taučer-Kapteijn et al., 2016), finding the source of *E. moraviensis* in this area is important. Mowing grass in the vicinity of the abstraction wells is part of the routine maintenance of the filtration area. During rainy periods, the soil may get saturated with nutrient rich water, which would promote growth or persistence of *E. moraviensis*. This species may benefit from nutrient-rich soil compartments where natural flora and predation are limited. Further investigations are needed to determine persistence and growth of *E. moraviensis* in the field. The results of this study are in line with studies on aquatic vegetation, which has been described as a habitat able to support the growth of enterococci (Byappanahalli et al., 2003). Persistence of enterococci on aquatic vegetation was observed in studies of *Cladophora*, in which enterococci survived for over 6 months in sun-dried *Cladophora* mats stored at 4 °C and which grew readily upon rehydration (Whitman et al., 2003). Furthermore, enterococci were shown to grow on *Cladophora* leachate exhibiting a similar growth profile to that observed on microbiological media (Byappanahalli et al., 2003). The possibility of growth on submerged aquatic vegetation (SAV) has also been suggested (Badgley et al., 2010b). Badgley et al. (Badgley et al., 2010a) found that enterococci survived longer and at much higher densities in mesocosms containing SAV than in those without SAV. Furthermore, the recovery of a dominant *E. casseliflavus* strain indicated that this species likely was adapted to, or naturalized on, this vegetation (Byappanahalli et al., 2012).

Aside from aquatic vegetation, enterococci have also been associated with zoo- or phyto-plankton communities. Mote et al. (2012) found that *E. faecalis* and *E. casseliflavus* were able to survive (and grow) in plankton suspension significantly longer than in artificial sea water. At both 10 ˚C and 30 ˚C they found significant differences between the maximum growth achieved by *E. faecalis* and *E. casseliflavus* in the 5% plankton treatment. *E. casseliflavus* grew to significantly higher densities than *E. faecalis* at both temperatures. These authors have indicated that plankton might serve as a reservoir for growth and persistence of enterococci.

The ability to grow on plant extract at 15 ºC could make enterococci less suitable as FIB. The comparative competition experiments in this study indicated however, that in the natural environment enterococci may not be able to compete for nutrients with the natural microbial population. Using filtered extract, the numbers of the natural community exceeded the numbers of enterococci within 3 days as a result of the competition between enterococci and the natural community. Growth of natural community (as measured by heterotrophic plate counts on PCA) occurred during the first 5 days, the point at which nutrients probably became limiting. In addition, a predation test showed that in a relatively short time (three days), the numbers of protozoa (especially the heterotrophic flagellates), present in unfiltered extract strongly increased in the bacteria-rich suspension, indicating that predation was occurring and that it probably was an important limiting factor for the concentration of enterococci as well as the natural community in our experiments. These observations are in line with the extended survival of enterococci as well as the natural community in filtered plant extract, in the absence of protozoa. It is likely that the protozoan grazing process results in a substantial portion of the organic carbon and other nutrients being recycled rendering them available for further bacterial growth (microbial loop). Enterococci were probably not able to compete with the more numerous natural community for these nutrients, which may explain the rapid decrease of enterococci in unfiltered extract. The numbers of protozoa in the soil, as well as the removal rates of enterococci by these organisms, are understudied. Pernthaler (2005) indicated that predation by ciliated and flagellated protists result in the consumption of microbial biomass at approximately the same rate as it is produced. However, it is necessary to consider the existence of special environmental conditions, which may promote the persistence or growth of enterococci in the environment; especially for species with higher growth capacity like *E. moraviensis* and *E. casseliflavus*.

CONCLUSIONS

Plant extracts of *Ammophila arenaria* promote dose-response related growth and long persistence of *E. moraviensis*, *E. casseliflavus*, *E. hirae*, *E. faecalis* and *E. faecium* at 15 ºC, when the natural microbial population is limited. *E. moraviensis* and *E. casseliflavus* were also able to grow at a concentration of filtered plant extract of 0.5 g L^{-1}, which was not observed for *E. faecium*, *E. faecalis* and *E. hirae*. *E. moraviensis* was the only species that was able to multiply in the presence of the indigenous micro-organisms. This indicates that the degree of growth on plant material may be species-specific, which could have implications for the suitability of certain species as a faecal indicator of water quality, especially in environments (suspensions) rich in plant material. The overall conclusion is that the growth of enterococci in the environments we tested depended on the amounts of available nutrients, the numbers of competing bacteria and predation (protozoa).

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Literature

Badgley, B.D., Thomas, F.I.M., & Harwood, V.J. 2010a. The effects of submerged aquatic vegetation on the persistence of environmental populations of Enterococcus spp. Environmental Microbiology, 12, 1271–1281.

Badgley, B.D., Nayak, B.S., & Harwood, V.J. 2010b. The importance of sediment and submerged aquatic vegetation as potential habitats for persistent strains of enterococci in a subtropical watershed. Water Research, 44, 5857–5866.

Byappanahalli M.N., Shively D.A., Nevers M.B., Sadowsky M.J. & Whitman R.L. 2003 Growth and survival of *Escherichia coli* and enterococci populations in the macro-alga *Cladophora* (Chlorophyta). *FEMS Microbiology Ecology*, 46 (2), 203-211.

Byappanahalli, M. & Fujioka, R. 2004. Indigenous soil bacteria and low moisture may limit but allow faecal bacteria to multiply and become a minor population in tropical soils Water Science and Technology, 50, 27–32.

Byappanahalli, M.N., Whitman, R.L., Shively, D.A., Ting, W.T., Tseng, C.C., & Nevers, M.B. 2006. Seasonal persistence and population characteristics of Escherichia coli and enterococci in deep backshore sand of two freshwater beaches Journal of Water Health, 4, 313–320.

Byappanahalli, M.N., Nevers, M.B., Korajkic, A., Staley, Z.R., & Harwood, V.J. 2012. Enterococci in the environment. Microbiology and Molecular Biology Reviews, 76, 685–706.

Carvalho, M.G.S., Lynn Shewmaker, P., Steigerwalt, A.G., Morey, R.E., Sampson, A.J., Joyce, K., Barrett, T.J., Texeira, L.M., & Facklam, R.R. 2006. *Enterococcus caccae* sp. nov., isolated from human stools. Int. Journal of Systematic and evolutionary Microbiology, 56, 1505-1508.

Chenoweth, C. & Schaberg, D. 1990. The epidemiology of enterococci. European Journal of Clinical Microbiology and Infectious Diseases, 9, 80–89.

Cotta, M.A., Whitehead, T.R., Falsen, E., Moore, E. & Lawson, P.A. 2013. Two novel species Enterococcus lemanii sp. nov. and Enterococcus eurekensis sp. nov., isolated from a swine-manure storage pit. Antonie van Leeuwenhoek,103(1):89-98.

Davies, C.M., Long, J.A.H., Donald, M., & Ashbolt, N.J. 1995. Survival of fecal microorganisms in marine and fresh water sediments Applied and Environmental Microbiology, 61, 1888–1896.

Frolková, P., Švec, P., Sedláček, I., Mašlaňová, I., Černohlávková, J., Ghosh, A., Zurek, L., Radiměřský, T., & Literák, I. 2013. Enterococcus alcedinis sp. nov., isolated from common kingfisher (Alcedo atthis). International Journal of Systematic and Evolutionary Microbiology, 63, 3069–3074.

Gelsomino, R., Vancanneyt, M., Cogan, T.M. & Swings, J. 2003. Effect of raw-milk cheese consumption on the enterococcal flora of human feces. Applied and Environmental Microbiology, 69, 312–319.

Hartz, A., Cuvelier, M., Nowosielski K., Bonilla, T.D., Green, M., Esiobu, N., McCorquodale, D.S., & Rogerson, A. 2008. Survival potential of Escherichia coli and enterococci in subtropical beach sand: Implications for water quality managers Journal of Environmental Quality, 37, 898–905.

Harwood, V.J., Delahoya, N.C., Ulrich, R.M., Kramer, M.F., Whitlock, J.E., Garey, J.R., & Lim, D.V. 2004. Molecular confirmation of *Enterococcus faecalis* and *E. faecium* from clinical, faecal and environmental sources. Letter in Applied Microbiology, 38 (6), 476–482.

He, J.W., & Jiang, S. 2005. Quantification of enterococci and human adenoviruses in environmental samples by real- time PCR. Appl. Environ. Microbiol., 71(5), 2250 - 2255.

ISO (7899-2:2000). Water quality. Detection and enumeration of intestinal enterococci. Part 2: Membrane filtration method.

Kay, D., Fleisher, J.M., Salmon, R.L., Jones, F., Wyer, M.D., Godfree, A.F., Zelenauch-Jacquotte, Z., & Shore, R. 1994. Predicting Likelihood of Gastroenteritis from Sea Bathing: Results from Randomised Exposure. Lancet 344(8927): 905-909.

Lee, C.M., Lin, T.Y., Lin, C.C., Kohbodi, G.A., Bhattl, A., Lee, R., & Jay, J. A. 2006. Persistence of fecal Indicator bacteria in Santa Monica Bay beach sediments Water Research, 40, 2593–2602.

Mallmann, W.L. & Litsky, W. 1951. Survival of selected enteric organisms in various types of soil American Journal of Public Health, 41, 38–44.

Manero, A., Vilanova, X., Cerda-Cuellar, M., & Blanch, A.R. 2002. Characterization of sewage waters by biochemical fingerprinting of Enterococci. Water Res., 36 (11), 2831-2835.

Mote, B.L., Turner, J.W., & Lipp, E.K. 2012. Persistence and growth of the fecal indicator bacteria enterococci in detritus and natural estuarine plankton communities. Appl. Environ. Microbiol. 78, 2569-2577.

Pernthaler, J., 2005. Predation on prokaryotes in the water column and its ecological implications. Nature reviews. Vol 3, 537.

Salminen, S., Wright, A.V. & Ouwehand, A. 2004. Lactic acid bacteria. Microbiological and functional aspects (Third ed.) New York: Mercel Dekker.

Schijven, J.F., Hoogenboezem, W., Nobel, P.J., Medema, G.J. & Stakelbeek, A. 1998. Reduction of FRNA-bacteriophages and faecal indicator bacteria by dune infiltration and estimation of sticking efficiencies. Water Science and Technology, 38 (12), 127-131.

Staley, C., Dunny, G.M., & Sadowsky, M.J. 2014. Environmental and Animal-Associated Enterococci. Advances in Applied Microbiology, 87, 147-186.

Stevens, M. Ashbolt, N. & Cunliffe, D. 2003. Recommendation to change the use of coliforms as microbial indicator of drinking water quality. Australian Government National Health and Medical Research Council.

Sukontasing, S., Tanasupawat, S., Moonmangmee, S., Lee, J.S. & Suzuki, K. 2007. Enterococcus camelliae sp. nov., isolated from fermented tea leaves in Thailand. International Journal of Systematic and Evolutionary Microbiology, 57, 2151–2154.

Švec P., Devriese L. A., Sedláček I., Baele M., Vancanneyt M., Haesebrouck F., Swings J. & Doškar J. 2001 *Enterococcus haemoperoxidus* sp. nov. and *Enterococcus moraviensis* sp. nov., new species isolated from water. Int. Journal of Systematic and Evolutionary Microbiology, 51, 1567-1574.

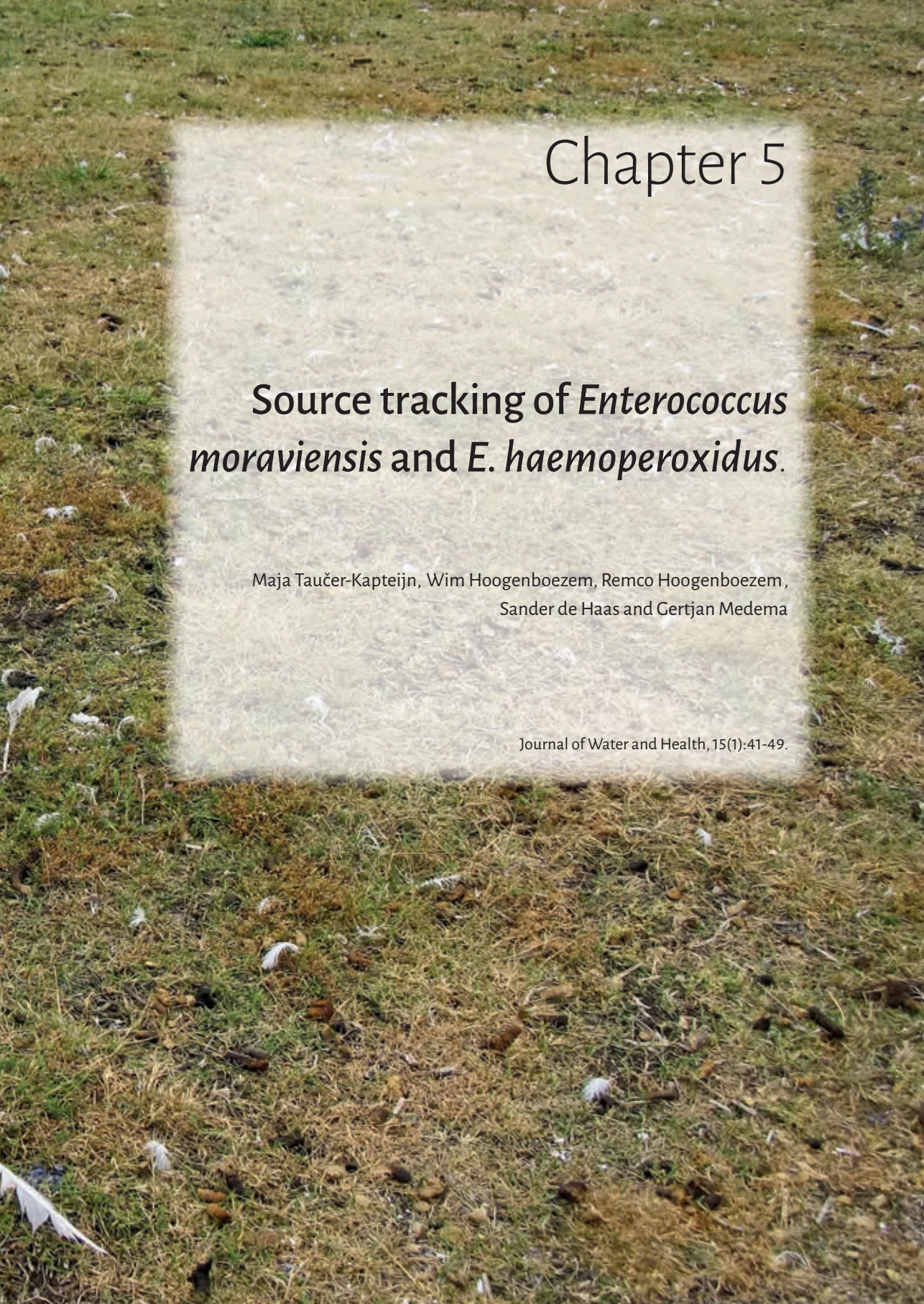
Švec, P., Vancanneyt, M., Devriese, L.A., Naser, S.M., Snauwaert, C., Lefebvre, K., Hoste, B. & Swings, J. 2005. Enterococcus aquamarinus sp. nov., isolated from sea water. International Journal of Systematic and Evolutionary Microbiology, 55, 2183–2187.

Taučer-Kapteijn, M., Medema, G.J, & Hoogenboezem, W. 2013. Comparison between Rapid ID 32 Strep System, Matrix Assisted Desorption Ionisation- Time of Flight Mass Spectrometry and 16S gene sequence analysis for the species identification of enterococcus spp. isolated from water. Water Science & Technology: Water supply 13(5): 1383-1389.

Taučer-Kapteijn, M., Hoogenboezem, W., & Medema, G.J. 2016. Environmental growth of the faecal indicator *Enterococcus moraviensis.* Water Science and Technology: Water Supply 16(4), 971-979.

Ulrich, A. & Müller, T. 1998. Heterogeneity of plant associated streptococci as characterized by phenotypic features and restriction analysis of PCR amplified 16S rDNA. J. Appl. Microbiol. 84, 293-303.

van der Kooij D. 2001. AOC Manual. BTO Report. 212 (S). Kiwa Water Research.



Chapter 5

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

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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. 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 & Bartley Agar. From these samples, enterococci isolates (1386 in total) were subsequently identified using MALDI-TOF MS. *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.

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 (FIB), 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 depends 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 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 and co-workers (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 three to 66 years were analysed for enterococci. The numbers of faecal samples per animal host are indicated in Table 5.1.

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 inner part taken for the analysis in order to exclude contamination from other sources (sand, grass, etc.). An amount of 0.5 g 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. The amount of 1ml of each dilution 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 (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 mass spectrometry (Biotyper, Bruker) in accordance with the manufacturer's instructions.

Abstracted water samples.

A total of 195 abstracted water samples (14 of 1L and 181 of 100L) were filtered at locations in the Castricum infiltration area between July 2012 and August 2014. A total of 5117 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 goose droppings per square metre and the average enterococci density (cfu/g faeces) measured in goose droppings.

RESULTS

Animal and human faecal samples (101 in total) were analysed for enterococci. A total of 1386 isolates were identified as *Enterococcus* species (Table 5.1).

Table 5.1. Number of isolates belonging to *Enterococcus* spp. isolated in faecal samples from different animal hosts.

Host species	No. of faecal samples	No. of *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	1386

The relative distribution of *Enterococcus* species among selected host species and in abstracted water samples is shown in Table 5.2. 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 species in other animal hosts. In order to verify these similarities, statistical methods were applied.

Table 5.2. 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	2.3		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

As shown in Figure 5.1, 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).

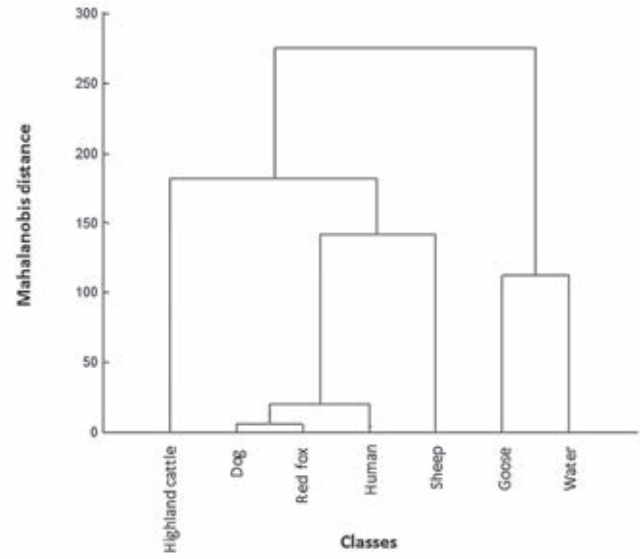

Figure 5.1. Relationships between different classes (animal hosts and abstracted water) based upon their bacterial composition, using Mahalanobis distance (MATLAB).

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 5.2). Higher numbers were observed in omnivores (dogs $1.6x10^6$/g, humans $7.7x10^5$/g and red foxes $4.4x10^5$/g) and geese ($3.1x10^5$/g), whereas lower numbers were observed in herbivorous mammals: sheep ($1.3x10^3$/g), rabbits (2.1^x10^2/g) and highland cattle ($2.9x10^1$/g).

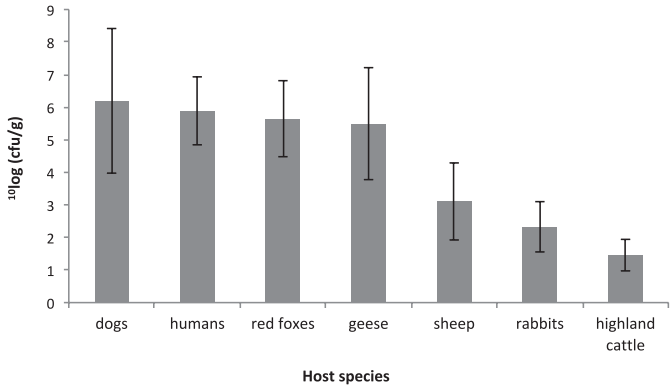

Figure 5.2. Average numbers of *Enterococcus* spp. per gram of faeces from selected hosts.

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 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 a 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 $3.48x10^5$ cfu/g. The faecal loads for enterococci at two locations (A and B) were almost the same: $1.9^{x}10^7$ cfu/m^2 and $1.8x10^7$ cfu/m^2 respectively (as shown in Table 5.3). 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 5.3. Faecal load number of goose droppings, goose 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	1,93E+07
B	1.19 (std=0.07)	51.8	1,80E+07

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 then 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 goose 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.

In the Guidelines for drinking water quality (2011) the intestinal enterococci group (*Enterococcus faecalis*, *E. faecium*, *E. durans* and *E. hirae*) is described as indicator of faecal pollution, because these species are typically excreted in the faeces of humans and other warm-blooded animals (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 measures: 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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REFERENCES

Aarestrup, F. M., Butaye, P. & Witte, W. 2002 Nonhuman Reservoirs of Enterococci. In: *The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance* (M. S. Gilmore *et al*., ed.). ASM Press, Washington, DC, USA, pp. 55-100.

Badgley, B. D., Thomas, F. I. M., Harwood, V. J. 2010 The effects of submerged aquatic vegetation on the persistence of environmental populations of *Enterococcus* spp. *Environ. Microbiol.* 42, 1271–1281.

Byappanahalli, M. N., Nevers, M. B., Korajkic, A., Staley, Z. R. & Harwood, V. J. 2012 Enterococci in the Environment. *Microbiol. Molec. Biol. Rev.* 76, 685-706.

Cai, Y. 1999 Identification and characterization of *Enterococcus* species isolated from forage crops and their influence on silage fermentation. *J. Dairy Sci.* 82, 2466 –2471.

Chvala, S., Fragner, K., Hackl, R., Hess, M. & Weissenböck, H. 2006 *Cryptosporidium* Infection in Domestic Geese (*Anser anser f. domestica*) Detected by In-situ Hybridization. *J. Compar. Pathol.* 134, 211–218.

Colles, F. M., Dingle, K. E., Cody, A. J. & Maiden, M. C. J. 2008 Comparison of *Campylobacter* Populations in Wild Geese with Those in Starlings and Free-Range Poultry on the Same Farm. *Appl. Environ. Microbiol.* 74, 3583-3590.

Devriese, L. L. A., van de Kerckhove, A., Kilpper-Baelz, R. & Schleifer, K. 1987 Characterization and identification of Enterococcus species isolated from the intestines of animals. *Int. J. Syst. Bacteriol.* 37, 257–259.

Fallacara, D. M., Monahan, C. M., Morishita, T. Y. & Wack, R. F. 2001 Fecal shedding and antimicrobial susceptibility of selected bacterial pathogens and a survey of intestinal parasites in free-living waterfowl. *Avian Dis.* 45, 128–135.

Fujioka, R.S., Sian-Denton, C., Borja, M., Castro, J. & Morphew, K. 1999 Soil: the environmental source of *Escherichia coli* and enterococci in Guam's streams. *J. Appl. Microbiol.* Symposium Supplement 85, 83S–89S.

Foulquie Moreno, M. R., Sarantinopoulos, P., Takalidou, E. & De Vuyst, L. 2006 The role and application of enterococci in food and health. *Int. J. Food Microbiol.* 106, 1–24.

Graczyk, T. K., Fayer, R., Trout, J. M., Lewis, E. J., Farley, C. A., Sulaiman, I. & Lal, A. A. 1998: *Giardia* sp. cysts and infectious *Cryptosporidium* parvum oocysts in the feces of migratory Canada geese (*Branta canadensis*). *Appl. Environ. Microbiol.* 64, 2736–2738.

Grant, S. B., Sanders, B. F., Boehm, A. B., Redman, J. A., Kim, J. H., Mrse, R. D., Chu, A. K., Gouldin, M., McGee, C. D., Gardiner, N. A., Jones, B. H., Svejkovsky, J., Leipzig, G. V. & Brown, A. 2001 Generation of enterococci bacteria in a coastal saltwater marsh and its impact on surf zone water quality. *Environ. Sci. Technol.* 35, 2407–2416.

ISO 7899-2:2000. Water quality. Detection and enumeration of intestinal enterococci. Part 2: Membrane filtration method.

Kassa, H., Harrington, B. J. & Bisesi, M. S. 2004 Cryptosporidiosis: a brief literature review and update regarding *Cryptosporidium* in feces of Canada geese (*Branta canadensis*). *J. Environ. Health* 66, 34–40.

Klein, G. 2003 Taxonomy, ecology and antibiotic resistance of enterococci from food and the gastro-intestinal tract. *Int. J. Food Microbiol.* 88, 123–131.

Kühn, I., Iversen, A., Burman, L. G., Olsson-Liljequist, B., Franklin, A., Finn, M., Aarestrup, F., Seyfarth, A. M., Blanch, A. R., Vilanova, X., Taylor, H., Caplin, J., Moreno, M. A., Dominguez, M., Herrero, I. A. & Möllby, R. 2003 Comparison of enterococcal populations in animals, humans, and the environment—a European study. *Int. J. Food Microbiol.* 88, 133–145.

Le Fevre, N. M. & Lewis, G. D. 2003 The role of resuspension in enterococci distribution in water at an urban beach. *Water Sci. Technol.* 47, 205–210.

Lillehaug A., Monceyron Jonassen, C., Bergsjø, B., Hofshagen, M., Tharaldsen, J., Nesse, L. L. & Handeland, K. 2005 Screening of Feral Pigeon (*Colomba livia*), Mallard (*Anas platyrhynchos*) and Graylag Goose (*Anser anser*) Populations for *Campylobacter* spp., Salmonella spp., Avian Influenza Virus and Avian Paramyxovirus. *Acta Vet. Scand.* 46, 193-202.

Mahalanobis, P. C. 1936 On the generalised distance in statistics. Proceedings of the National Institute of Sciences of India 2, 49–55.

Manero, A., Vilanova, X., Cerda-Cuellar, M. & Blanch, A. R. 2002 Characterization of sewage waters by biochemical fingerprinting of enterococci. *Water Res.* 36, 2831–2835.

Martin, J. D. & Mundt, J. O. 1972 Enterococci in insects. *Appl. Microbiol.* 24, 575–580.

Moriarty, E. M., Karki, N., Mackenzie, M., Sinton, L. W., Wood, D. R. & Gilpin, B. J. 2011 Faecal indicators and pathogens in selected New Zealand waterfowl. *N. Z. J. Mar. Fresh. Res.* 45, 679–688.

Müller, T., Ulrich, A., Ott, E. M. & Müller, M. 2001 Identification of plant-associated enterococci. *J. Appl. Microbiol.* 91, 268–278.

Mundt, J. O., Coggins, J. H., Johnson, L. F. 1962 Growth of *Streptococcus faecalis* var. *liquefaciens* on plants. *Appl. Microbiol.* 10, 552–555.

Mundt, J. O. 1963 Occurrence of enterococci in animals in a wild environment. *Appl. Environ. Microbiol.* 11, 136–140.

Murray, B. E. 1990 The life and times of the *Enterococcus*. *Clin. Microbiol. Rev.* 3, 46–65.

Ott, E. M., Müller, T., Müller, M., Franz, C. M., Ulrich, A., Gabel, M. & Seyfarth, W. 2001 Population dynamics and antagonistic potential of enterococci colonizing the phyllosphere of grasses. *J. Appl. Microbiol.* 91, 54–66.

Pacha, R. E., Clark, G. W., Williams, E. A. & Carter, A. M. 1988 Migratory birds of central Washington as reservoirs of *Campylobacter jejuni*. *Can. J. Microbiol.* 34, 80–82.

Plutzer, J. & Tomor, B. 2009 Role of aquatic birds in the environmental dissemination of human pathogenic *Giardia duodenalis* cysts and *Cryptosporidium* oocysts in Hungary) *Parasitol. Int.* 58, 227–231

Ruoff, K. L., de la Maza, L., Murtagh, M. J., Spargo, J. D. & Ferraro, M. J. 1990 Species identities of enterococci isolated from clinical specimens. *J. Clin. Microbiol.* 28, 435–437.

Simpson, E. H. 1949 Measurement of diversity. *Nature* 163, 688.

Švec, P., Devriese L. A., Sedláček, I., Baele, M., Vancanneyt, M., Haesebrouck, F., Swings, J. & Doskar, J. 2001 *Enterococcus haemoperoxiudus* sp. nov. and *Enterococcus moraviensis* sp. nov., new species isolated from water. *Int. J. Syst. Evol. Microbiol.* 51, 1567-1574.

Švec, P., Devriese, L. A., Sedláček, I., Baele, M., Vancanneyt, M., Haesebrouck, F., Swings, J. & Doskar, J. 2002 Characterization of yellow-pigmented and motile enterococci isolated from intestines of the garden snail Helix aspersa. *J. Appl. Microbiol.* 92, 951–957.

Taučer-Kapteijn, M., Medema, G., Hoogenboezem, W. 2013 Comparison between Rapid ID 32 Strep System, Matrix Assisted Laser Desorption Ionisation–Time of Flight Mass Spectrometry and 16S rRNA gene sequence analysis for the species identification of *Enterococcus* spp. isolated from water. *Water Sci. Techol.:Water Supply* 13, 1383-1389.

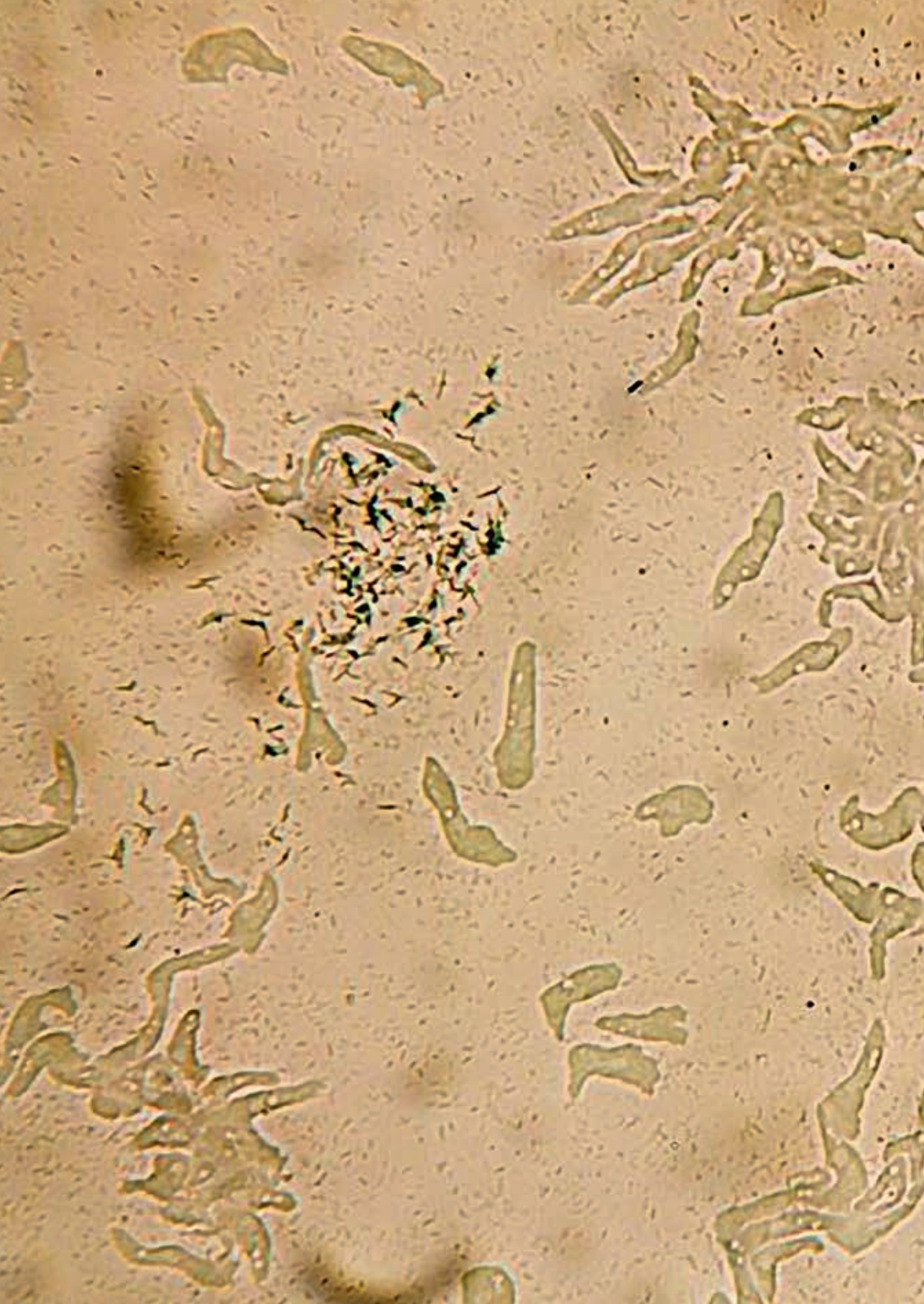
Taučer-Kapteijn, M., Hoogenboezem, W., Medema, G. 2016 Environmental growth of the faecal indicator *Enterococcus moraviensis*. *Water Sci. Techol.:Water Supply* 16(4), 971-979.

Wahlstrom, H., Tysen, E., Olsson Engvall, E., Brandstrom, B., Eriksson, E., Morner, T. & Vagsholm, I. 2003 Survey of *Campylobacter* species, VTEC O157 and *Salmonella* species in Swedish wildlife. *Vet. Rec.* 153, 74–80.

Whitman, R. L., Shively, D. A, Pawlik, H., Nevers, M. B. & Byappanahalli, M. N. 2003 Occurrence of *Escherichia coli* and enterococci in *Cladophora* (Chlorophyta) in nearshore water and beach sand of Lake Michigan. *Appl. Environ. Microbiol.* 69, 4714–4719.

World Health Organisation (WHO). 2011 Guidelines for Drinking-water Quality. Fourth edition.

Zhou, L., Kassa, H., Tischler, M. L. & Xiao, L. 2004 Host adapted *Cryptosporidium* spp. in Canada geese (*Branta canadensis*). *Appl. Environ. Microbiol.* 70, 4211–4215.



Chapter 6

Cellular slime moulds as regulators of bacterial numbers in faecal droppings and soil- are they important to the drinking water company?

Wim Hoogenboezem, Maja Taučer-Kapteijn and Gertjan Medema

This chapter has been submitted for publication.

ABSTRACT

Microbial contamination of drinking water abstracted from infiltration areas and water bodies in general may occur via faecal contaminations, containing large numbers of faecal indicators and possibly also pathogens, on the surface near shallow abstraction wells or receiving water bodies. A reduction of bacterial numbers in faecal droppings can be associated with several physical factors (temperature, drought, UV (sunlight), etc.) and biological factors, such as predation by protozoans. The importance of protozoan grazing in aquatic ecosystems is well documented. In terrestrial systems, amoebae are considered important predators of bacteria. Many of these amoebae belong to the slime moulds. This study confirmed the presence of cellular slime moulds (dictyostelids) in both soil and dung obtained from an infiltration area. The ability of *Dictyostelium mucoroides* to feed on several species of indicator bacteria (*E. coli*, *Clostridium bifermentance* and five species of *Enterococcus*) was experimentally demonstrated. In order to evaluate the possible impact of these grazers on the microbial abundance in soil and dung, the maximal growth rate of *D. mucoroides* was determined using *Pseudomonas fluorescens* as food. It was found that *D. mucoroides* is able to grow within three days up to 1.6×10^5 amoebae in a single test area of c. 10 cm^2. It is concluded that cellular slime moulds can indeed have a considerable influence on the microbial community in both soil and dung; therefore, these processes should be included in the risk assessments of shallow abstraction wells for drinking water production.

INTRODUCTION

Infiltration areas used for the production of drinking water in the Netherlands are usually protected nature reserves in the dunes. To assure a good quality of vegetation it is important to have a more or less natural grazing pressure from several species of mammals. Sheep, goats or cattle are therefore brought into the areas to feed on the vegetation. However, the droppings of these animals and feral animals (deer, rabbits, geese, etc.) can pose a contamination risk to the recharged water from shallow recharge wells (1). Risk assessment of faecal droppings in the direct vicinity of drinking water abstraction wells requires an understanding of the survival times of pathogens and indicator bacteria in the faecal dropping environment. The influence of UV radiation from the sun is known to reduce numbers of bacteria (2). A sufficient water content in soil or faeces is crucial for the survival of bacteria (2). However, a microbial ecological approach, such as predation by free-living protozoa, has not yet been applied to address this issue.

Protozoan grazing on the aquatic prokaryote community has been addressed in many studies and has been shown to have an important impact on the prokaryote community. In the aquatic environment, heterotrophic flagellates, amoebae and ciliated protozoa play an important role in these interactions. These studies are thoroughly reviewed by Pernthaler (3) and have shown a distinct influence of protozoa on the bacterial community. In water, heterotrophic nanoflagellates appear to be the most important grazers on the prokaryote community. In response, various anti-predation strategies have been developed in various bacterial species, for instance size reduction, formation of exopolymer production (EPS), toxin release, formation of long filamentous cells and adaptations to the cell wall structure (3). Some of these adaptations may also be the result of other causes; for instance, filament formation may also occur in starvation situations (4). Faeces dropped on the ground in the neighbourhood of abstraction wells in infiltration areas are usually not immersed in water and water protozoans are not likely to exploit the rich bacterial community in the faeces of mammals and birds. The question is whether and, if so, how bacterial communities in such an environment are exploited. Information on the microbial ecology in faecal droppings is needed in order to evaluate the risk of faecal droppings in the vicinity of drinking water installations. The question is generic and applies to all faecal droppings on land; here, the focus is on shallow aquifers and land near the recharge wells of superficially infiltrated surface water.

Mycetozoa.

According to Fenchel (5), terrestrial protozoa have to cope with strong fluctuations in both moisture content and food resources (bacteria). Soil protozoa survive periods of drought or a shortage of food as cysts. Amoebae are by far the most abundant soil protozoans; they are best adapted to live in thin water films around soil particles (5). Many terrestrial amoebae belong to the Mycetozoa or slime moulds and are important grazers on microbial communities. They feed on bacteria, yeasts, spores, hyphae of filamentous fungi, algae and other protozoa, and are found in a variety of terrestrial environments, such as dead plant material, litter, soil, bark, dung and rotten wood from boreal to tropical regions (6, 7). The lifecycle of Mycetozoa consists of two important stages: a trophic stage, characterized by feeding amoeboid cells (in some groups, flagellated amoeboid cells) and, when the food resources are depleting, a stage during which these cells refrain from feeding and aggregate to form a large 'body' of cells (pseudoplasmodium in Protostelia, Dictyostelids and Guttulinia). Or the cells fuse into a large multi-nucleate cell, a plasmodium (syncytium) in Myxogastria (formerly Myxomycetes). In Myxogastria, the plasmodium is still consuming food particles (8, 7, 9). These pseudo-plasmodia migrate towards a place in the light where fruiting bodies (a kind of sporangia in Myxogastria and sorocarps in cellular slime moulds) are formed. In many species, these fruiting bodies are stalked to increase the chance of transportation by passing invertebrate animals or dispersion by the wind (Fig. 6.1).

Species of five orders of Myxogastria are associated with dung and some of those are obligate coprophilous (10). Cellular slime moulds (CSMs) are very abundant on dung (7). Their abundance makes CSMs quite interesting for a study on the effect of Mycetozoa on bacterial survival in dung. Therefore, the primary focus in this study was on CSMs. Most coprophilous Mycetozoa are found on herbivore dung, although some observations on carnivore dung have been reported (7). The names of two genera in the Guttulinia, *Copromyxa* and *Copromyxella*, refer to faeces as the preferred substrate of these species (11, 6). Thus, the Mycetozoa have an important potential as bacteria-consuming protozoans and are therefore an important subject for further investigation.

As far as we know, the present study was the first to be carried out on the microbial ecology of Mycetozoa in relation to the survival of bacteria that are relevant to the hygienic quality of drinking water. Mycetozoa may significantly reduce the survival times of bacteria in faeces or soil. The main goal of this study was to investigate whether the common *Dictyostelium mucoroides,* isolated from the dune infiltration

area, is able to consume the common faecal indicator bacteria (FIB), namely *Escherichia coli*, *Clostridium perfringens* and several species of enterococci. The second goal was to estimate the growth rate of the amoebae in the presence of an excess of food bacteria, in order to estimate the consumption rate of these protozoans. The third goal was to establish the presence of Mycetozoa spores in or on faecal samples aseptically collected in the infiltration area.

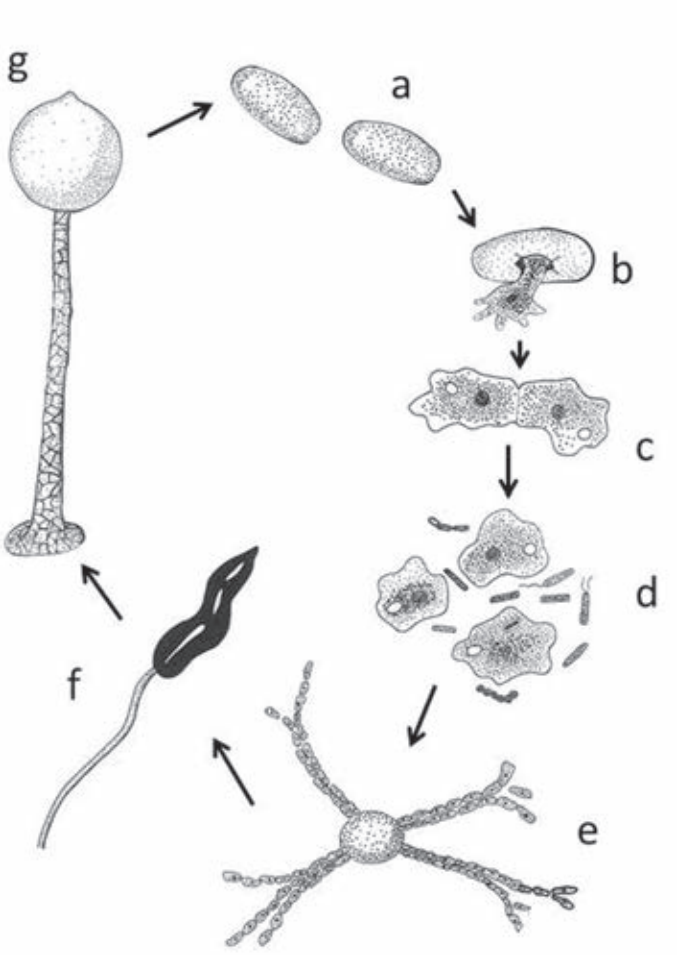


FIG 6.1 Scheme of the lifecycle of a Dictyostelid slime mould: (a) spores, usually elliptic in form; (b) amoeba emerging from a germinating spore; (c and d) vegetative growth consuming bacteria and multiplying by binary fission; (e) when food sources are depleted, the amoebae aggregate and form a grex or slug; (f) the grex finally develops into a stalked sorocarp (g), which contains a large number of spores that can easily be dispersed due to its elevated position above the substrate. Under laboratory conditions (22 °C), this cycle is usually completed in two to five days, depending on food and moisture conditions.

MATERIALS AND METHODS

Isolation of *Dictyostelium mucoroides* from soil.

Soil samples containing some decaying plant material were collected from the Dutch dune area. Some material (c. 0.25 – 0.5 g) was spread on a prepared water agar (15.0 g technical agar (Oxoid, UK) per litre of tap water) plate (plastic Petri-dish Ø 10 cm), covered with a thin layer of food bacteria (*Pseudomonas fluorescens*, strain P17). One or several colonies of this bacterium were spread over the surface using a Drigalski spatula (Sarstedt, Germany). The *P. fluorescens* was cultivated on a non-selective medium (Lab-Lemco Agar, Oxoid, England) to prevent growth inhibition of the target organism. After seven days of incubation at room temperature (c. 21 °C), each plate was inspected for the presence of typical stalked sorocarps. Typical sorocarps were used to inoculate a new plate with a lawn of *P. fluorescens* P17. These plates were inspected regularly using a compound microscope (magnification 100× and 250×) to determine the presence of amoebae, the grex and later the formation of new sorocarps (see Fig. 6.1).

Predation experiments.

Predation on FIB was studied by applying a comparable method. Here, the FIB were used as bait for the *Dictyostelium mucoroides*. These tests were incubated for five days at 22 °C. *Escherichia coli*, *Clostridium perfringens*, *Enterococcus faecalis*, *E. faecium*, *E. moraviensis* and *E. hirae* were used in individual plates as food source. The used FIB strains were identified using Matrix Assisted Laser Desorption Ionisation Time Of Flight Mass Spectrometry (MALDI-TOF MS) (12). Formation of sorocarps provided evidence for the growth of *D. mucoroides* on the particular food source.

Growth curves of *Dictyostelium mucoroides* amoebae.

A 2 mm thick layer of water agar (12.0 g technical agar (Oxoid, England) per litre of tap water) 31.5 mm square was made using a Perspex frame and then mounted on a large microscope slide (40 mm × 76 mm). Molten agar was poured into the frame and immediately covered with a large coverslip (46 mm × 46 mm, Menzel-Gläser, Germany). After congelation, the coverslip was removed and the thin layer of water agar was ready and suited for use under a compound microscope. A thoroughly mixed suspension of food bacteria (*P. fluorescens* P17) in tap water was spread over the surface of the agar square with a Drigalski spatula. To ensure an inoculation with a low number of spores, the layer of bacteria was inoculated with a diluted suspension of a thoroughly mixed sorocarp. To establish the presence of spores in the suspension, a

drop of the diluted mixture was examined microscopically (phase contrast microscopy, Leitz Ortholux magnification: 250×) before being incubated at room temperature. These preparations were examined microscopically twice a day and the numbers of amoebae were counted in 20 areas (arranged in four rows and five columns). For each count, a microscopic field of view was randomly selected and counted in each of these 20 areas. Counts were carried out using a Carl Zeiss Standard 18 microscope, 25× objective (magnification 250×). The examined surface for each microscopic field of view was 0.44 mm^2.

Isolation of Mycetozoa from faecal samples.

The presence of Mycetozoa in faeces was studied by incubating faecal samples of domestic sheep (*Ovis aries*) and goat (*Capra hircus)* and dung of feral rabbit *(Oryctolagus cuniculus*) that had been aseptically collected in the dune area. The faecal samples were placed on pieces of water-soaked filter paper or absorbent paper towel in 17 cm glass petri dishes (9), and these moist chambers were incubated at room temperature (c. 21 °C). The cultures were inspected every two days, using a stereo microscope (Olympus SZ 11). Presumptive sporangia or sorocarps were transferred to a water agar plate with a lawn of *P. fluorescens* P17, in order to make a pure culture and to confirm the nature of the presumed isolate by observing foraging amoebae and sorocarp formation via grex to new sorocarp.

RESULTS

The CSM that was isolated from a soil sample from the dune area was identified as *Dictyostelium mucoroides* by the presence of a stalk with a framework of empty cells, a globose sorocarp unbranched stalk with terminal sorocarp, containing elliptical spores. Tests with indicator bacteria as food bacteria and the tests for the growth curves were carried out using this strain. Predation on FIB was tested by applying spores of *D. mucoroides* on a water agar plate covered with a lawn of each of the seven FIB, namely *Escherichia coli*, *Clostridium bifermentans*, *Enterococcus hirae*, *E. moraviensis*, *E. faecalis*, *E. faecium* and *E. casseliflavus*. Duplicate tests showed the formation of sorocarps in all cases, indicating that the applied *Dictyostelium* spores had developed into amoebae and multiplied in the presence of a single food bacterium species and ultimately formed new sorocarps. This implies that the isolated *D. mucoroides* strain is able to feed on all indicator bacteria used in this study. However, the sorocarps formed in the tests

using the *C. bifermentans* strain were smaller compared to the other tested species, an indication that *C. bifermentans* is not the preferred food species of *D. mucoroides*.

To test whether *D. mucoroides* is able to grow on faeces, some faecal pellets of a domestic rabbit were inoculated with spores of *D. mucoroides* and incubated at 22 °C for five days in a moist chamber culture to ensure sufficient humidity. After five days, the surfaces of the pellets were covered with typical sorocarps of our test species (Fig. 6.2 left). The isolated *D. mucoroides* strain from the soil was able to grow on the bacterial flora in domestic rabbit and sheep dung.

Next, the presence of spores or amoebae of CSMs in faecal samples was tested for in faecal samples of domestic sheep (*Ovis aries*) and goat (*Capra hircus)* and dung of feral rabbit *(Oryctolagus cuniculus*) collected in the infiltration area. These samples were incubated in a moist chamber culture for five days at 22 °C. All samples developed sorocarps of CSMs belonging to the genera *Dictyostelium* and/or *Polysphondylium*. In another test in the three species, a sample of the faecal dropping was aseptically taken from the interior to exclude external contamination with spores from the soil. The internal fragments also developed sorocarps, indicating that spores (and/or amoebae) were present in the interior of the faecal droppings, where contamination from the soil can be excluded. These results showed the presence of CSMs in the infiltration area in soil and faecal material.

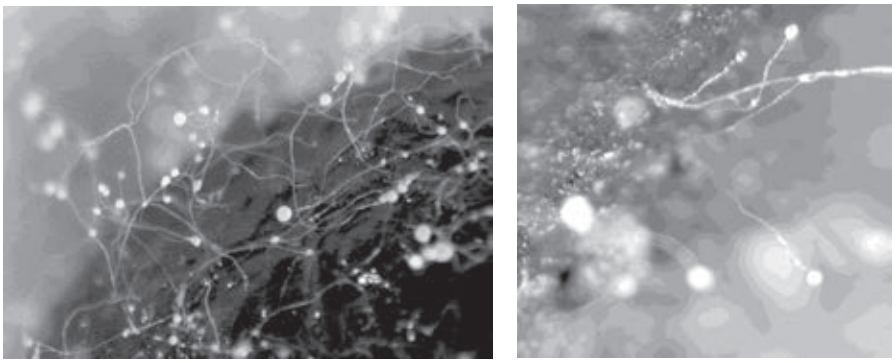


FIG 6.2 Faecal dropping of a rabbit (*Oryctolagus cuniculus*) artificially infected with spores of *Dictyolstelium mucoroides*. A large number of sorocarps developed in five days (left). *Dictyostelium* sp. sorocarps developed on a sheep (*Ovis aries*) dropping from the infiltration area, after five days of incubation (right).

In order to estimate the number of amoebae that can develop in a few days, two growth experiments were carried out (one duplicate test and one four-fold test). The development of amoebae was used to estimate the number of bacteria consumed in that period. The numbers of amoebae were counted four or five times between six and 60 hours after inoculation with a relatively small (<100) number of spores per experiment (Fig. 6.3).

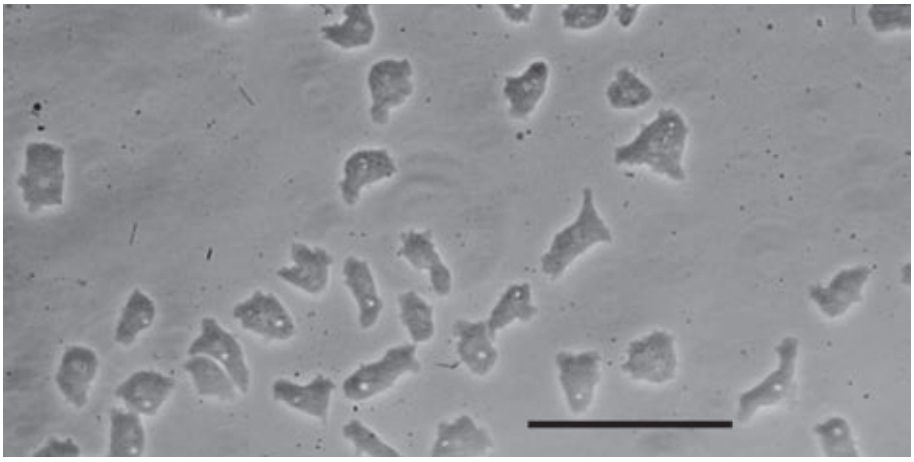


FIG 6.3 Foraging amoebae on an agar plate (Leitz Orthoplan, phase contrast, magnification 250×; size bar indicates 100 µm).

The spores and amoebae were rather heterogeneously distributed in the experimental agar preparation. Most of the first counts found only one or a few amoebae in 20 microscopic fields of view. Throughout the experiment, microscopic fields of view without any amoebae occurred even when other fields in the same count contained over 100 cells. However, counting 20 fields of view every interval yielded average counts that resulted in adequate duplicates counts. The observed number of amoebae was calculated as number per square millimetre and plotted in Fig. 6.4. In the experimental setup, the amoebae reached a maximum concentration in 48–72 hours, when the cells started aggregating. In that period, the amoebae divided c. 10 times, resulting in a mean generation time of 4.8 hours. The growth curve was distinctly exponential (curve A: $Y = 0.24*e^{0.15t}$; $R^2 = 0.99$ and curve B: $Y = 0.23*e^{0.15t}$; $R^2 = 0.98$, t in hours). The actual counted surface of the culture slide was very small (8.8 mm^2, microscope objective 25×) compared to the total experimental surface (31.5×31.5 = 992 mm^2). Based on the numbers counted in the microscopic fields of view, the total number of amoebae in the experimental slide was estimated to be as high as $1.6×10^5$.

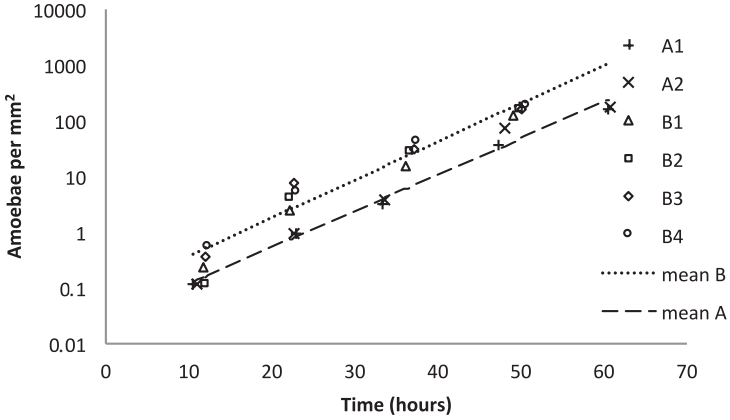


FIG 6.4 Growth curves of *Dictyostelium* amoebae cultured on water agar with an abundance of *P. fluorescence* P17 as food bacterium. The dashed line shows the average curve of regression duplicates tests A1 and A2; the dotted line shows the average curve of regression of four-fold measurement B1, B2, B3 and B4. The number of food bacteria was not standardized; this may have caused the differences between test A and test B.

DISCUSSION

In this study on CSMs in a natural area, it turned out to be relatively easy to demonstrate their presence in soil and dung samples from the study area. According to the literature, these organisms are found in many types of soil samples; the highest densities (up to 20,000 g^{-1} soil) were observed in forest soils (6, 7, 8, 11, 13, 14, 15, 16). But the densities are usually considerably lower ($10 - 10^3$ g^{-1} soil). Forest soil and leaf litter are rich in organic matter and bacteria, and consequently contain the highest numbers of these organisms (11). These studies demonstrated the ubiquitous occurrence of spores of CSMs in the soil environment and their ability to multiply rapidly once food bacteria becomes available. In our experiments with several individual indicator bacteria strains as food source, the development of fully developed and abundant sorocarps was observed, a clear indication that they feed on these bacteria. Smaller and less abundant sorocarps were observed in the experiments using *Clostridium bifermentans* as food bacterium, perhaps indicating that they are less adapted to that type of food bacterium. Horn (16) found various species of slime mould that were able to feed on a wide range of bacterial species.

The occurrence of CSMs in faeces is not exceptional, as the first CSM that Breveld discovered (17) was isolated from horse dung. Later, several studies on the dispersion

of dictyostelids showed their presence in invertebrate droppings (18), in the droppings of amphibians, mammals and birds (19), and in the droppings of ground-feeding migrating songbirds (20). In the last-mentioned study, as many as 11 species of dictyostelids were found in the droppings. These studies confirm our observations of the presence of CSMs in mammal dung (sheep, goat and rabbit) and indicate that various groups of animals play a role in the short-distance dispersal (invertebrates) and the long-distance dispersal (birds and mammals) of CSMs. The dune area is an important route for migratory birds in the Netherlands, and many birds stay for a shorter or longer time in the dune area, leaving faecal material in the vicinity of abstraction wells (1). Thus, dung contains not only bacteria (FIB and possibly zoonotic pathogens), but also potential bacteria-grazing CSMs. The fact that sorocarps are formed on droppings indicates a considerable consumption of bacteria, probably including FIB. It is recommended for future studies to investigate CSM predation on FIB in the presence of many other bacterial species.

The consumption capacity of slime moulds can be important in the regulation of bacterial numbers. Raper (11) mentions that an amoeba consumes on average 1100 bacteria prior to cell division, and the figure reported by Ashworth and Dee (21) is c. 1000 bacteria. The growth curves in the present study show a considerable potential: amoebae grew in two to three days to up to 1.6×10^5 cells on a surface of 992 mm^2. At this density, they started to build sorocarps and apparently depleted their food resource. Considering each generation consumed c. 1000 bacteria per cell, 1.6×10^5 cells consumed 1.6×10^8 bacteria. In this experimental setup it is estimated, these amoebae may have reduced the number of bacteria by eight log units in only 60 hours. Although the setup represented ideal circumstances (moisture, the availability of food bacteria and no competition from other consumers), these characteristics indicate that CSMs have a considerable grazing capacity.

Assessing the contamination risk of groundwater abstracted from shallow aquifers requires quantitative information about the sources, occurrence, fate and transport of pathogenic microorganisms in the abstraction area (22, 23, 24). One of the elements is the occurrence and survival of pathogens (and FIB) in droppings from animals in the area. Studies on the survival of microorganisms in manure have been conducted, but they focused on physicochemical conditions, such as temperature or moisture content. Such studies indicate that FIB tend to grow at high (≥ 80%) water content while decay starts already at < 70% water content (25). Other studies looked at the effect of solar

radiation and found a reduction in numbers in radiated ground samples compared to samples not exposed to solar radiation (25, 26). However, no attention had been paid to the ability of CSMs to reduce bacterial numbers. The present results show that FIB are consumed by *D. mucoroides*. CSMs are ubiquitously present in faeces and soil. During their rapid growth, they consume huge numbers of bacteria. It is therefore important to include these organisms in studies on the environmental fate of pathogens and FIB in faecal droppings and in risk evaluations of faecal droppings near abstraction wells. Based on our findings, we conclude that Mycetozoa, which are abundantly present in the environment, help to substantially reduce the number of indicator and pathogenic bacteria in faecal droppings and in the soil.

Conclusion.

This first exploration in the field of cellular slime moulds (CSMs) in relation to the production of drinking water via shallow recharge wells has shown the presence in the recharge area of both dictyostelids and faecal droppings containing spores and/or amoebae of these organisms. Laboratory experiments demonstrated the ability of CSMs to consume faecal indicator bacteria (FIB). Moreover, growth experiments showed that these organisms are able to multiply massively in a relatively short period of time. Such large numbers can consume substantial numbers of bacteria. Consequently, CSMs are likely to have an important impact on the microbial ecology in both faecal droppings and the soil. These organisms may considerably reduce the survival times of FIB and pathogens in faeces and soil in the vicinity of shallow drinking water installations. It is therefore recommended to include the possible impact of CSMs in microbial risk evaluations in infiltration areas and recharge installations for the production of drinking water.

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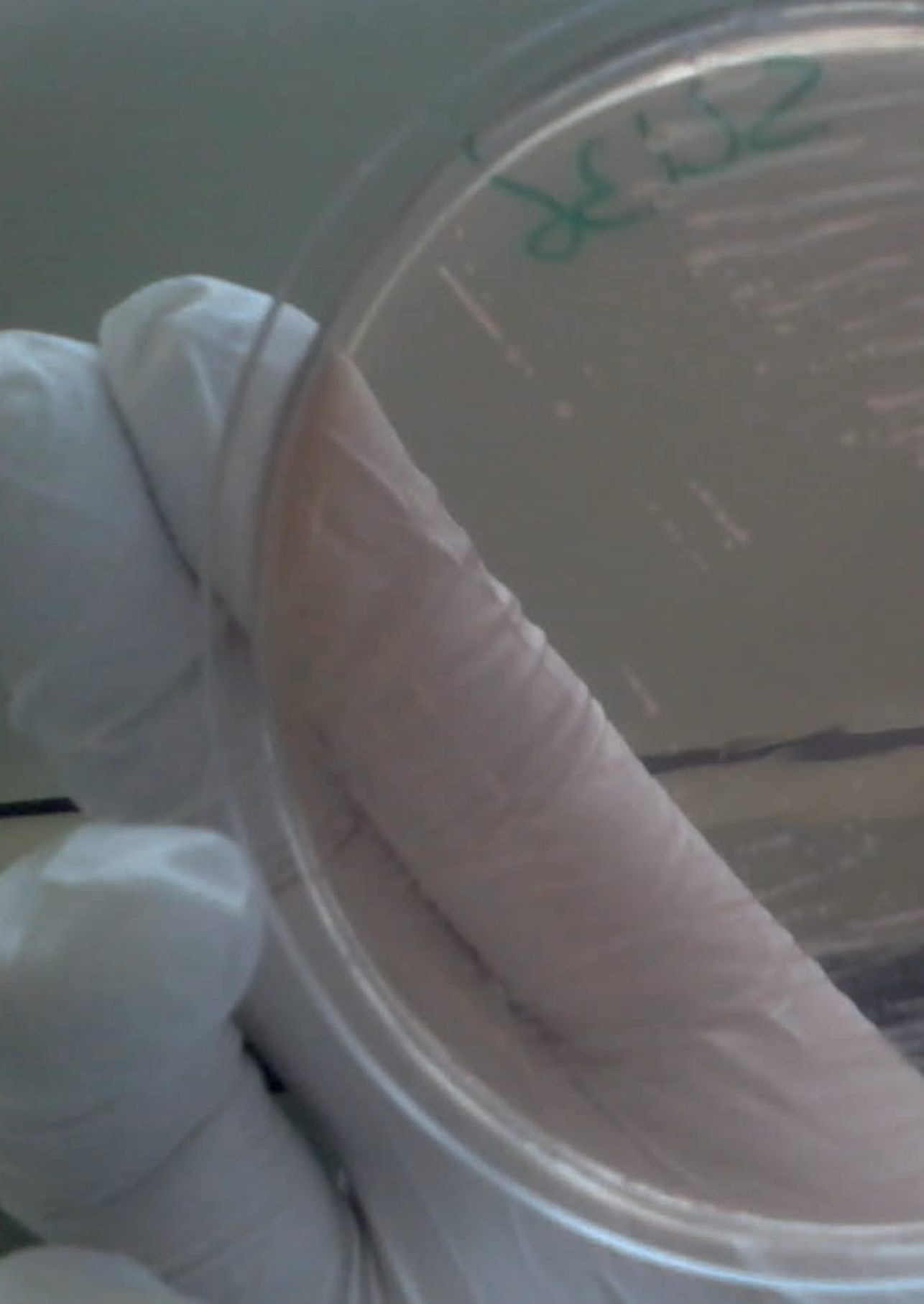
REFERENCES

1. Taučer-Kapteijn M, Hoogenboezem W, Hoogenboezem R, de Haas S, Medema GJ. 2016. Source tracking of *Enterococcus moraviensis* and *E. haemoperoxidus*. In press: accepted on 17 Jul 2016, Journal of Water and Health.
2. Staley C, Dunny GM, Sadowsky MJ. 2014 Chapter four–Environmental and Animal-Associated Enterococci. Advances in applied microbiology. Vol 87: 147-186.
3. Pernthaler J. 2005. Predation on prokaryotes in the water column and its ecological implications. Nature reviews. Vol 3: 537-546.
4. McArthur JV. 2006. Microbial ecology an evolutionary approach. Academic Press/ Elsevier pp: i-xii+ 1- 416.
5. Fenchel T. 1987. Ecology of protozoa. The ecology of free-living Phagotrophic Protists. Sc. Tech Publishers and Springer Verlag.
6. Dijkstra MJ, Keller HW. 2000. In Lee JJ, Leedale GF, Bradbury P. An illustrated guide to the Protozoa (2nd edition). Soc. of Protozoologists Kansas.
7. Spiegel FW, Stephenson SL, Keller HW, Moore DL, Cavender JC. 2004. 547-576. In Mueller GM, Bills GF, Foster MS. 2004. Biodiversity of fungi, inventory and monitoring methods Elsevier pp I – xviii; 1 – 777.
8. Olive LS. 1975. The Mycetozoans. Academic Press, New York, pp. i-ix; 1 – 293.
9. Stephenson SL, Stempen H. 1994. Myxomycetes: A handbook of slime molds. Timber Press, Portland, Oregon.
10. Krug JC, Benny GL, Keller HW. 2004. Coprophilous fungi (467-499). In Mueller GM, Bills GF, Foster MS Biodiversity of fungi, inventory and monitoring methods Elsevier pp i – xviii; 1 – 777.
11. Raper KB. 1984. The Dictyostelids. Princeton University Press, Princeton, New Jersey.
12. Taučer-Kapteijn M, Medema GJ, Hoogenboezem W. 2013. Comparison between Rapid ID 32 Strep System, Matrix Assisted Desorption Ionisation- Time of Flight Mass Spectrometry and 16S gene sequence analysis for the species identification of enterococcus spp. isolated from water. Water Science & Technology: Water supply 13(5): 1383-1389.
13. Bonner JT. 2009. The social amoebae. The biology of cellular slime molds. Princeton University Press. pp. i – ix; 1 – 144.
14. Ketcham RB, Levitan DR, Shenk MA, Eisenberg RM. 1988. Do interactions of cellular slime mold species regulate their densities in soil? Ecology 69(1): 193–199.
15. Kuserk FT. 1980. The relationship between cellular slime molds in forest soil. Ecology 61:1474–1485.
16. Horn EG. 1971. Food competition among the cellular slime molds. Ecology 52: 475–484.
17. Breveld O. 1869. *Dictyostelium mucoroides* Ein neue oranismus und der Verwandschaft der Myxomyceten. Abh. SchenkenbergNaturforsch. Ges. 7:85 – 107
18. Huss MJ. 1989. Dispersal of cellular slime molds by two soil invertebrates. Mycologia 81: 677-682.
19. Stephenson SL, Landolt J. 1992. Vertebrates as vectors of cellular slime molds in temperate forests. Mycological Research 96: 670-672.
20. Suthers HB. 1985. Ground-feeding migratory songbirds as cellular slime mold distribution vectors. Oecologia 65: 526-530.
21. Ashworth JM, Dee J. 1975. The biology of slime moulds. Studies in Biology no 56; pp 1 – 67.
22. Bradford SA, Schijven J. 2002. Release of *Cryptosporidium* and *Giardia* from dairy calf manure: impact of solution salinity. Envron. Sci. Technol. 3916-3923.
23. Schijven J, Bradford SA, Yang S. 2004. Release of Cryptosporidium and Giardia from dairy cattle manure: physical factors. J Environ Qual 33(4): 1499-1508.
24. Blaustein RA, Pachepsky YA, Hill RL, Shelton DR. 2015. Solid manure as a source of faecal indicator microorganisms: release under simulated rainfall. Environ. Sci. Technol. 49(13): 7860-7869.
25. Sinton L, Hall C, Braithwaite R. 2007. Sunlight inactivation of *Campylobacter jejuni* and *Salmonella enterica* compared with *Escherichia coli*, in seawater and river water. J Water Health 5(3): 357-365.
26. Reed RH. 1997. Solar inactivation of faecal bacteria in water: the critical role of oxygen. Letters in Applied Microbiology 24: 276-280.



Chapter 7

Screening municipal wastewater effluent and surface water used for drinking water production for the presence of ampicillin and vancomycin resistant enterococci.

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ABSTRACT

The emergence of clinical enterococcal isolates that are resistant to both ampicillin and vancomycin is a cause of great concern, as therapeutic alternatives for the treatment of infections caused by such organisms are becoming limited. Aquatic environments could play a role in the dissemination of antibiotic resistant enterococci. This study investigated the presence of ampicillin and vancomycin resistant enterococci in the treated effluent of six wastewater treatment plants (WWTPs) and in surface water used as a source for drinking water production in the Netherlands. Membrane filtration in combination with selective media with ampicillin or vancomycin was applied to determine the presence of ampicillin resistant *Enterococcus* (ARE) and vancomycin resistant *Enterococcus* (VRE) species. Ampicillin resistant *E. faecium* (minimal inhibitory concentration (MIC) >16 µg/ml; n=1033) was observed in all studied WWTP effluents. In surface water used for drinking water production (intake locations), no ARE or VRE were observed. At both types of location, intrinsic vancomycin resistant *Pediococcus* spp., *Leuconostoc* spp. and *Lactobacillus* spp. were isolated with the vancomycin medium. The ampicillin resistant *E. faecium* (ARE*fm*) isolates (n=113) did not contain the *vanA* or *vanB* gene, but MIC testing for vancomycin showed intermediate vancomycin resistance (2 to 8 µg ml^{-1}) to occur in these ARE*fm* strains. This study documents the discharge of ampicillin resistant *E. faecium* strains with intermediate vancomycin resistance by the WWTPs into the surface water, but no presence of these strains downstream at intake locations for drinking water production.

INTRODUCTION

Enterococcus species are part of the natural intestinal flora of both humans and animals. Because of their abundance in the faeces of warm-blooded animals and their long-term survival in the environment, they have traditionally been used as indicators of faecal contamination in the aquatic environment, including sewage, rivers and coastal areas (Anonymus, 1986; Haach et al., 2003; Nishiyama et al., 2015; Shibata et al., 2004), where they are ubiquitously detected (Lleo et al., 2005; Murray, 1990).

Some of the *Enterococcus* species have also been widely reported as opportunistic pathogens causing infections of the urinary tract, the bloodstream or skin wounds of immunocompromised persons (Jett et al., 1994) in healthcare settings. Ampicillin and vancomycin are important antibiotics in the treatment of those infections. In infections with ampicillin resistant enterococci (ARE), vancomycin can still be used, but that has led to the development of *Enterococcus* strains that are not susceptible to vancomycin; these are known as vancomycin resistant enterococci (VRE). Thus, therapeutic options for ARE and VRE infections are becoming limited.

It has been discovered that the phenotypic association of ampicillin and vancomycin resistance is often due to a genetic linkage and co-transfer of determinants responsible for resistance to both antibiotics (Shepard and Gilmore, 2002). A polyclonal outbreak of VRE, 95% of which were *E. faecium*, in several hospitals in northeast Ohio led to the identification of transferable ampicillin and vancomycin resistance among many of the isolates (Donskey et al., 1999; Shepard and Gilmore, 2002). An analysis of several of the VRE strains isolated during the outbreak revealed the presence of a novel Tn*916*-like transposon (Tn*5382*) encoding the *vanB* resistance. The transposon was integrated within a larger transferable element that also contained a gene encoding an alternate PBP5 with decreased affinity for binding to ampicillin (Carias et. al, 1998; Shepard and Gilmore, 2002).

Enterococci possesses a natural, low-level intrinsic resistance to β-lactam antibiotics (including ampicillin), which is due to the low affinity of their penicillin binding proteins (PBPs) for the β-lactam agents (Kak and Chow, 2002). This natural resistance was mainly found in *E. faecium* and *E. faecalis*, but was also described in *E. raffinosus* strains (Facklam et al., 2002). On top of the intrinsically present resistance to beta-lactams

and aminoglycosides, hospital-derived *E. faecium* has acquired resistance to high levels of aminoglycosides and beta-lactams (including ampicillin) through a combination of mutations and horizontal gene transfer (Top et al., 2008a).

VRE infections are caused mostly by *E. faecalis* and *E. faecium* (Cetinkaya et al., 2000; Jett et al., 1994; Marothi et al., 2005). Nine gene clusters associated with vancomycin resistance have been identified in *Enterococcus* species: *vanA* to *vanN* (Table 7.1).

Table 7.1. Overview of vancomycin resistance genotypes. MIC: Minimal Inhibitory Concentration.

Genotype	Location	Vancomycin MIC (µg/ml)	Most frequent enterococcal species	Transferable	Reference
VanA	on chromosome/ plasmid	64 - >1000	*E. faecium, E. faecalis*	Yes	Cetinkaya et al., 2000
VanB	on chromosome/ plasmid	4 - 1024	*E. faecium, E. faecalis*	Yes	Cetinkaya et al., 2000
Van C1/C2/C3	on chromosome	2-32	*E. gallinarum, E. casseliflavus E. flavescens, E. faecalis and E. faecium*	No	Kak and Chow, 2002; Nishiyama et al., 2015
VanD	on chromosome	64-128	*E. faecium*	No	Kak and Chow, 2002; Klare et al., 2003
VanE	?	8-32	*E. faecalis*	No	Kak and Chow, 2002; Courvalin, 2006; Klare et al., 2003
VanG	?	8-32	*E. faecalis*	No	Kak and Chow, 2002; Courvalin, 2006; Klare et al., 2003
VanL	on chromosome	*8*	*E. fa*ecalis	?	Boyd et al., 2008
VanM	*?*	*>256*	*E.* faecium	by conjugation to other *E. faecium*	Lebreton et al., 2011
VanN	?	16	*E. faecium*	by conjugation to other *E. faecium*	Xu et al., 2010

VanA and *vanB* are clinically the most important genotypes (Arias and Murray, 2013; Cetinkaya et al., 2000; Klare et al., 2003). The *vanA* resistance operon is acquired through the Tn*1546* transposon, and the *vanB* resistance operon is acquired through the exchange of transposons Tn*1547* and/or Tn*5382* (Kak and Chow, 2002). Vancomycin

intrinsically resistant species do not cause the same infection control concerns as *E. faecium* or *E. faecalis* VRE, as their resistance is chromosomal rather than plasmid mediated (Griffin et al., 2012).

The transfer of resistant bacteria from environmental compartments to humans may occur through contaminated food (Perreten et al., 1997), manure (if used as a fertilizer) and contaminated surface water used for irrigation or as recreational water. Wastewater and sludge from municipal sewage water treatment plants have been reported as favourable environments, consisting of variable mixtures of bacteria, nutrients and antimicrobial agents, for both survival and gene transfer (Lindberg et al., 2004), spreading resistant bacteria in both aquatic and terrestrial environment (Iversen et al., 2004). An additional concern is the possible presence of resistant enterococci in surface water used as a source for the production of drinking water.

The presence of a large reservoir of VRE in the environment could pose a threat for the transmission of vancomycin resistant bacteria to humans, either of enterococcal strains harbouring vancomycin-resistance genes, or via the horizontal spread of the genetic elements. This study investigated the presence of enterococci that are resistant to ampicillin and vancomycin in effluent from wastewater treatment plants (WWTPs) and in the surface water used for drinking water production in the Netherlands, applying membrane filtration and Slanetz and Bartley agar (SBA) complemented with ampicillin or vancomycin.

MATERIAL AND METHODS

Sampling and sampling locations

In September 2014, 1-litre sample was collected in sterile bottles at six WWTP effluent locations and at four locations (one river, two canals and one lake) where surface water is used as a drinking water production source (intake locations). All sampling points were located in the western part of the Netherlands. At the municipal WWTPs, the amount of the influent wastewater and the amount of treated wastewater that was discharged were similar. The flow rates at the WWTPs 1 to 6 were $2x10^3$ m^3 d^{-1}, $3x10^3$ m^3 d^{-1}, $8x10^3$ m^3 d^{-1}, $8x10^3$ m^3 d^{-1}, $1.3x10^4$ m^3 d^{-1} and $2.8x10^4$ m^3 d^{-1} respectively. Beside domestic waste water and storm water, WWTPs 2 and 4 also received hospital waste water. Treatment steps in these WWTPs are comparable and consist of: bar screens, grit chambers,

primary sedimentation, aeration, activated sludge and a second sedimentation step. At the WWTPs, samples were collected over a 24-hour period, harvesting 45 ml every 60 minutes. All samples were transported at 4 ^{0}C to the laboratory and analysed within 24 hours after sampling.

Isolation, enumeration and identification

Filtration was performed (ISO 7988-2:2000) using a nitrate membrane filter (0.45 µm, Sartorius, Germany) and Slanetz and Bartley agar (SBA, Oxoid, England). Additionally, SBA with the addition of 16 µg ml^{-1} of ampicillin (Sigma Aldrich, A9393-5G, USA) or the addition of 16 µg ml^{-1} of vancomycin (Sigma Aldrich, 75423-5VL, USA) were used for the detection and enumeration of ARE and VRE, respectively. The concentrations of the antibiotics were based on clinical breakpoints indicated by the Clinical and Laboratory Standards Institute (CLSI). In order to provide the correct enumeration of the colonies in 1 litre, sub-samples of 50-100 ml were filtered. Petri dishes of the three different media were incubated for 48 hours at 37 ^{0}C. After incubation, filters with pink, red, maroon or brown colonies were removed from the SBA agar and placed on Bile-esculin-azide agar (BEAA, Merck, Germany) and incubated for another 2 hours at 44 ^{0}C. After incubation, dark brown to black colonies were considered as enterococci. Ten percent of the isolates obtained on ampicillin-SBA for each location and the selection of isolates obtained on vancomycin-SBA (all colonies found at intake locations and a few morphologically different colonies per WWTP) were freshly cultured on SBA without supplement and subsequently identified (n=1033) using matrix-assisted laser desorption ionization – time of flight mass spectrometry (MALDI-TOF MS, Software version 3.0, Microflex series, Bruker Daltonics Inc., Germany), following the manufacturer's instructions. Score values of ≥ 2.0 were considered as reliable identifications. Ampicillin and vancomycin resistant *E. faecium* strain (AVRE) was used as a positive control, and ampicillin and vancomycin sensitive *E. faecalis* strain (ATTC 27270) was used as a negative control. The AVRE strain was kindly provided by the microbiology laboratory of the University Medical Centre Groningen (UMCG).

Antibiotic susceptibility testing

As a quality control, the selectivity of SBA supplemented with ampicillin was tested first. The MICs of two randomly chosen isolates per WWTP location (in total 12 isolates) were analysed by the Vitek 2 system (version 6.01, bioMérieux, France). In short, a 0.5 McFarland (McF) bacterial suspension was prepared using fresh colonies and then the AST-P586 card (bioMérieux, France) was used for susceptibility measurements

according to the manufacturer's instructions. An MIC of ≥ 16 µg ml^{-1} was considered as resistant (CLSI guidelines, 2014).

Secondly, 20 randomly chosen ARE isolates per WWTP were investigated for their ability to grow on SBA supplemented with vancomycin and the MIC values were determined using the Epsilometer test (Etest) (bioMérieux, France). Selected isolates were streaked on SBA supplemented with vancomycin and incubated for 48 hours at 37 °C. In order to determine the MIC, first a suspension of 2.0 McF in 0.45% saline solution was prepared by emulsifying freshly grown colonies using a sterile swab. Brain–heart infusion (BHI) agar (Oxoid, England) was used as medium for Etest. It was inoculated with the 2.0 McF suspension and the Etest strip vancomycin (range of 0.016 to 256 µg ml^{-1}) was applied to the inoculated BHI plate using sterile forceps. BHI plates were subsequently incubated for 48 hours at 37 °C. After the incubation, the MIC was determined based on the ellipse that intersects the MIC reading scale where the vancomycin inhibits the growth of enterococci. The MIC clinical breaking points for vancomycin Etests were read to the nearest two-fold concentration on the Etest strip. The selection of 120 isolates was stored in cryopreservative (Microbank Vials, Pro-Lab Diagnostics Inc., Canada) at -80 °C until further use by PCR method.

VanA and vanB PCR

Of the 120 stored isolates, 113 were successfully cultured on SBA and tested for the presence of *vanA* and *vanB* genotype. The DNA extraction and the PCR were performed as described by Fang and colleagues (Fang et al., 2012). Phocine Herpes Virus (PhHV) was added to the bacterial cells as an isolation- and an amplification control prior to DNA-extraction. A PCR to detect the target gene of the PhHV was performed in a multiplex assay simultaneously with the PCRs detecting *vanA* and *vanB*. In each PCR run positive controls consisting of clinical isolates of confirmed *vanA* and *vanB* positive *E. faecium* strains were used.

RESULTS

Presumptive enterococci (grown on SBA without antibiotic) were detected in WWTP effluent and in surface water at intake locations for drinking water production. The concentrations of presumptive enterococci grown on SBA without supplement were higher in the WWTP effluents (up to $3.7x10^4$ cfu L^{-1}) than in the samples from the intake

locations (up to $3.8x10^2$ cfu L^{-1}). Of the intake locations, number 2 (large lake) yielded the lowest number of presumptive enterococci. The concentrations of confirmed enterococci (BEAA) in WWTP effluents were also higher than the concentrations found at intake locations (Table 7.2). Applying ampicillin SBA, high concentrations of presumptive ampicillin resistant enterococci ($0.4x10^3$ to $4.4x10^3$ cfu L^{-1}) were observed in WWTP effluents, while no isolates were found in samples taken at intake locations. Using vancomycin SBA, more colonies were present in the WWTP effluents ($1.1x10^3$ – $1.5x10^4$ cfu L^{-1}) than in the samples from the intake points (0 – $1.0x10^2$ cfu L^{-1}). With the exception of WWTPs 4 and 6, the number of colonies grown on vancomycin SBA was higher than the number grown on ampicillin SBA in WWTP effluents. At WWTP 4, the number of colonies observed on the ampicillin SBA ($4.4x10^3$ cfu L^{-1}) was four times greater than the number observed on the vancomycin SBA ($1.1x10^3$ cfu L^{-1}).

Table 7.2. Concentration of bacteria (cfu L^{-1}) observed at sampling locations using membrane filtration and different media

Location	SBA	BEAA	SBA+Amp16	SBA+Van16
WWTP 1	1.6×10^4	1.6×10^4	1.5×10^3	1.8×10^3
WWTP 2	2.9×10^4	2.9×10^4	2.5×10^3	1.5×10^4
WWTP 3	3.7×10^4	3.7×10^4	6.0×10^2	2.0×10^3
WWTP 4	2.4×10^4	2.4×10^4	4.4×10^3	1.1×10^3
WWTP 5	2.5×10^4	2.5×10^4	4.0×10^2	1.2×10^3
WWTP 6	2.9×10^4	2.9×10^4	9.0×10^2	8.0×10^2
Intake point 1	1.8×10^2	9.0×10^1	0	1.0×10^2
Intake point 2	2.0×10^1	2.0×10^1	0	0
Intake point 3	3.8×10^2	3.8×10^2	0	7.0×10^1
Intake point 4	1.0×10^3	2.0×10^2	0	2.0×10^1

No ampicillin resistant isolates were observed at the intake locations; therefore, only ARE isolated from WWTP effluents were identified. For the WWTP effluents, the percentages of ARE among the total number of enterococci were 9.4%; 8.6%; 1.6%; 18.3%; 1.6% and 3.1%, respectively. All ARE isolates were identified as *E. faecium* (ARE*fm*) (Table 7.3).

Table 7.3. ARE species in the WWTP effluents

Location	No. of ARE identified cfu*	MALDI-TOF identification	(%)
WWTP 1	152	*E. faecium*	100%
WWTP 2	253	*E. faecium*	100%
WWTP 3	56	*E. faecium*	100%
WWTP 4	440	*E. faecium*	100%
WWTP 5	44	*E. faecium*	100%
WWTP 6	88	*E. faecium*	100%
Total	1033		

*10% of all colonies counted on ampicillin medium

For the 12 selected isolates grown on ampicillin SBA, ampicillin MICs were ≥ 32 µg ml^{-1}, which confirmed the selectivity of this medium.

Although the colony morphology and the colour of isolates obtained on vancomycin medium did not differ from enterococci, none of these colonies was identified as a member of the genus *Enterococcus*. The majority of species found at the intake locations were *Leuconostoc citreum*, *Lactobacillus plantarum* and species belonging to the genus *Pediococcus* spp. (Table 7.4). Comparable results were obtained for WWTPs 1, 3 and 4. Although *Pediococcus* spp. was also observed in WWTPs 5 and 6, too few isolates were identified to obtain a good impression of the species distribution. For intake location 2, no presumptive colonies were observed on vancomycin medium. The numbers of colonies at this sampling point were low for all media used. This result remains unexplained.

Because no enterococci were observed on vancomycin medium, a selection of 120 ampicillin resistant *E. faecium* (ARE*fm*) colonies obtained from WWTP effluents (20 per location) were cultured on vancomycin SBA and SBA without antibiotics, screened for the presence of the *vanA* or *vanB* gene (n=113), and had their vancomycin MIC determined by an Etest (n=120). None of 120 selected ARE*fm* isolates grew on vancomycin medium, which indicated that the vancomycin MIC values were < 16 µg ml^{-1}. Growth was observed for the positive control strain. The vancomycin MIC test (Table 7.5) showed that the majority (82%) of the selected ampicillin resistant *E. faecium* had displayed vancomycin resistance with a MIC of 4 µg ml^{-1}. At WWTPs 1, 3, 5 and 6, ARE*fm* were found with intermediate vancomycin resistance (MIC of 8 µg ml^{-1}). At WWTP 6, the majority (75.0%) of analysed isolates were ARE*fm* with elevated MICs. WWTPs 2 and 4 receive hospital effluent. None of the tested ARE*fm* isolates showed a MIC test result

of ≥16 µg ml^{-1}. The concentration of the ARE*fm* with corresponding vancomycin MICs was calculated from the concentrations found on SBA with ampicillin. The percentage of resistant enterococci was calculated by comparing these concentrations against the concentrations found on SBA (confirmed with BEAA) per WWTP (Table 7.5). In none of 113 ARE*fm* isolates was the *vanA* or *vanB* gene detected.

Table 7.4. Species distribution at sampling locations isolated using vancomycin SBA

Location	No. of colonies identified	Species identifications	%
Intake location 1	100	*Leuconostoc citreum*	47.0 %
		Pediococcus pentosaceus	27.0 %
		*Unidentified	26.0 %
Intake location 3	74	*Lactobacillus plantarum*	56.8 %
		Leuconostoc citreum	16.2 %
		Pediococcus pentosaceus	5.4 %
		Leuconostoc pseudomesenteroides	1.4 %
		*Unidentified	20.3 %
Intake location 4	20	*Leuconostoc citreum*	30.0 %
		Lactobacillus plantarum	25.0 %
		Pediococcus pentosaceus	15.0 %
		Leuconostoc lactis	5.0 %
		Pediococcus acidilactici	5.0 %
		Lactobacillus fermentum	5.0 %
		*Unidentified	15.0 %
WWTP 1	41	*Leuconostoc citreum*	53.7%
		Pediococcus pentosaceus	39.0%
		*Unidentified	7.3%
WWTP 3	43	*Lactobacillus plantarum*	48.8%
		Pediococcus pentosaceus	9.3%
		Leuconostoc citreum	9.3%
		*Unidentified	32.6%
WWTP 4	12	*Lactobacillus plantarum*	25.0%
		Pediococcus pentosaceus	16.7 %
		Leuconostoc citreum	16.7%
		Pediococcus acidilactici	8.3%
		*Unidentified	33.3%
WWTP 5	5	*Pediococcus acidilactici*	60%
		Pediococcus pentosaceus	40%
WWTP 6	5	*Pediococcus pentosaceus*	40%
		Pediococcus acidilactici	40%
		*Unidentified	20%

* Identification not reliable (score < 2.0)

Table 7.5. Etest vancomycin MIC value distributions in percentages (%) and concentration estimates of ARE*fm* with associated vancomycin MICs per WWTP

	MIC distributions µg ml^{-1} (%)						ARE*fm* vancomycin MIC/enterococci (cfu L^{-1})		
Locations	1	2	4	8	16	≥ 32	2 µg ml^{-1}	4 µg ml^{-1}	8 µg ml^{-1}
WWTP 1 (n = 19*)	0	0	89.5 (17)[1]	10.5 (2)	0	0	0	1.3E+03 (8.4%)	1.6E+02 (0.99%)
WWTP 2 (n = 19*)	0	0	100.0 (19)	0	0	0	0	2.5E+03 (8.6%)	0
WWTP 3 (n = 20)	0	0	95.0 (19)	5.0 (1)	0	0	0	5.7E+02 (1.5%)	2.0E+01 (0.08%)
WWTP 4 (n = 20)	0	5.0 (1)	95.0 (19)	0	0	0	2.2E+02 (0.91%)	4.2E+03 (17.4%)	0
WWTP 5 (n = 20)	0	0	90.0 (18)	10.0 (2)	0	0	0	3.6E+02 (1.4%)	4.0E+01 (0.16%)
WWTP 6 (n = 20)	0	0	25.0 (5)	75.0 (15)	0	0	0	2.3E+02 (0.77%)	6.8E+02 (2.3%)

*Etest of one isolate could not be read properly. 1 (no. of isolates).

DISCUSSION

Using membrane filtration in combination with the selective media, relatively high concentrations of ARE*fm* were found in WWTP effluents, whereas ARE and VRE were absent from the surface water used as a source for drinking water production. This indicates that the dilution and the inactivation of bacteria reduce the concentrations of antibiotic resistant enterococci in an aquatic environment. Of the total enterococci in the effluent of WWTPs, between 1.6 % and 18.3 % were found to be ARE. The percentage of ARE at the intake points was <0.3 – 5%, suggesting differences in the inactivation of total enterococci and ARE, or input from other enterococci sources than domestic wastewater. Applying ampicillin SBA, *E. faecium* was the only species observed in this study, which is in line with previous investigations by Anastasiou and Schmitt (2011), who found that a high percentage (88 – 100%) of ARE present in surface water was ARE*fm*.

Considering that ARE have emerged in Europe as a frequent cause of invasive enterococcal infections even in countries with a low prevalence of VRE, such as the Netherlands (Top et al., 2007; Top et al., 2008b), the numbers of ARE*fm* in WWTP effluent observed in this study indicate a reservoir of ARE*fm* outside the healthcare setting. To confirm that the ARE*fm* found in this study are hospital-associated *E. faecium* (HA-E*fm*), additional analysis on the presence of enterococcal surface protein (*esp*) gene

can provide additional information. HA-E*fm* isolates are genotypically distinct from community-derived human or animal strains, and the majority of HA-E*fm* has been reported to contain the *esp* gene, which encodes for an enterococcal surface protein (Anastasiou and Schmitt, 2011; Leavis et al., 2004). The presence of the *esp* gene was reported previously by Anastasiou and Schmitt (2011) in other WWTP effluents in the Netherlands.

Studies in which enterococci isolated from surface water were analysed for their vancomycin resistance levels are scarce. In the investigation by Blaak et al. (2010), *E. faecium* and *E. faecalis* were isolated from surface water in an area with a high density of animal farms. In these isolates, 4 µg ml^{-1} was the highest MIC value observed, using the same method. A MIC value of 8 µg ml^{-1} was observed in only two isolates of *E. casseliflavus*, which were presumed to possess intrinsic resistance (*vanC*). In another study by Blaak et al. (2011), ARE*fm* with the highest vancomycin MIC values of 4 µg ml^{-1} were isolated from three large Dutch rivers (Meuse, Rhine and New Meuse). The vancomycin MICs observed in our study exceed the maximum previously observed in the Netherlands. Since we did not observe ARE*fm* in surface water at intake locations, it is likely that the concentration of enterococci and that of antibiotic resistant enterococci may vary depending on the sampling site. Blaak et al. (2011) selected surface water sampling sites in large rivers affected by wastewater discharges from large urban and agricultural areas. The concentration of total enterococci at these sites was also higher (1.6×10^3 cfu/l) compared to that at the intake points in the present study. The detection of ARE in more contaminated surface water indicates that, depending on the disappearance rate of the ARE, low levels of ARE could be present also at intake points. Compared with the results obtained in Portugal and the USA, where VRE were found in the effluent of WWTPs (da Costa et al., 2006; Rosenberg Goldstein et al., 2014), the vancomycin resistance levels found in this study are low. Interestingly, vancomycin MICs of 8 µg ml^{-1} in ARE*fm* were observed at all WWTP effluent locations, except for the two WWTPs that also receive hospital effluents. Further investigations are needed to estimate the significance of hospital effluents as a source of vancomycin resistant strains reaching the WWTPs.

Because there is little knowledge of the acquisition or transfer of antibiotic resistance in enterococci that are discharged into the aquatic environment, the understanding of the mechanisms regulating low-level vancomycin resistance in the ARE*fm* strains found in this study is incomplete. The absence of high-level *vanA* and *vanB* resistance

genes indicates that these strains do not belong to the clinically most important genotypes. According to the literature, low-level vancomycin resistance (8-32 µg ml^{-1}) was observed in *vanC*, *vanE*, *vanG*, *vanL* and *vanN* genotypes. Until now, *vanE*, *vanG* and *vanL* were observed only in *E. faecalis*. *VanN* has been discovered in two clinical isolates of *E. faecium* (vancomycin MIC 16 µg ml^{-1}), both susceptible to ampicillin, but transferable by conjugation to *E. faecium* (Lebreton et al., 2011). *VanC* resistance, which has been shown to be intrinsic to *E. gallinarum*, *E. casseliflavus* and *E. flavescens*, has recently been identified in *E. faecium* and *E. faecalis* isolated from sewage and river water in the provincial city of Miyazaki, Japan (Nishiyama et al., 2015). Nishiyama et al. (2015) reported that these ampicillin susceptible *E. faecalis* and *E. faecium* possessing *vanC2/3* were isolated along with *E. casseliflavus/gallinarum* from the aquatic environment, and suggested that these strains may have acquired *vanC2/3* by horizontal gene transfer in the aqueous environment or through horizontal gene transfer prior to entering the aquatic environment. Although the strains observed in our study were not screened for the presence of *vanC* and *vanN* and the vancomycin resistance mechanisms are not clear, relatively high numbers of *E. faecium* with a combination of ampicillin resistance and low vancomycin resistance, may be clinically significant and need further investigation.

This study and other studies mentioned earlier have shown that antibiotic resistant bacteria can reach surface waters via WWTPs. The biological treatment process in conventional WWTPs may result in a selective increase in the antibiotic resistant bacteria population and the increased occurrence of multidrug resistant bacteria (Zhang et al., 2009). Although the mechanisms that contribute to a selective increase in antibiotic resistant bacteria in WWTPs remain undefined, a number of studies have shown that the conditions in WWTPs favour antibiotic resistant bacteria (Iwane et al., 2001; Schwartz et al., 2003). Also Anastasiou and Schmitt (2011) indicated that ARE*fm* might survive sewage treatment slightly better than non-resistant enterococci. Consequently, WWTPs do discharge resistant bacteria into surface waters. When people come into contact with contaminated water, for instance during recreation or irrigation, they risk being exposed to bacteria that are resistant to one or more antibiotics. To estimate the magnitude of the exposure risk via surface water and the health consequences, more studies are needed to clarify the persistence of resistant bacteria and their genes in surface waters, the frequency of horizontal gene transfer in water, etc. Taking into account the dilution of numbers of resistant strains discharged into surface waters, possibly low numbers of resistant strains at the intake locations and a high level of enterococci removal during the drinking water production process, the

concern about the presence of ARE and VRE in surface water regarding drinking water production in the Netherlands may be insignificant. However, it is desirable to carry out further monitoring to evaluate the numbers of and possible increase in antibiotic resistance levels in enterococci and other clinically relevant microorganisms in WWTP effluents and surface waters used for irrigation or recreation, as well as gene transfer in the aquatic environment.

Conclusions

In this study, the use of SBA complemented with ampicillin or vancomycin was shown to be a suitable two-step screening method for enterococci with clinically relevant antibiotic resistance in water. The growth of *Leuconostoc* spp., *Pediococcus* spp. and *Lactobacillus* spp., which are intrinsically resistant to glycopeptides, makes SBA complemented with vancomycin less suitable for the isolation of VRE from water using membrane filtration. Rapid identification techniques, such as MALDI-TOF MS (Taučer-Kapteijn et al., 2013), may serve as a screening tool for the confirmation and speciation of presumptive enterococci colonies on ampicillin SBA and vancomycin SBA. Using these methods, no VRE were found in WWTP effluents or at intake locations used for drinking water production. Ampicillin resistant *E. faecium* isolates with low-level resistance to vancomycin (MICs 2 - 8 mg L^{-1}) were observed in all studied WWTP effluents. Considering that also in the Netherlands ARE have emerged as a frequent cause of invasive enterococcal infections, further investigation is required to determine whether the ARE*fm* found in this study resemble the ARE*fm* found in those infections. No ARE were detected at intake locations at total enterococci concentrations of 20 – 380 cfu L^{-1}. To further evaluate surface waters as a transmission route for antibiotic resistant bacteria from the environment to humans, more investigations are required to understand the fate of resistance genes once discharged into surface water.

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REFERENCES

Anastasiou, I. and Schmitt, H., 2011. Hospital-associated *Enterococcus faecium* in the water chain, Rhine Water Works The Netherlands.

Anonymus, 1986. Ambient Water-Quality Criteria for Bacteria -1986. Office of Water Regulations and Standards, EPA 440/5-84-002. U.S. Environmental Protection Agency: Washington DC.

Arias, C.A. and Murray B.E., 2013. The rise of the *Enterococcus:* beyond vancomycin resistance. Nat. Rev. Microbiol. 10(4), 266-273.

Blaak, H., Schets, F.M., Italiaander, R., Schmitt, H., de Roda Husman, A.M., 2010. Antibioticaresistente bacteriën in Nederlands oppervlaktewater in veeteeltrijk gebied. RIVM rapport 703719031.

Blaak, H., van Rooijen, S.R., Schuijt, M.S., Docters van Leeuwen, A.E., Italiaander, R., van den Berg, H.H.J.L., Lodder-Verschoor, F., Schets, F.M., de Roda Husman, A.M, 2011. Prevalence of antibiotic resistant bacteria in the rivers Meuse, Rhine and New Meuse. National Institute for Public Health and the Environment.

Boyd, D.A., Willey, B.M., Fawcett, D., Gillani, N., Mulvey, M.R., 2008. Molecular Characterization of Enterococcus faecalis N06-0364 with Low-Level Vancomycin Resistance Harboring a Novel D-Ala-D-Ser Gene Cluster, *vanL*. Antimicrob. Agents Ch. 52(7), 2667-2672.

Carias, L.L., Rudin, S.D., Donskey, C.J., Rice, L.B., 1998. Genetic linkage and cotransfer of a novel, *vanB*-containing transposon (*Tn5382*) and a low-affinity penicillin-binding protein 5 gene in a clinical vancomycin-resistant *Enterococcus faecium* isolate. J. Bacteriol. 180, 4426-4434.

Cetinkaya, Y., Falk, P., Mayhall, C.G., 2000. Vancomycin-Resistant Enterococci, Clin. Microbiol. Rev. 13(4), 686-707.

CLSI (Clinical and Laboratory Standards Institute), 2014. Performance Standards for Antimicrobial Susceptibility Testing: Twenty-Fourth Informational Supplement. M100-S24.

da Costa, P.M., Vaz-Pires, P., Bernardo, F., 2006. Antimicrobial resistance in *Enterococcus* spp. isolated in inflow, effluent and sludge from municipal sewage water treatment plants. Water Res. 40, 1735-1740.

Courvalin, P., 2006. Vancomycin resistance in gram-positive cocci. Clin. Infect. Dis. 42(1), S25-S34.

Donskey, C.J., Schreiber, J.R., Jacobs, M.R., Shekar, R., Salata, R.A., Gordon, S., Whalen, C.C., Smith, F., Rice, L.B., 1999. A polyclonal outbreak of predominantly VanB vancomycin-resistant enterococci in northeast Ohio. Northeast Ohio Vancomycin-Resistant Enterococcus Surveillance Program. Clin. Infect. Dis. 29, 573-579.

Facklam, R.R, Carvalho, M.S., Teixeira, L.M., 2002. History, Taxonomy, Biochemical Characteristics, and Antibiotic Susceptibility Testing of Enterococci, in: Gilmore, M.S., Clewell, D.B., Courvalin, P., Dunny, G.M., Murray, B.E., Rice, L.B. (Eds), The Enterococci: Pathogenesis, Molecular Biology and Antibiotic Resistance. ASM Press, Washington, pp. 1-54.

Fang, H., Ohlsson, A.-K., Jiang G.-X., Ullberg M., 2012. Screening for vancomycin resistant enterococci: an efficient and economical laboratory-developed test. Eur. J. Clin. Microbiol. 31(3), 261-265

Griffin, P.M., Price, G.R., Schooneveldt, J.M., Schlebusch, S., Tilse, M.H., Urbanski, T., Hamilton, B., Venter, D., 2012. Use of Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry To Identify Vancomycin-Resistant Enterococci and Investigate the Epidemiology of an Outbreak. J. Clin. Microbiol. 50(9), 2918-2931.

Haach, S.K., Fogarty, L.R., Wright, C., 2003. Escherichia coli and Enterococci at beaches in the Grand Traverse Bay, Lake Michigan: Sources, Characteristics and Environmental Pathways. Environ. Sci. Technol. 37, 3275-3282.

ISO (7899-2: 2000). Water quality. Detection and enumeration of intestinal enterococci. Part 2: Membrane filtration method.

Iversen, A., Kühn, I., Rahman, M., Franklin, A., Burman, L.G., Olsson-Liljequist, B., Torell, E., Möllby, R., 2004. Evidence for transmission between humans and the environment of a nosocomial strain of *Enterococcus faecium*. Environ. Microbiol. 6(1), 55-61.

Iwane, T., Urase, T., Yamamoto, K., 2001. Possible Impact of Treated Wastewater Discharge on Incidence of Antibiotic Resistant Bacteria in River Water. Water Sci. Technol. 43, 91-99.

Jett, B.D., Huycke, M.M., Gilmore, M.S., 1994.Virulence of Enterococci. Clin. Microbiol. Rev. 7(4), 462-478.

Kak, V. and Chow, J.W., 2002. Acquired Antibiotic Resistances in Enterococci, in: Gilmore, M.S., Clewell, D.B., Courvalin, P., Dunny, G.M., Murray, B.E., Rice, L.B. (Eds), The Enterococci: Pathogenensis, Molecular Biology and Antibiotic Resistance. ASM Press, Washington, pp. 355-383.

Klare, I., Konstabel, C., Badstübner, D., Werner, G., Witte, W., 2003. Occurrence and spread of antibiotic resistance in *Enterococcus faecium*. Int. J. Food Microbiol. 88, 269-290.

Leavis, H., Top, J., Shankar, N., Borgen, K., Bonten, M., van Embden, J., Willems, R.J., 2004. A Novel Putative Enterococcal Pathogenicity Island Linked to the *Esp* Virulence Gene of *Enterococcus Faecium* and Associated with Epidemicity. J. Bacteriol. 186, 672-682.

Lebreton, F., Depardieu, F., Bourdon, N., Fines-Guyon, M., Berger, P., Camiade, S., Leclercq, R., Courvalin, P., Cattoir, V., 2011. D-Ala-D-Ser VanN-Type Transferable Vancomycin Resistance in *Enterococcus faecium*. Antimicrob. Agents Ch. 55(10), 4606-4612.

Lindberg, R., Jarnheimer, P., Olsen, B., Johansson, M., Tysklind, M., 2004. Determination of antibiotic substances in hospital sewage water using solid phase extraction and liquid chro- matography/mass spectrometry and group analogue internal standards. Chemosphere. 57, 1479–1488.

Lleo, M.M., Bonato, B., Benedetti, D., Canepari, P., 2005. Survival of enterococcal species in aquatic environments. FEMS Microbiol. Ecol. 54, 189–196.

Marothi, Y.A., Agnihotri, H., Dubey, D., 2005. Enterococcal Resistance – An Overview. Indian J. Med. Microbi. 23(4), 214-219.

Murray, B.E., 1990. The life and times of the Enterococcus. Clin. Microbiol. Rev. 3, 46-65.

Nishiyama, M., Iguchi, A., Suzuki, Y., 2015. Identification of Enterococcus faecium and Enterococcus faecalis as vanC-type Vancomycin- Resistant Enterococci (VRE) from sewage and river water in the provincial city of Miyazaki, Japan. J. Environ. Sci. Heal. A. 50, 16-25.

Perreten, V., Schwarz, F., Cresta, L., Boeglin, M., Dasen, G., Teuber, M., 1997. Antibiotic Resistance Spread in Food. Nature. 389, 801-802.

Rosenberg Goldstein, R.E., Micallef, S.A., Gibbs, S.G., George, A., Claye, E., Sapkota, A., Joseph, S.W., Sapkota, A.R., 2014. Detection of vancomycin –resistant enterococci (VRE) at four U.S. wastewater treatment plants that provide effluent for reuse. Sci. Total Environ. 466-467 (404-411).

Schwartz, T., Kohnen, W., Jansen, B., Obst, U., 2003. Detection of Antibiotic-Resistant Bacteria and their Resistance Genes in Wastewater, Surface Water, and Drinking Water Biofilms. FEMS Microbiol. Ecol. 43, 325-335.

Shepard, B.D. and Gilmore, M.S., 2002. Antibiotic-Resistant Enterococci: The Mechanisms and Dynamics of Drug Introduction and Resistance. Microb. Infect. 4, 215-224.

Shibata, T., Solo-Gabriele, H.M., Fleming, L.E., Elmir, S., 2004. Monitoring marine recreational water quality using multiple microbial indicators in an urban tropical environment. Water Res. 38, 3119–3131.

Taučer-Kapteijn, M., Medema, G., Hoogenboezem, W., 2013 Comparison between Rapid ID 32 Strep System, Matrix Assisted Laser Desorption Ionisation–Time of Flight Mass Spectrometry and 16S rRNA gene sequence analysis for the species identification of *Enterococcus* spp. isolated from water. Wa. Sci. Technol. 13(5), 1383-1389.

Top, J., Willems, R., Blok, H., De Regt, M., Jalink, K., Troelstra, A., Goorhuis, B., Bonten, M., 2007. Ecological replacement of *Enterococcus faecalis* by multiresistant clonal complex 17 *Enterococcus faecium*. Clin. Microbiol. Infect. 13(3), 316-319.

Top, J., Willems, R., Bonten, M., 2008a. Emergence of CC17 *Enterococcus faecium:* from commensal to hospital-adapted pathogen. FEMS Immunol. Med. Microbiol. 52(3), 297-308.

Top, J., Willems, R., van der Velden, S., Asbroek, M., Bonten, M., 2008b. Emergence of clonal complex 17 *Enterococcus faecium* in The Netherlands. J. Clin. Microbiol. 46(1), 214-9.

Xu, X., Lin, D., Yan, G., Ye, X., Wu, S., Guo, Y., Zhu, D., Hu, F., Zhang, Y., Wang, F., Jacoby, G.A., Wang, M., 2010. VanM, a New Glycopeptide Resistance Gene Cluster Found in *Enterococus faecium*. Antimicrob. Agents Ch. 54(11), 4643-4647.

Zhang, Y., Marrs, C.F., Simon, C., Xi, C., 2009. Wastewater Treatment Contributes to Selective Increase of Antibiotic Resistance among Acinetobacter Spp. Sci. Total Environ. 407, 3702-3706.

Chapter 8

General discussion

The investigations described in this thesis were carried out to analyse the occurrence and fate of enterococci in the environment, and the implications for their use as faecal indicators in (drinking) water analysis and the occurrence and fate of vancomycin-resistant enterococci in the water environment. The specific objectives of the research were:

- To examine the suitability of MALDI-TOF MS technique to identify enterococci isolated from water compared to biochemical and molecular identification techniques (Chapter 2).

- To examine the growth of *E. moraviensis* under non-enteric conditions (abstracted water, biofilms in the abstraction wells, sediments from the wells and humus and on plant extract). (Chapter 3).

- To compare the ability of *E. moraviensis* to grow on plant extract with that of other *Enterococcus* species (*E. casseliflavus*, *E. hirae*, *E. faecalis*, *E. faecium*) and to examine whether these species are able to grow in competition with natural microbial flora (Chapter 4).

- To evaluate the possible faecal sources of *E. moraviensis* and *E. haemoperoxidus* in the vicinity of the abstraction wells and to find a possible link between the *Enterococcus* species distribution found in faecal samples and the water samples (Chapter 5).

- To investigate whether the cellular slime moulds (dictyostelids) can be isolated from soil and dung from the dune infiltration area and to test their potential ability to substantially reduce the numbers of indicator bacteria (Chapter 6).

- To evaluate the presence of ampicillin- (ARE) and vancomycin (VRE)-resistant enterococci in treated wastewater and in surface water used for the production of drinking water using a modified version of the Slanetz & Bartley medium (enriched with 16 mg L^{-1} ampicillin or 16 mg L^{-1} vancomycin) (Chapter 7).

The outcomes of the research served to: a) explain the occurrence of enterococci in abstracted water (after dune passage) in the Castricum dune infiltration area (The Netherlands), b) to evaluate the use of these *Enterococcus* strains as faecal indicators, and c) to investigate the possible reservoir of clinically relevant ampicillin- and vancomycin-resistant *Enterococcus* strains in Waste Water Treatment Plant (WWTP) effluents and surface water used for the drinking water production in The Netherlands.

Identification of enterococci using Slanetz & Bartley medium and MALDI-TOF MS

For the evaluation of the diversity and the incidence of enterococcal species detected in abstracted water and their potential sources, the identification of *Enterococcus* isolates was needed. The identification of 101 isolates using Rapid 32 ID Strep system, MALDI-TOF MS and 16S rRNA gene sequence analysis has demonstrated that MALDI-TOF MS provides reliable identification results of enterococci isolated from water samples. Variation of the scores obtained in 20 consecutive runs for one randomly chosen isolate, showed good repeatability. The use of two different culture media, Slanetz & Bartley and Columbia Sheep-Blood agar, did not influence the MALDI-TOF MS identification. Additionally, false-positive test results using ISO 7899-2:2000 were highlighted by the identification of *Aerococcus viridans* strains, which are common in the environment, but are not related to faecal contamination. According to the confirmation test according to ISO 7899-2: 2000 (Anonymous, 2000), some of the *A. viridans* strains are also bile-esculin positive. This indicates that this detection method may not be ideal for the enumeration of enterococci in water as indicators of faecal pollution. This finding is in line with earlier observations that the genera *Pediococcus*, *Lactococcus*, *Aerococcus* and *Leuconostoc* may also occasionally result in positive reactions on bile-esculin-agar, which may further lead to unfavourable judgements about the quality of the water and unnecessarily restrictive control measures (Devriese et al. 1993; Leclerc et al. 1996; Pinto et al., 1999). In addition to the reliability of the results and the efficiency of the identification using MALDI-TOF MS, the possibility of identifying the species, and therefore also false positive strains, is another benefit of this technique in the routine analysis of enterococci (Chapter 2).

The ability to grow on plant extracts

The study described in Chapter 3 aimed to establish whether the repeated presence of *E. moraviensis* in abstracted water was associated with its potential ability to grow in the habitats present in the dune passage process (sediment from the abstraction wells,

biofilm developed using abstracted water and soil) or on an extract of dune vegetation collected in the vicinity of the abstraction wells. The experiments demonstrated that *E. moraviensis* was not able to grow or persist in these dune passage process habitats. For all three types of habitats no growth or long persistence, but die-off of *E. moraviensis* was observed. In the experiment using sediment as a growth promotor, temperature-dependent decay was observed, with higher inactivation rates at higher temperatures, probably due to the increase in the rate of biochemical reactions at higher temperatures. These results indicated that these habitats do not support growth or persistence of *E. moraviensis*. This encouraged further investigations which have shown that various concentrations of boiled and filtered plant extracts (derived from the dune vegetation) supported growth of *E. moraviensis* (increasing in numbers for up to 6 log), with maximum concentrations after four to six days at 15 ˚C. The observation that *E. moraviensis* was not able to grow in the examined dune passage habitats indicated that *E. moraviensis* can serve as a faecal indicator in these habitats. However, concern was growing that this species, after it was shed in faeces and deposited on the ground, could grow on nutrients derived from (decaying) dune vegetation during heavy rains, especially after mowing. Byappanahalli et al. (2003) observed that algal (*Cladophora*) leachate readily supported in-vitro multiplication of enterococci (100-fold increase in concentration in undiluted algal leachate at 35 °C for 24 h). They suggested that leachates contain the necessary growth-promoting substances to sustain these bacteria (Byappanahalli et al., 2003). We examined if the ability of *E. moraviensis* and other *Enterococcus* species to grow on plant extract was affected by the abundance of indigenous bacteria (and other micro-organisms) that create competitive conditions for nutrients and predation.

The experiments presented in Chapter 4 demonstrated that filtered plant extract of *Ammophila arenaria* (a common dune plant) promoted nutrient concentration dependent growth of all five selected *Enterococcus* species (*E. moraviensis*, *E. casseliflavus*, *E. hirae*, *E. faecalis* and *E. faecium*) at 15 °C, when the indigenous microbial community was reduced by membrane filtration prior to the inoculation with enterococci. At lower plant extract concentrations (5 gL^{-1} and 0.5 gL^{-1}) *E. moraviensis* and *E. casseliflavus* reached higher concentrations compared to *E. hirae*, *E. faecalis* and *E. faecium*. Reduced ability to grow at different doses of nutrients has also been observed for *E. faecium* in this study. This indicated that even though all tested species are able to grow on filtered plant extract, *E. moraviensis* and *E. casseliflavus* may be better adapted to the conditions tested. Similar experiments, but without removal of the indigenous microbial

community, aimed to simulate the fate of enterococci under natural conditions. When plant extract was not filtered, enterococcal growth was absent or very limited. Instead, a rapid decrease of enterococcal numbers was observed for all five species (*E. moraviensis*, *E. casseliflavus*, *E. hirae*, *E. faecalis* and *E. faecium*). These results suggest that competition for nutrients limits the success of enterococci on plant material. This is in line with results reported by Desmarais et al. (2002), who demonstrated that competition for nutrients hinder replication of enterococci in the environment and concluded that inactivating the indigenous microbes in beach sand provides a favourable environment for *E. coli* and enterococci to grow, whether through increased nutrient availability or decreased predation. However, the affinity for substrates, such as plant material, may be species-specific. *E. moraviensis* showed an initial increase in numbers even in the presence of the indigenous microbial flora, which was not the case for any other species tested. From this point of view, *E. moraviensis* may be better suited to multiply in the presence of (decaying) plant material indicating it may be a less suitable indicator of faecal pollution in pristine, plant material-rich environments than *E. faecium*. Similarly, Badgley et al. (2010) suggested that *E. casseliflavus* might have environmental adaptations in submerged aquatic vegetation (Badgley et al., 2010).

Identification of enterococci isolated from abstracted water and from faecal samples

In a study described in Chapter 5, the MALDI-TOF MS was applied for the identification of enterococci isolated from large volume samples (100 L) of water abstracted from the dune infiltration area (Castricum, The Netherlands). *E. moraviensis* was the most frequently observed *Enterococcus* species in these samples and the faecal origin of these species was not yet described. An extensive survey of faecal droppings of animals living in the vicinity of the abstraction wells and human faecal samples, with enumeration, isolation and identification of enterococcal isolates from excreta revealed that *E. moraviensis* has a faecal origin: it was frequently present in geese faeces. A few *E. moraviensis* isolates have also been found in red foxes and rabbits. Among seven animal species, geese were found to be the only carriers of *E. haemoperoxidus*. This is the first report on the faecal sources of *E. moraviensis* and *E. haemoperoxidus*. It has been suggested that dominant presence of particular *Enterococcus* species in environmental samples may provide indications for the specific source of the pollution. In our setting, *E. moraviensis* and *E. haemoperoxidus* presence is indicative of faecal contamination by geese. The statistical analysis of the composition of enterococcal species in faeces of hosts and the composition of enterococci found in abstracted water suggested

that geese droppings are a source of contamination, since the species composition in abstracted water samples and in geese droppings is very similar. The route of the contamination of water with enterococci is not yet fully understood. However the species distribution in water and geese faeces, frequent presence of *E. moraviensis* in geese droppings, significant numbers and frequent identification of *E. moraviensis* in abstracted water, the presence of geese (moulting in the summer) in specific parts of the dune infiltration area close to the abstraction wells and the evidently high faecal load contributed by geese, all indicate a probable influence of geese droppings on the quality of the abstracted water. Our results also show that *Enterococcus* species of which faecal sources are not (yet) identified, may have faecal sources and that the identification of *Enterococcus* species isolated from different enteric and extra-enteric environments may help to clarify their role as faecal indicators and point towards contamination sources. Given that geese can be carriers of *Giardia* spp., zoonotic strains of *Cryptosporidium parvum* (Graczyk et al., 1998, Zhou *et al.*, 2004), *Cryptosporidium hominis* (Zhou *et al.*, 2004), *Salmonella* spp. (Feare et al., 1999) and *Campylobacter jejuni* (Moriarty et al., 2011), this strengthens the hygienic importance of *E. moraviensis* and *E. haemoperoxidus* and their significance as faecal indicators. Medema et al. (1999) indicated earlier that waterfowl is a potentially significant source of water contamination with oocysts of *Cryptosporidium* spp. and cysts of *Giardia* spp. in pre-treatment storage reservoirs of river water in The Netherlands. Geese, but also ruminants (deer, cattle, sheep) can be infected with *C. parvum* (Angus, 1990). These animals are prevalent in dune infiltration areas in The Netherlands and are part of the natural environmental management of these areas with a high nature value. The implications of human illness due to contamination from animal faeces and zoonotic pathogens should not be underestimated. Olsen et al. (2002) reported an outbreak of *Escherichia coli* O157:H7 infections, where unchlorinated municipal water supply became contaminated when surface water, containing deer and elk faeces, leached into the town's unconfined aquifer. A large waterborne outbreak of *E. coli* O157:H7 infections occurred also at a county fair in New York (Centres for Disease Control and Prevention, 1999). In that outbreak, the drinking water was likely contaminated when cow manure seeped into a shallow, unchlorinated well after a heavy rainstorm (Olsen et al., 2002). Furthermore, an estimated 2,300 people became seriously ill and seven died from exposure to microbial contaminated drinking water in the town of Walkerton, Ontario, Canada in May, 2000. The pathogens *E. coli* 0157:H7 and *Campylobacter jejuni* causing the outbreak were attributed to contamination of the shallow (5–8 m) well arising from cattle manure from a nearby farm following a period of heavy spring rainfall (Hrudey et

al., 2003). Other outbreaks of illness as a consequence of drinking water contamination by bird faeces were reported. Palmer et al. (1983) reported an outbreak of *Campylobacter* gastroenteritis affecting 257 persons who consumed water taken from an open-topped storage tank which supplied predominantly unchlorinated water contaminated by faecal material from birds or bats. Similarly, Koplan et al. (1978) reported an outbreak of salmonellosis as a consequence of the consumption of water supplied by a storage tank of roof-collected rainwater most likely contaminated with bird faeces.

Craun et al. (2010), who reviewed drinking water related outbreaks in the US, indicated that groundwater contamination can have broad public health impact and supported the conclusion by Curriero et al. (2001) that severe weather should demand increased awareness and active inquiry by the responsible staff into the risk of contamination.

These outbreaks of drinking water related illness indicate that different animals can be carriers of pathogens which may pose a threat to humans and that the consumption of water contaminated with faeces of these animals may lead to numerous cases of disease. They highlight the importance of exercising extreme caution when water is assumed to be contaminated by animal faeces.

During heavy rainfall faecal flora (including *E. moraviensis*) may travel with percolating rainwater to the groundwater. The presence of these species in abstracted water indicates a breakthrough of this faecal flora, potentially including zoonotic pathogens, from the topsoil towards the abstraction wells. Therefore, *E. moraviensis* cannot be neglected as a faecal indicator, indicating the presence of zoonotic pathogens in faeces of geese. If growth in topsoil by *E. moraviensis* is mirrored by growth of pathogenic bacteria, this may even strengthen its role as a faecal indicator. Whitman et al. (2003) showed that the algal mats collected along shorelines of southern and northern Lake Michigan in the Great Lakes were a significant source of FIB (*E. coli* and enterococci), with densities often exceeding 100,000 CFU/g (dry weight) (Whitman et al., 2003). Aside from FIB, enteric pathogens, such as Shiga toxin-producing *E. coli* (STEC), *Shigella*, *Salmonella*, and *Campylobacter*, have also been isolated from these mats (Byappanahalli et al., 2009), though it is not clear whether *Cladophora* promoted the growth or persistence of these pathogens.

The occasional occurrence of enterococci in abstracted water in the dune area may be the result of the transport of microorganisms from top soil towards the abstraction

wells. Rewetting of the contaminated soil (heavy rain or high groundwater level) may promote their survival and transport in soil. Also, stopping the abstraction of groundwater results in higher groundwater levels where the groundwater level may even reach the surface. Since the transport of microorganisms through saturated soil is faster than through the unsaturated zone, this would increase the probability of the presence of enterococci and other microorganisms in abstracted water. From this perspective, it would be desirable to keep the groundwater level in the abstraction area as low as possible.

Among other micro-organisms, species belonging to the *Enterococcus* genus have been used as an indicator of faecal contamination by humans and warm-blooded animals. The standard method (ISO 7899-2:2000) has been used to detect species belonging to genus *Enterococcus* in water. As mentioned earlier, this method in not highly specific, as occasionally also other genera are detected using this method. Identification techniques, such as MALDI-TOF MS can be applied as a rapid confirmation and identification test for *Enterococcus*. The number of newly described *Enterococcus* species increases rapidly and testing for their presence in faecal matter of many different potential hosts lags behind. Consequently, each discovery of a novel *Enterococcus* species isolated from non-faecal sources creates the possibility to bring its suitability as a faecal indicator into question. However, forming judgement on the suitability of particular species should also take into account the possibility that newly described *Enterococcus* species have been isolated from faecal matter previously, but were identified as a different species, possibly belonging to the group named 'intestinal'(*E. faecium*, *E. faecalis*, *E. hirae*, *E. durans*) of which the suitability as faecal indicator is not doubtful. As the faecal source of *E. moraviensis* and *E. haemoperoxidus* in which human pathogens may be present has been demonstrated, this suggests the importance of extreme caution when enterococci (including *E. moraviensis*) are detected in drinking water distribution systems. As the growth of enterococci on the plant material from the Castricum dune infiltration area is very limited, enterococci (including *E. moraviensis*) are still sensitive indicators of faecal contamination. *E. moraviensis* also points towards the contamination source as they were frequently isolated from geese droppings, and indicated the route of faecal contamination of the abstracted groundwater. If *E. moraviensis* can reach the abstracted water, it is possible that also other micro-organisms (bacteria and especially viruses) can reach abstracted water via the same route. In addition, *E. moraviensis* might be used as a model organism in further investigations on the removal capacity of the vertical route (from topsoil towards abstracted water) for different microorganisms.

The results of this study encouraged preventive measures being taken in direct vicinity of the abstraction wells and call for a multidisciplinary approach in the management of these areas, to meet the needs of nature management as well as the needs of safe drinking water production (see below).

Predation

When faecal droppings containing enterococci and other FIB and potentially enteric pathogenic bacteria are deposited on the ground, the conditions change and organisms adapted to the intestinal tract of warm-blooded animals tend to die-off. Several physical factors like temperature, drought, UV (sunlight), etc. cause or affect the reduction of bacterial numbers in faecal droppings. An element that is not well studied is that this reduction can also be caused by predation by protozoa. Our study described in Chapter 6 confirmed the presence of cellular slime moulds (dictyostelids) in both soil and faeces obtained from an infiltration area. The ability of *Dictyostelium mucoroides* to feed on several species of indicator bacteria (*E. coli*, *Clostridium bifermentans* and five species of *Enterococcus*) was demonstrated in feeding experiments. In order to evaluate the possible impact of these grazers on the microbial abundance in soil and faeces, the growth rate of *D. mucoroides* was determined using a *Pseudomonas fluorescens* culture as food. It was found that *D. mucoroides* is able to grow within three days to 1.6×10^5 amoebae in a single test area of c. 10 cm^2, which indicates that cellular slime moulds may have a considerable influence on the bacterial numbers in both soil and faecal droppings. In our study, the sorocarps formed in the tests using the *C. bifermentans* strain as food were smaller compared to the other tested bacterial species. The explanation of this result may be the predilection of *D. mucoroides* for the prey with certain morphology and that *C. bifermentans* is not the preferred food of *D. mucoroides*. The characteristics of prey such as cell wall morphology and the physiological state were earlier observed to influence the magnitude of protozoan grazing in water ecosystems (Beardsley et al., 2003; Gonzalez et al., 1990; Matz et al., 2002; Simek et al., 1994; Verity, 1991). A better understanding of the predilection of different types of terrestrial protozoa for particular organisms is needed to estimate the magnitude of grazing FIB but also for pathogens in faeces and soil. Further investigations are also needed to determine whether the magnitude of protozoan grazing in faeces and soil is temperature dependent. For protozoa grazing in aquatic ecosystems, a direct correlation between rates of predation and temperature was found. Higher rates of grazing and an increase in protozoan concentrations were observed at higher temperatures (An et al, 2002; Anderson et al., 1983; Barcina et al., 1991; McCambridge & McMeekin, 1980; Sherr et al., 1988). The

factors determining the magnitude of grazing by protozoa in terrestrial ecosystems are not fully understood, but our study indicated that these organisms have the potential to considerably reduce the numbers of FIB and pathogens in faeces and soil. Therefore, it is suggested to characterize this activity of cellular slime moulds in more detail and to evaluate both their significance as a "pathogen barrier" and their place in microbial risk assessment. Further investigations on protozoan grazing in soil might include the activity of protozoa in different soil layers. If protozoa are most active in the rhizosphere and if the effect of grazing decreases with the depth, then the microorganisms that have penetrated deeper into the soil have more chance to survive. When the activity of cellular slime moulds is neglected, the load of faecal contamination in the vicinity of the abstraction wells may be overestimated in the risk assessments applied by drinking water companies. This is of particular relevance to shallow abstraction wells, where contamination routes from topsoil to the abstracted water can occur. However, further experiments are needed to estimate the magnitude of the reduction of FIB numbers in faeces due to cellular slime mould activity under different conditions.

The significance of ampicillin and vancomycin resistance in enterococci isolated from water

Beside their role as faecal indicators, enterococci have been associated with infections in humans. Though enterococci generally display low levels of virulence (Arias & Murray, 2012), they have emerged as one of the leading causes of healthcare-associated infections in recent decades. This trend has been attributed to increasing resistance to antibiotics (Heimer et al., 2015). Antibiotic resistance (ABR) in general has become a serious public health concern. Today, 700,000 people die of resistant infections every year and without policies to stop the spread of ABR, the estimations for 2050 are 10 million mortalities per year, which is more than currently die from cancer (O'Neill, 2016). Beside the impact on human health, ABR also has a large economic cost. For the US, it has been estimated that the annual cost of ABR is 55 billion dollars, however these estimates have been challenged and indicated as low (Smith & Coast, 2013). The genus *Enterococcus* includes some of the most important nosocomial multidrug-resistant organisms, and these pathogens usually affect patients who are debilitated by other, concurrent illnesses and undergoing prolonged hospitalization (Arias & Murray, 2012). The emergence of clinical enterococcal isolates that are resistant to both ampicillin (ARE) and vancomycin (VRE) is a great concern. VRE infections lead to increased morbidity and mortality (Malathum & Murray, 1999) as therapeutic alternatives for the treatment of infections caused by such organisms are becoming limited. At present,

only two compounds have been approved by the Food and Drug Administration for the treatment of ampicillin-resistant VRE infections, linezoid and quinupristin/dalfopristin, both reporting 65% overall success rates (Heimer et al., 2015). Infections with linezolid-resistant VRE strains have been reported, with *Enterococcus faecium* being the species most commonly associated with these cases (Gonzales et al. 2001; Bersos et al., 2004; Bae et al., 2006; De Almeida et al., 2014). Additionally, the potential for enterococci to transmit resistance genes, including vancomycin resistance, to other more pathogenic organisms such as *Staphylococcus aureus* (Chang et al., 2003), makes it of paramount importance to limit the dissemination of these resistant strains (Heimer et al., 2015). Compared to the very large number of studies of enterococci of human origin, there are only limited studies on occurrence of antimicrobial resistance and resistance genes among enterococci isolated from animals, food, or the environment (Aarestrup *et al*., 2002). The relationship between the non-therapeutic use of antibiotics and the occurrence of enterococci in various non-human habitats is of substantial interest (Lebreton et al., 2014).

Aquatic environments could play a role in the dispersion of antibiotic resistant enterococci. The co-mixture of antibiotics, antibiotic resistance genes, bacteria carrying these genes and other indigenous bacteria in environments provide an ideal opportunity for antibiotic-resistance genes to develop and disseminate among the bacterial community (Wellington et al. 2013). These bacteria and antibiotic-resistance genes can be dispersed or released through runoff directly into receiving surface waters. The correlation has been shown wherever antibiotics are spread in this manner, resistant bacteria follow the same route of dispersal (Baquero et al. 2008). These types of water bodies are likely to be hotspots where antibiotic-resistance genes proliferate and new resistant strains or pathogens are formed through horizontal gene transfer (Seyedmonir et al. 2015). Understanding the role of such environments in the dissemination of antibiotic-resistance genes is essential to combat the rising threat of antibiotic resistance effectively (Berglund 2015; Nakipoğlu et al. 2016).

In the study described in Chapter 7, we investigated the presence of ampicillin- (ARE) and vancomycin-resistant enterococci (VRE) in the effluent of six (secondary) wastewater treatment plants (WWTPs) and in receiving surface waters that are used as a source for drinking water production in The Netherlands. The use of ampicillin SBA (Slanetz & Bartley agar modified with 16 µg ml^{-1} ampicillin) has been demonstrated as a suitable screening method for ARE in water. Screening of VRE on vancomycin

SBA was not feasible; *Leuconostoc* spp. *Pediococcus* spp. and *Lactobacillus* spp. are intrinsically resistant to vancomycin (Nicas et al., 1989) and are commonly found in surface waters. They also grow on SBA and as a result they emerge as false positives on the SBA-medium complemented with vancomycin. The MALDI-TOF MS served as a screening tool for the confirmation and speciation of presumptive enterococci colonies on ampicillin SBA and vancomycin SBA. Relatively high concentrations of ampicillin resistant isolates ($0.4x10^3$ to $4.4x10^3$ cfu L^{-1}) were found, but no clinically important VRE were found in WWTP effluents. At intake locations used for drinking water production ARE and VRE were not detected, which indicates that dilution and inactivation reduce the concentrations of ARE discharged via WWTP into the aquatic environment. Ampicillin-resistant *E. faecium* (ARE*fm*) was the only ARE species observed in this study, which is in line with previous investigations by Anastasiou & Schmitt (2011), who found that a high percentage (88–100%) of ARE present in surface water was ARE*fm*. Considering that ARE have emerged in Europe as a frequent cause of invasive enterococcal infections even in countries with a low prevalence of VRE, such as The Netherlands (Top et al., 2007; 2008), the numbers of ARE*fm* in WWTP effluent observed in this study indicate a reservoir of ARE*fm* outside the healthcare setting. To confirm that the ARE*fm* found in this study are hospital-associated *E. faecium* (HA-E*fm*), additional analysis on the presence of enterococcal surface protein (*esp*) gene can provide additional information. HA-E*fm* isolates are genotypically distinct from community-derived human or animal strains, and the majority of HA-E*fm* has been reported to contain the *esp* gene, which encodes for an enterococcal surface protein (Anastasiou & Schmitt, 2011; Leavis et al., 2004). The presence of the *esp* gene was reported previously by Anastasiou & Schmitt (2011) in other WWTP effluents in The Netherlands. The vancomycin Minimum Inhibitory Concentrations (MICs) of ARE*fm* observed in our study were low ($2 - 8$ μg ml^{-1}), but exceed the maximum previously observed (Blaak et al., 2010, 2011) in surface water in The Netherlands. Compared with the results obtained in Portugal and the USA, where VRE were found in the effluent of WWTPs (da Costa et al., 2006; Rosenberg Goldstein et al., 2014; Young et al., 2016), the vancomycin resistance levels found in this study are low. Because little is known about the acquisition or transfer of antibiotic resistance in enterococci that are discharged into the aquatic environment, the understanding of the mechanisms regulating low-level vancomycin resistance in the ARE*fm* strains found in this study is incomplete. The absence of high-level *vanA* and *vanB* resistance genes indicates that the strains found in water do not belong to the clinically most important genotypes. However, relatively high numbers of *E. faecium* with a combination of ampicillin resistance and low vancomycin

resistance may be clinically significant and need further investigation. This study confirmed earlier findings that antibiotic resistant bacteria can reach surface waters via WWTPs. The biological treatment process in conventional WWTPs may result in a selective increase in the antibiotic-resistant bacteria population and the increased occurrence of multidrug resistant bacteria (Zhang et al., 2009). Although mechanisms that contribute to a selective increase in antibiotic resistant bacteria in WWTPs remain undefined, a number of studies have shown that the conditions in WWTPs favour antibiotic-resistant bacteria (Iwane et al., 2001; Schwartz et al., 2003). Possible low numbers of resistant strains at the intake locations and a high level of bacterial removal during the drinking water production process, make the concern about the use of surface water at intake locations regarding drinking water production in The Netherlands low. However, it is desirable to carry out further monitoring to evaluate the numbers of and possible increase in antibiotic resistance levels in enterococci and other clinically relevant microorganisms, as well as the occurrence of gene transfer, in WWTP effluents and surface waters used for irrigation or recreation.

RESEARCH NEEDS

There is a need to further evaluate the risk to human health associated with faecal contamination by specific wild animals. Field evaluations should evaluate the zoonotic potential and survival (or lack thereof) of pathogens such as *Salmonella*, *Shigella*, *Campylobacter*, *Giardia*, and *Cryptosporidium*. Since the FIB such as enterococci are still widely used as indicators of microbial (un)safety of water, the relationship between (species of) enterococci and pathogens in these sources and environments also needs to be characterized.

Future research initiatives may focus on the use of genetic techniques for the evaluation of enterococci populations recovered from the soil to determine if these populations are clonal. In case enterococci are presumed to be a result of growth in a non-enteric environment, such techniques may provide better insight into the genetic diversity among different isolates belonging to the same species. Such information might help to better understand the occurrence and fate of enterococci in non-enteric environments.

The results presented in this thesis suggest that the occasional detection of enterococci in abstracted water is likely to be the result of breakthrough of the microorganisms from

geese droppings on the topsoil, however actual routes are not fully understood and need to be examined. Studying these routes may demonstrate the extent of removal of enterococci during vertical soil passage. Breakthrough of enterococci indicates that other (pathogenic) bacteria from droppings in the vicinity of the abstraction wells may also occasionally reach the abstracted water.

The reliability of identification using MALDI-TOF MS depends mainly on the number of mass spectra belonging to each individual species. The extension of MALDI-TOF MS database with more environmental strains of FIB is needed to increase the reliability of the identification of environmental strains. As the number on the newly described *Enterococcus* species is still increasing, it is important to add those to the database.

The faecal contamination load delivered by the geese in the vicinity of the abstraction wells called for measures to keep geese away from these vulnerable parts of the dune filtration area. In the winter of 2015 and 2016 these parts of the area have been selected and fenced in by nets. In this two year period no enterococci have been observed in water abstracted from this area, which strengthen the hypothesis that the contamination route from the top soil towards the abstracted water exists. It is therefore recommended to continue to use fencing nets for at least 4 years and also to continue with large volume sampling of abstracted water in order to confirm the effectiveness of this preventive measure.

Because it has been demonstrated that *E. moraviensis* is able to grow on plant material and because geese feed on plants, growth of this indicator might also occur in geese faeces, consisting of poorly digested grass leaves. Applications of new techniques like whole genome sequencing might facilitate the estimation of risks to human health if they could determine the difference in genetic characteristics between the isolates grown under enteric or environmental conditions (environmental growth).

The investigation of the ARE and VRE has demonstrated a reservoir of ampicillin resistant *E. faecium* (ARE*fm*) outside the healthcare setting. Because some of ARE*fm* isolates have also shown low resistance to vancomycin (2-8 $\mu g ml^{-1}$), there is a need to study mechanisms like gene transfer between bacteria belonging to the same species or between more distantly related bacteria present in water. These may regulate low-level vancomycin resistance in ARE*fm*. As these mechanisms are not well understood, further monitoring of the extent of vancomycin resistance in ARE*fm* entering surface

waters at WWTP's, in recreational waters and at the intake locations used for drinking water production is desirable. The monitoring of other types of antibiotic resistance in bacteria isolated from surface waters, such as Extended Spectrum Beta-Lactamase (ESBL) and carbapenemase producing Enterobacteriaceae is of similar importance.

Practical implications for the drinking water supply companies

1. Source and health significance of contamination can be and was identified

Our studies showed that *E. moraviensis* occurs in faecal droppings of geese, foxes and rabbits, *E. haemoperoxidus* occurs in droppings of geese. Finding these *Enterococcus* species in water samples should therefore be regarded as indication of faecal contamination. Since geese have also been shown to carry and excrete zoonotic, enteric pathogens, these also indicate a potential risk to health. Our studies also showed how typing of the enterococci from water samples and potential contamination sources may help to identify the origin of faecal contamination events. The faecal source of the most frequently isolated *Enterococcus* species (*E. moraviensis*) in abstracted water (after dune passage) in the Castricum dune infiltration was identified with a high probability. The results show that geese from the infiltration area are carriers of *E. moraviensis*, as well as being carriers of other enterococci species. It has been suggested that the geese faecal material may reach the abstracted water. That implies that also pathogens that may be present in (geese) droppings may occasionally reach the abstracted water. The occurrence of *E. moraviensis* may therefore indicate the possible presence of pathogens; its role as an indicator of faecal contamination should not be ignored.

2. Control measure was shown to be effective

These findings encouraged the design of preventive measures (fencing off vulnerable parts of the Castricum dune infiltration area) to reduce the risk of faecal contamination of the abstracted water. The absence of contamination of abstracted water after these measures were implemented suggests that these measures were effective. However, it is desirable to continue with fencing in following years to further test its effectiveness.

3. Environmental growth of enterococci was not significant in the study site

Enterococci are able to grow on plant material. However, the growth of enterococci in the environment is limited (and the inactivation intensified) by the competition for nutrients with the indigenous microbial community and by the predation of protozoa. These findings, and those demonstrating that *E. moraviensis* was not able to multiply

in the habitats present in the dune passage process (sediment from abstraction wells, biofilm developed using abstracted water and soil), indicate that the growth of enterococci was not a significant factor in enterococci occurrence in water in these environments. This implies that bacteria from the topsoil are able to reach the ground water through a relatively short unsaturated and saturated zone. The barrier function of the short soil passage is therefore limited, so it is important to avoid the presence of faecal droppings from animals (and sometimes maybe humans) in the direct vicinity of the abstraction wells and to assure low levels of groundwater.

4. Contamination events can be confirmed rapidly

The MALDI-TOF MS is a rapid identification method with many advantages, even though the purchase costs are high. A valuable benefit of reliable identification of enterococci in water samples is the possibility to avoid false positive test results, such as detection of some *Aerococcus viridans* strains, which are common in the environment, do not indicate faecal contamination, but are also bile-esculin positive (confirmation test according to ISO 7899-2:2000), and can therefore be confused with *Enterococcus* spp. in water testing. Validation and application of the system for the enterococci typing initiated the validation of this system also for the rapid identification of other faecal bacteria (*E. coli*, *Campylobacter* spp.) and other micro-organisms like *Legionella* spp., which accelerated the confirmation of these organisms and therefore the availability of confirmed test results for at least 24 hours. Besides rapid identification there are further applications of MALDI-TOF MS, which are more suitable for research purposes than for routine use. When enterococci and coliforms are detected in water samples, the identification of species may provide some additional information on the contamination event. The evaluation of the variety and incidence of species detected at different sampling points can be used as a source-tracking tool. Also, such evaluations may contribute to a better understanding of the microbial ecology and the role of particular species as faecal indicators.

5. A simple screening method for antibiotic-resistant enterococci showed presence in effluent of wastewater treatment plants, but absence at intake of drinking water supplies. Vigilance is required.

In chapter 7 it has been shown that antibiotic-resistant enterococci can enter the surface waters via the WWTP effluents. Relatively high numbers of *E. faecium* with a combination of ampicillin resistance and low vancomycin resistance were found in these effluents. There may be evolution towards clinical significance of these

strains, whereby the level of resistance may increase over time, and this needs further investigation. Monitoring potential evolution and spread of vancomycin resistance in antibiotic-resistant *E. faecium* entering surface waters is needed. Even though the drinking-water treatment systems in The Netherlands are capable of removing or inactivating ampicillin- and vancomycin-resistant enterococci if present in surface water, it is important to monitor the presence of clinically significant *Enterococcus* strains in surface water used for recreation or irrigation and to understand whether resistance genes may be transferred and remain present in aquatic and water treatment environments.

References

Aarestrup FM, Butaye P, Witte W. 2002. Non-human reservoirs of enterococci. *In*. Gilmore MS, Clewell DB, Courvalin P, Dunny GM, Murray BE, Rice LB (Eds.), The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance (pp. 55-100). Washington, District of Columbia: ASM Press.

An YJ, Kampbell DH, Breidenbach GP. 2002. *Escherichia coli* and total coliforms in water and sediments at lake marinas. *Environ. Pollut*. 120:771–778.

Anastasiou I and Schmitt H. 2011. Hospital-associated *Enterococcus faecium* in the Water Chain. Rhine Water Works The Netherlands.

Anderson IC, Rhodes MW, Kator HI. 1983. Seasonal variation in survival of *Escherichia coli* exposed in situ in membrane diffusion chambers containing filtered and nonfiltered estuarine water. *Appl. Environ. Microbiol*. 45:1877–1883.

Angus KW. 1990. Cryptosporidiosis in ruminants. In: Dubey JP, Speer CA, Fayer R (eds.). Cryptosporidiosis of man and animals. CRC Press, Boca Raton, USA. pp. 83-104.

Anonymous. 2000. ISO 7899-2:2000. Water quality -- Detection and enumeration of intestinal enterococci -- Part 2: Membrane filtration method. Geneva, Switzerland.

Arias CA and Murray BE. 2012. The rise of the *Enterococcus*: beyond vancomycin resistance. *Nature Reviews. Microbiology*, 10(4), 266–278.

Badgley BD, Thomas FIM, Harwood VJ. 2010. The effects of submerged aquatic vegetation on the persistence of environmental populations of *Enterococcus* spp. *Environ. Microbiol*. 42:1271–1281.

Bae HG, Sung H, Kim MN, Lee EJ, Koo Lee S. 2006. First report of a linezolid- and vancomycin-resistant *Enterococcus faecium* strain in Korea. *Scand. J. Infect. Dis*. 38:383–386.

Baquero F, Martinez JL, Canton R. 2008. Antibiotics and antibiotic resistance in water environments. *Curr. Opin. Biotechnol*. 19:260–265.

Barcina I, Gonzalez JM, Iriberri J, Egea L. 1991. Role of protozoa in the regulation of enteric bacteria populations in seawater. *Mar. Microb. Food Webs* 5:179–188.

Beardsley C, Pernthaler J, Wosniok W, Amann R. 2003. Are readily culturable bacteria in coastal North Sea waters suppressed by selective grazing mortality? *Appl. Environ. Microbiol*. 69: 2624–2630.

Berglund B. 2015. Environmental dissemination of antibiotic resistance genes and correlation to anthropogenic contamination with antibiotics. *Infect. Ecol. Epidemiol*. 8(5):28564.

Bersos Z, Maniati M, Kontos F, Petinaki E, Maniatis AN. 2004. First report of a linezolid-resistant vancomycin-resistant *Enterococcus faecium* strain in Greece. *J. Antimicrob. Chemother*. 53:685–686.

Blaak H, Schets FM, Italiaander R, Schmitt H, de Roda Husman AM. 2010. Antibioticaresistente bacteriën in Nederlands oppervlaktewater in veeteeltrijk gebied. RIVM rapport 703719031. (in Dutch)

Blaak H, van Rooijen SR, Schuijt MS, Docters van Leeuwen AE, Italiaander R, van den Berg, HHJL, Lodder-Verschoor F, Schets FM, de Roda Husman AM. 2011. Prevalence of Antibiotic Resistant Bacteria in the Rivers Meuse Rhine and New Meuse. National Institute for Public Health and the Environment.

Byappanahalli MN, Shively DA, Nevers MB, Sadowsky MJ, Whitman RL. 2003. Growth and survival of *Escherichia coli* and enterococci populations in the macro-alga *Cladophora* (Chlorophyta). *FEMS Microbiol. Ecol*. 46(2):203–211.

Byappanahalli MN, Sawdey R, Ishii S, Shively DA, Ferguson JA, Whitman RL, Sadowsky M J. 2009. Seasonal stability of *Cladophora*-associated *Salmonella* in Lake Michigan watersheds. *Water Res*. 43:806–814.

Centres for Disease Control and Prevention. 1999. Outbreak of *E. coli* O157:H7 and *Campylobacter*—New York, 1999. *Morb. Mortal. Wkly. Rep*. 48:803-4.

Chang S, Sievert DM, Hageman JC, Boulton ML, Tenover FC, Downes FP, Shah S, Rudrik JT, Pupp GR, Brown WJ, Cardo D, Fridkin SK. 2003. Infection with vancomycin-resistant *Staphylococcus aureus* containing the vanA resistance gene. *N. Engl. J. Med*. 348(14):1342-1347.

Craun GF, Brunkard JM, Yoder J S, Roberts VA, Carpenter J, Wade T, Calderon RL, Roberts JM, Beach M J, Roy SL. 2010. Causes of Outbreaks Associated with Drinking Water in the United States from 1971 to 2006. *Clin. Microbial. Rev.* 23(3): 507–528.

Curriero FC, Patz JA, Rose JB, Lele S. 2001. The association between extreme precipitation and waterborne disease outbreaks in the United States, 1948–1994. *Am. J. Publ. Hlth*. 91(8):1194–1199.

da Costa PM, Vaz-Pires P, Bernardo F. 2006. Antimicrobial resistance in *Enterococcus* spp. isolated in inflow, effluent and sludge from municipal sewage water treatment plants. *Water Res*. 40:1735–1740.

De Almeida LM, de Araújo MRE, Iwasaki MF, Sacramento AG, Rocha D, da Silva LP, Pavez M, de Brito AC, Ito LCS, Gales AC, Lincopan N, Sampaio JLM, Mamizukaa EM. 2014. Linezolid Resistance in Vancomycin-Resistant *Enterococcus faecalis* and *Enterococcus faecium* Isolates in a Brazilian Hospital. *Antimicrobial Agents and Chemotherapy*. 58(5):2993-2994.

Desmarais, TR, Solo-Gabriele HM, Palmer CJ. 2002. Influence of soil on fecal indicator organisms in a tidally influenced subtropical environment. *Appl. Environ. Microbiol.* 68(3): 1165-1172.

Devriese LA, Pot B, Collins MD. 1993. Phenotypic identification of the genus *Enterococcus* and differentiation of phylogenetically distinct enterococcal species and species group. *J. Appl. Bacteriol*. 75:399–408.

Feare CJ, Sanders MF, Blasco R, Bishop JD. 1999. Canada goose (*Branta canadensis*) droppings as a potential source of pathogenic bacteria. *The journal of the Royal Society for the Promotion of Health*, 119(3):146-155.

Gonzalez JM, Sherr EB, Sherr BF. 1990. Size-selective grazing on bacteria by natural assemblages of estuarine flagellates and ciliates. *Appl. Environ. Microbiol*. 56:583–589.

Gonzales RD, Schreckenberger PC, Graham MB, Kelkar S, DenBesten K, Quinn JP. 2001. Infections due to vancomycin-resistant *Enterococcus faecium* resistant to linezolid. *Lancet* 357:1179.

Graczyk TK, Fayer R, Trout JM, Lewis EJ, Farley CA, Sulaiman I, Lal AA. 1998. *Giardia* sp. cysts and infectious *Cryptosporidium parvum* oocysts in the feces of migratory Canada geese (*Branta canadensis*). *Appl. Environ. Microbiol*. 64: 2736–2738.

Heimer SR, Morrison D, Gilmore MS. 2015. Chapter 39 The Enterococci. In: Molecular Medical Microbiology. DOI: http://dx.doi.org/10.1016/B978-0-12-397169-2.00039-1.

Hrudey SE, Payment P, Huck PM, Gillham RW, Hrudey EJ. 2003. A fatal waterborne disease epidemic in Walkerton, Ontario: comparison with other waterborne outbreaks in the developed world. *Water. Sci. Technol.* 47(3):7-14.

Iwane T, Urase T, Yamamoto K, 2001. Possible impact of treated wastewater discharge on incidence of antibiotic resistant bacteria in river water. *Water Sci. Technol*. 43:91–99.

Koplan JP, Deen RD, Swanston WH, Tota B. 1978. Contaminated roof-collected rainwater as a possible cause of an outbreak of salmonellosis. *J of Hyg*. 81(2):303-309.

Leavis H, Top J, Shankar N, Borgen K, Bonten M, van Embden J, Willems RJ. 2004. A novel putative enterococcal pathogenicity island linked to the Esp virulence gene of *Enterococcus faecium* and associated with epidemicity. *J. Bacteriol*. 186:672–682.

Lebreton F, Willems RJL, Gilmore MS. 2016. *Enterococcus* Diversity, Origins in Nature, and Gut Colonization in Enterococci: From Commensals to Leading Causes of Drug Resistant Infection (Internet, November, 2016).

Leclerc H, Devriese LA, Mossel DAA. 1996. Taxonomical changes in intestinal (faecal) enterococci and streptococci: consequences on their use as indicators of faecal contamination in drinking water. *J. Appl. Bacteriol*. 81:459–466.

Malathum K and Murray BE. 1999. Vancomycin-resistant enterococci: recent advances in genetics, epidemiology and therapeutic options. *Drug Resist. Updates* 2:224-243.

Matz C, Boenigk J, Arndt H, Jurgens K. 2002. Role of bacterial phenotypic traits in selective feeding of the heterotrophic nanoflagellate *Spumella* sp. *Aquat. Microb. Ecol*. 27:137–148.

McCambridge J and McMeekin TA. 1980. Relative effects of bacterial and protozoan predators on survival of Escherichia coli in estuarine water samples. *Appl. Environ. Microbiol*. 40:907–911.

Medema GJ, Ketelaars H, de Bruin J, Kruidenier L, Engels G, de Bruin R. 1999. The significance of wildlife as source of *Cryptosporidium* and *Giardia* in pre-treatment reservoirs. In *Cryptosporidium* and *Giardia*: new challenges to the water industry. *PhD Thesis*. Utrecht University. pp. 87-98.

Moriarty EM, Karki N, Mackenzie M, Sinton LW, Wood DR, Gilpin BJ. 2011.

Faecal indicators and pathogens in selected New Zealand waterfowl. *New Zealand Journal of Marine and Freshwater Research*, 45(4):679-688.

Nakipoğlu M, Yilmaz F, Icgen B. 2016. *van*A Gene Harboring Enterococcal and Non-enterococcal Isolates Expressing High Level Vancomycin and Teicoplanin Resistance Reservoired in Surface Waters. *Bull. Environ. Contam. Toxicol*. https://dx.doi.org/10.1007/s00128-016-1955-8

Nicas TI, Cole CT, Preston DA, Schabel AA, Nagarajan R. 1989. Activity of glycopeptides against vancomycin-resistant gram-positive bacteria. *Antimicrobial agents and chemotherapy*, 33(9):1477-1481.

Olsen SJ, Miller G, Breuer T, et al. 2002. A Waterborne Outbreak of *Escherichia coli* O157:H7 Infections and Hemolytic Uremic Syndrome: Implications for Rural Water Systems. Emerging Infectious Diseases. 8(4):370-375.

O'Neill J. (Chair). 2016. The review on antimicrobial resistance: Tackling drug-resistant infections globally. Final report and recommendations. May 2016.

Palmer SR, Gully PR, White JM, Pearson AD, Suckling WG, Jones DM, Rawes JCL, Penner JL. 1983. Water-borne outbreak of *Campylobacter* gastroenteritis. *The Lancet*. 1(8319):287-290.

Pinto B, Pierotti R, Canale G, Reali D.1999. Characterization of "faecal streptococci" as indicators of faecal pollution and distribution in the environment. *Lett. Appl. Microbiol*. 29:258–263.

Rosenberg Goldstein RE, Micallef SA, Gibbs SG, George A, Claye E, Sapkota A, Joseph SW, Sapkota AR. 2014. Detection of vancomycin-resistant enterococci (VRE) at four U.S. wastewater treatment plants that provide effluent for reuse. *Sci. Total Environ*. 466–467:(404–411).

Schwartz T, Kohnen W, Jansen B, Obst U. 2003. Detection of antibiotic-resistant bacteria and their resistance genes in wastewater surface water, and drinking water biofilms. *FEMS Microbiol. Ecol*. 43: 325–335.

Seyedmonir E, Yilmaz F, Icgen B. 2015. *mec*A gene dissemination among staphylococcal and non-staphylococcal isolates shed in surface waters. *Bull. Environ. Contam. Toxicol*. 95:131–138.

Sherr BF, Sherr EB, Rassoulzadegan F. 1988. Rates of digestion of bacteria by marine phagotrophic protozoa: temperature dependence. *Appl. Environ. Microbiol*. 54:1091–1095.

Simek K, Vrba J, Hartman P. 1994. Size-selective feeding by *Cyclidium* sp on bacterioplankton and various sizes of cultured bacteria. *FEMS Microbiol. Ecol*. 14:157–167.

Smith R and Coast J. 2013. The true cost of antimicrobial resistance. BMJ (Clinical research ed), 346. f1493. ISSN 0959-8138 DOI: 10.1136/bmj.f1493.

Top J, Willems R, Blok H, De Regt M, Jalink K, Troelstra A, Goorhuis B, Bonten M. 2007. Ecological replacement of *Enterococcus faecalis* by multiresistant clonal complex 17 *Enterococcus faecium*. *Clin. Microbiol. Infect*. 13(3):316–319.

Top J, Willems R, van der Velden S, Asbroek M, Bonten M. 2008. Emergence of clonal complex 17 *Enterococcus faecium* in The Netherlands. *J. Clin. Microbiol*. 46(1):214–219.

Verity PG. 1991. Feeding in planktonic protozoans—evidence for nonrandom acquisition of prey. *J. Protozool*. 38:69–76.

Wellington EM, Boxall AB, Cross P, Feil EJ, Gaze WH, Hawkey PM, Johnson-Rollings AS, Jones DL, Lee NM, Otten W, Thomas CM, Williams AP. 2013. The role of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria. *Lancet Infect. Dis*. 13:155–165.

Whitman RL, Shively DA, Pawlik H, Nevers MB, Byappanahalli MN. 2003. Occurrence of *Escherichia coli* and enterococci in *Cladophora* (Chlorophyta) in nearshore water and beach sand of Lake Michigan. *Appl. Environ. Microbiol*. 69:4714–4719.

Young S, Nayak B, Sun S, Badgley B, Rohr J, Harwood VJ. 2016. Vancomycin resistant enterococci and bacterial community structure following a sewage spill into an aquatic environment. *Appl. Environ. Microbiol*. doi:10.1128/AEM.01927-16.

Zhang Y, Marrs CF, Simon C, Xi C. 2009. Wastewater treatment contributes to selective increase of antibiotic resistance among *Acinetobacter* spp. *Sci. Total Environ*. 407:3702–3706.

Zhou L, Kassa H, Tischler ML, Xiao L. 2004. Host adapted *Cryptosporidium* spp. in Canada geese (*Branta canadensis*). *Appl. Environ. Microbiol*. 70:4211-4215.

Summary

Testing the microbiological quality of water for the presence of a broad spectrum of human pathogens would be a time-consuming and an expensive task. Therefore, the detection of indicator microorganisms has been applied, using relatively fast and inexpensive methods, based on the principle that faecal contaminated water may also contain water borne pathogens, which may cause an outbreak of illness in humans, where unboiled water is consumed. Beside *E. coli*, other bacteria like coliforms and enterococci, which commensally inhabit the intestinal tract of humans and warm-blooded animals, have also been used as indicators of faecal contamination in water. Enterococci are also frequently detected in soil and water. Their persistence and growth under certain extra-enteric conditions may confound their use as indicator of actual risk to public health. Enterococci have been demonstrated as agents of human illnesses and nosocomial infections and there is growing concern regarding the incidence of antibiotic resistance in enterococci and the resistant genes in the environment. Aquatic environments may play a role in the dispersion of antibiotic-resistant strains in the environment. The investigations described in this thesis focused on a better understanding of the sources and fate of enterococci in the environment, in order to evaluate their role as indicators of faecal pollution in drinking water production processes and to examine the potential reservoir of clinically relevant antibiotic-resistant enterococci in treated waste water, and surface waters used for the production of drinking water in The Netherlands. In comparison with biochemical and molecular techniques, Matrix Assisted Laser Desorption Ionisation-Time of Flight Mass Spectrometry (MALDI-TOF MS) has been shown as a reliable and efficient technique for the identification of *Enterococcus* species isolated from surface water, processed water and drinking water samples (Chapter 2). Therefore, MALDI-TOF MS has been applied for the identification of enterococci in large volume (100 L) samples, occasionally isolated from abstracted water (after dune passage) in the Castricum dune infiltration area. *E. moraviensis* was the most frequently observed species in these samples. Because faecal contamination of abstracted water was considered unlikely and the faecal sources of *E. moraviensis* have not been reported yet, research has further focused on the source tracking of this species in the infiltration area and on the growth abilities of this species under certain non-enteric conditions. Source tracking of *E. moraviensis* in faecal samples of animals living in the vicinity of abstraction wells and in human faecal samples revealed that *E. moraviensis* was frequently present in geese droppings. Geese were found to be the only carriers of *E. haemoperoxidus*, which was also observed in the abstracted water whereby the faecal source has not been yet demonstrated. These results suggest that high incidence of particular *Enterococcus*

species may indicate the specific type of pollution, in this case emanating from geese. Because the species composition of *Enterococcus* spp. isolated from all the abstracted water samples was similar to the composition found in the geese droppings and because a sanitary survey showed that geese provide a substantial faecal load in the vicinity of the abstraction wells, it has been suggested that geese may influence the quality of abstracted water in these parts. According to the literature, geese faeces may also contain human pathogens, this supports the significance of *E. moraviensis* and *E. haemoperoxidus* as faecal indicators (Chapter 5). Another study demonstrated that *E. moraviensis* was not able to grow in habitats present in the dune passage process (sediment from the abstraction wells, biofilm developed using abstracted water and soil) but filtered plant extracts obtained from dune vegetation supported growth of *E. moraviensis* up to 6 log-units (Chapter 3). These results led to further experiments, where the extent of growth on filtered plant extract was compared for *E. moraviensis*, *E. casseliflavus*, *E. hirae*, *E. faecalis* and *E. faecium*. When the major part of the microbial community was removed from the plant extract, a distinct nutrient dependent growth was observed in all five species. However, in presence of the natural microbial community, only limited or no growth was observed. High concentration of the plant extract supported initial growth of *E. moraviensis*, which was not the case in the other species studied. For *E. faecium*, the reduced ability to grow on plant extract has been observed in both experiments. It is considered that competition for the available nutrients hindered the replication and persistence of enterococci in this experiments, which may also be the situation in the environment (Chapter 4). Furthermore, *Dictyostelium mucoroides* (a species of cellular slime moulds) isolated from soil and dung collected in the infiltration area, has been shown to be able to consume different faecal indicator bacteria (FIB). Laboratory experiments showed a high reproductive capacity. Their bacterial consumption and reproduction rate imply a potentially significant reduction of bacterial numbers, including FIB and possibly pathogens in soil and faeces (Chapter 6). It has been concluded that there are pathways which might be involved in the transport of enterococci from the topsoil towards abstracted water, which stimulated preventive measures and further investigations on the transport and survival of enterococci in this area. The absence of enterococci in abstracted water during 2015 and 2016 suggests that the preventive measures taken (fencing to keep geese away from the abstraction wells) were effective, however it is advisable to continue with these measures to confirm their effectiveness. Screening for the presence of ampicillin- and vancomycin-resistant enterococci in the treated effluent of wastewater treatment plants (WWTPs) and in surface water used as a source for drinking water production in The Netherlands demonstrated the discharge of ampicillin-

resistant *E. faecium* strains with low level vancomycin resistance by the WWTPs into the surface water, when no strains with these characteristics were observed at intake locations for drinking water production. The results suggested that because of the issues such as the dilution of numbers of resistant strains discharged into surface waters, possible low numbers of resistant strains at the intake locations and a high level of enterococci removal during the drinking water production process, concern about the presence of ARE and VRE in surface water regarding drinking water production in The Netherlands may be insignificant. However, it is desirable to carry out further monitoring to evaluate the numbers of and possible increase in antibiotic-resistance levels in enterococci and other clinically relevant microorganisms in WWTP effluents and surface waters used for irrigation or recreation, as well as gene transfer in the aquatic environment (Chapter 7).

This thesis concludes with a General discussion. In this chapter the most important findings of this thesis and their practical implications for the water companies are discussed. Finally, the gaps in the current knowledge are appointed.

Overall conclusions and practical implications

The MALDI-TOF MS is a reliable and efficient method for the identification of enterococci isolated from water. Use of this method makes it possible to avoid false positive enterococci test results when ISO 7899-2:2000 has been applied for in the detection of enterococci in water. Validation and application of the system for the identification of enterococci initiated the validation of this system, also for the rapid identification of other faecal bacteria (*E. coli*, *Campylobacter* spp.) and other micro-organisms like *Legionella* spp., which accelerated the confirmation of these organisms and therefore the availability of confirmed test results for at least 24 hours. Beside rapid identification, there are also other possible applications for MALDI-TOF MS, which are more suitable for research purposes than for routine use. The evaluation of the variety, the incidence and the type of species detected at different sampling points can be used as a source tracking tool. (Chapter 2 and 5).

The competition for nutrients with indigenous flora has been shown to significantly limit the growth of enterococci and inhibit their survival in extract obtained from a common dune plant (*Ammophila arenaria)*. Differences based on the ability to grow on plant extract have been observed among five *Enterococcus* species, which suggests that *E. moraviensis* may be a less suitable predictor of recent faecal pollution than *E. faecium* in plant-rich environments (Chapter 3 and 4).

The faecal source of the most frequently isolated *Enterococcus* species (*E. moraviensis*) from abstracted water (after dune passage) in the Castricum dune infiltration area was identified. Geese were found to be carriers of *E. moraviensis* and *E. haemoperoxidus*, which is the first reporting on the faecal source of these species. Findings that this species has frequently been found in geese droppings, that geese were regularly observed in the vicinity of the abstraction wells and that they have been observed to generate a substantial faecal load in specific parts of this area (especially during warmer periods of the year) all point towards the conclusion that geese droppings are an important source of the *Enterococcus* species found in the abstracted water. These findings led to preventive measures - fencing off the vulnerable parts of the area. Since geese can be carriers of pathogens that may also affect humans and as it has been shown that the competition with other microorganisms for nutrients and protozoan predation negatively influence the fate of enterococci in the environment, the occurrence of *E. moraviensis* may indicate the possible presence of pathogens. Its role as indicator of faecal contamination should not be ignored (Chapter 5).

The growth of enterococci in the environment is limited (and the inactivation intensified) by the competition for nutrients with the indigenous microbial community and by the predation of protozoa. These findings, and those demonstrating that *E. moraviensis* was not able to multiply in the habitats present in the dune passage process (sediment from abstraction wells, biofilm developed using abstracted water and soil), indicate that bacteria from the topsoil are able to reach the ground water through relatively short unsaturated and saturated zones. The barrier function of the short soil passage is therefore limited, so it is important to avoid the presence of faecal droppings from animals (and sometimes maybe humans) in the direct vicinity of the abstraction wells (Chapter 3, 5 and 6).

In Chapter 7 it has been shown that antibiotic-resistant enterococci can enter the surface waters via the WWTP effluents. Relatively high numbers of *E. faecium* with a combination of ampicillin resistance and low vancomycin resistance are found in these effluents and further investigations are needed. Even though the drinking-water treatment systems in The Netherlands are capable of removing or inactivating ampicillin- and vancomycin-resistant enterococci if present in surface water, it is important to monitor the presence of clinically significant *Enterococcus* strains in surface water, especially when used for recreation or irrigation.

Research needs

- There is a need to further evaluate the survival of pathogens such as *Salmonella*, *Shigella*, *Campylobacter*, *Giardia*, and *Cryptosporidium* when associated with plant-rich environment as well as the relationship between enterococcal species and pathogens in such environments.

- The use of genetic techniques for the evaluation of enterococci populations recovered from soil in order to determine if these populations are clonal.

- To study actual routes of the breakthrough of the microorganisms from the topsoil towards the abstraction wells and the removal of microorganisms during vertical routes.

- The extension of MALDI-TOF MS database with more environmental strains of FIB and newly described species.

- To continue with fencing (preventive measures to keep geese away) off the vulnerable parts of the dune filtration area for at least 5 years to evaluate the effectiveness of this preventive measure.

- To apply whole genome sequencing to differentiate between strains derived from recent faecal event or as a result of growth in non-enteric environment.

- To further monitor low resistance to vancomycin (2-8 $\mu g ml^{-1}$) in ARE*fm* isolated from WWTP' s effluents, recreational water and water used for the production of drinking water (intake locations).

- To study mechanisms regulating low-level vancomycin resistance in ARE*fm*.

- To study horizontal gene transfer between bacteria that are present in water.

Samenvatting

Om de microbiologische betrouwbaarheid van water te controleren door detectie van alle mogelijke voor mensen pathogene organismen, zou tijdrovend zijn en duur. In plaats daarvan wordt water gecontroleerd op de aanwezigheid van (fecale) indicator organismen, die relatief snel en goedkoop kunnen worden bepaald. Het gebruik van indicator organismen is gebaseerd op het principe dat fecaliën die in het water terecht zijn gekomen, mogelijk ook voor mensen pathogene organismen bevatten. De consumptie van ongekookt fecaal verontreinigd water kan tot ziekte bij mensen leiden. Behalve *Escherichia coli* worden bacteriën van de coligroep en enterokokken gebruikt als indicatoren voor de fecale verontreiniging van water, omdat deze bacteriën als commensaal voorkomen in de darmen van mens en warmbloedige dieren. Enterokokken worden ook vaak aangetroffen in grond en water. De persistentie en mogelijke groei onder bepaalde milieuomstandigheden buiten de darmen, kan de bruikbaarheid van de enterokokken als fecale indicator en als indicatie voor potentieel gezondheidsrisico verminderen. Hoewel enterokokken worden gerekend tot ongevaarlijke darmflora, zijn bepaalde soorten /stammen enterokokken bekend als veroorzakers van infecties, vooral binnen zorginstellingen. Het aantal infecties met enterokokken die resistent zijn voor bepaalde soorten antibiotica, vormt een toenemende zorg binnen de medische sector, evenals de verspreiding van resistentie-genen in het milieu. Beide aspecten verdienen een bredere maatschappelijke aandacht. Water zou kunnen dienen als de verspreidingsroute van antibiotica resistente stammen in het milieu.

Doelen van dit onderzoek zijn: 1) de evaluatie van enterokokken als fecale indicator organismen tijdens de bereiding van drinkwater (bronopsporing en groei onder milieuomstandigheden buiten de darmen), 2) het vinden van een mogelijk reservoir van klinisch relevante antibioticaresistente enterokokken in gezuiverd afvalwater en in water dat gebruikt wordt als bron voor de productie van drinkwater in Nederland. Uit vergelijkend onderzoek naar de identificatie van enterokokken soorten met behulp van een biochemische methode, een moleculaire methode en de Matrix Assisted Laser Desorption Ionization –Time of Flight Mass Spectrometry (MALDI-TOF MS) is gebleken, dat MALDI-TOF MS een betrouwbare en efficiënte identificatietechniek is voor de identificatie van enterokokken soorten geïsoleerd uit oppervlaktewater, proceswater en drinkwater (Hoofdstuk 2). Daarom werd deze techniek ingezet voor de identificatie van enterokokken stammen die incidenteel geïsoleerd werden uit groot volume monsters (100 L) van het onttrokken water (na de duinpassage) in infiltratiegebied Castricum. In deze monsters was *Enterococcus moraviensis* de meest voorkomende *Enterococcus* soort. Omdat de fecale besmetting van onttrokken water

niet aannemelijk was en omdat fecale bronnen van *E. moraviensis* in de literatuur niet waren beschreven, werd het onderzoek gericht op de bronopsporing van deze soort in het infiltratiegebied en op het groeivermogen van deze soort onder verschillende milieuomstandigheden. Uit de bronopsporing van *E. moraviensis* in humane feces en in feces van geselecteerde diersoorten in dit duingebied is gebleken, dat deze soort veel voorkwam in feces van ganzen. Bovendien bleek de gans de enige gastheer waar *Enterococcus haemoperoxidus* werd gevonden, een soort die in ons onderzoek ook uit onttrokken water werd geïsoleerd. Uit de literatuur waren nog geen fecale bronnen van *E. haemoperoxidus* bekend. Deze resultaten laten zien dat het vaak aantreffen van een bepaalde *Enterococcus* soort op een specifieke fecale bron kan wijzen, in dit geval op ganzen. De statistische vergelijking (cluster-analyse) van de soortensamenstelling van enterokokken in alle monsters van het onttrokken water en die van de feces monsters van verschillende gastheren, laat zien dat de samenstelling van soorten in de watermonsters het best overeenkomt met de soortensamenstelling in ganzenfeces. Uit het veldonderzoek is gebleken, dat in bepaalde delen van het infiltratiegebied ganzen substantieel bijdragen aan de fecale belasting op het maaiveld in de directe omgeving van de winputten. Het vermoeden ontstond dat in deze delen van het infiltratiegebied de uitwerpselen van ganzen een invloed hebben op de kwaliteit van onttrokken water. Uit literatuuronderzoek is gebleken, dat ganzen dragers kunnen zijn van voor mensen pathogene organismen. Dit toont de waarde van *E. moraviensis* en *E. haemoperoxidus* als fecale indicator organismen heel duidelijk aan (Hoofdstuk 5). Een andere studie liet zien dat *E. moraviensis* niet kon groeien in de habitats aanwezig in het duinpassage proces (sediment uit de winputten, biofilm ontwikkeld uit onttrokken water en grond), maar wel op gefilterd plantenextract (tot 6 log toename). Hierbij waren de meeste concurrerende flora en predatie afwezig (Hoofdstuk 3). Dit resultaat initieerde het vervolgonderzoek, waarin het vermogen van *E. moraviensis* om te groeien op gefilterd plantenextract werd vergeleken met dat van vier andere enterokokken soorten: *E. casseliflavus*, *E. faecium*, *E. faecalis* en *E. hirae*. In het experiment waar het grootste gedeelte van de natuurlijke microbiologische populatie door middel van filtratie uit het plantextract was verwijderd, toonde alle vijf enterokokken soorten een nutriëntafhankelijke groei. Echter, in een serie parallelle experimenten waarbij de natuurlijke microbiële populatie niet verwijderd was, was er geen of zeer gelimiteerde groei aantoonbaar. Een hoge concentratie van plantenextract bevorderde initiële groei bij *E. moraviensis*. Dat was niet het geval bij de andere onderzochte soorten. *E. faecium* toonde in beide experimenten een verminderde groeivermogen op plantenextract. Er werd aangenomen dat in deze experimenten competitie om de beschikbare

voedingsstoffen de groei en de persistentie van enterokokken heeft gehinderd en dat deze situatie ook in het milieu (zoals maaiveld) voorkomt (Hoofdstuk 4). Verder onderzoek was gericht op de predatie van fecale indicator organismen in het milieu. Experimenten met *Dictyostelium mucoroides* (slijmzwam) geïsoleerd uit grond en uitwerpselen verzameld in het infiltratiegebied, hebben aangetoond dat *D. mucoroides* in een relatief korte tijd in staat is om verschillende soorten fecale indicatoren te consumeren en zich snel kan vermenigvuldigen. Dit impliceert dat *D. mucoroides* een belangrijke rol kan spelen in de reductie van aantallen fecale indicator organismen en mogelijk pathogenen op feces en op het maaiveld (Hoofdstuk 6). Er is geconcludeerd dat er routes bestaan, die een rol spelen in het verticale transport van enterokokken vanaf het maaiveld richting onttrokken water. Dat heeft vanaf 2015 geleid tot preventieve maatregelen en verder onderzoek naar de transportroute en overleving van enterokokken in het infiltratiegebied. De afwezigheid van enterokokken in grootvolume monsters van het onttrokken water in 2015 en 2016, doet vermoeden dat de preventieve maatregelen (plaatsen van netten die ganzen weren rondom de winputten, etc.) effectief waren. Ten behoeve van een verdere evaluatie van genomen preventieve maatregelen is het wenselijk om deze maatregelen te continueren.

Het onderzoek naar de aanwezigheid van ampicilline (ARE) en vancomycine resistente enterokokken (VRE) in effluent van rioolwaterzuiveringsinstallaties (RWZI's) en in oppervlaktewater dat in Nederland als productie bron van drinkwater wordt gebruikt (inname locaties), laat zien dat ampicilline resistente *E. faecium* met een lage vancomycine resistentie met het effluent van RWZI's in het oppervlaktewater terecht komen. Enterokokken met deze eigenschappen werden niet gevonden in oppervlaktewater bij innamelocaties voor drinkwaterproductie. Rekening houdend met een sterke verdunning van het RWZI-effluent na lozing op het oppervlaktewater is het waarschijnlijk dat er resistente stammen in (zeer) kleine aantallen bij innamepunten van drinkwaterbedrijven kunnen voorkomen.

De Nederlandse drinkwaterbedrijven hebben allemaal een aanzienlijke verwijderings-capaciteit voor micro-organismen, waardoor de zorg over het verspreiden van antibiotica-resistentie via deze drinkwaterbedrijven waarschijnlijk veel minder groot is dan aanvankelijk werd gedacht. Toch verdient het aanbeveling om resistente enterokokken en andere klinisch relevante resistente bacteriën in RWZI-effluenten en in oppervlakte-, recreatie- en irrigatiewater te monitoren. Het is van belang hierover informatie te verzamelen om een eventuele toename van resistente bacteriën of

toename van de mate van resistentie (MIC-waarden) te bepalen. Behalve het monitoren is het wenselijk om te weten of gen-overdracht een rol speelt in het verspreiden van antibiotica resistentie in aquatische systemen.

Dit proefschrift eindigt met een algemene discussie, waarin een kort overzicht van de belangrijkste resultaten wordt gegeven en hun implicaties voor drinkwater-productiebedrijven. Als laatste worden de hiaten in de huidige kennis benoemd.

Algemene conclusies en betekenis van dit onderzoek voor drinkwater-productiebedrijven

MALDI-TOF MS is een betrouwbare en efficiënte techniek voor de identificatie van enterokokken geïsoleerd uit water. Met gebruik van deze methode kunnen eventuele vals-positieve resultaten worden vermeden, zoals die werden verkregen met gebruik van de enterokokken methode (ISO 7899-2:2000). Validatie en de toepassing van deze techniek voor de identificatie van enterokokken heeft geleid tot de validatie van het systeem ook voor andere fecale indicatoren (*E. coli*, *Campylobacter* spp.) en andere micro-organismen zoals *Legionella* spp. MALDI-TOF MS identificatie van geïsoleerde micro-organismen heeft de tijd voor de bevestigingsanalyse minimaal met 24 uur verkort. Naast de snelle identificatie van micro-organismen in routinematig gebruik, biedt MALDI-TOF MS nog andere applicaties die geschikt zijn voor onderzoeksdoeleinden. Deze techniek maakt het mogelijk om de diversiteit aan soorten en de frequentie waarmee deze worden aangetroffen op een bepaald bemonsteringspunt te evalueren en kan dienen als een instrument in de bronopsporing (Hoofdstuk 2 en 5).

Het is aannemelijk gemaakt dat competitie met natuurlijke flora voor de beschikbare voedingstoffen de groei van enterokokken beperkt en hun overleving remt in plantextract verkregen uit de meest voorkomende plant in de duinvegetatie, helmgras (*Ammophila arenaria*). Er zijn verschillen waargenomen in het groeivermogen van 5 verschillende *Enterococcus* soorten op plant-extract. De resultaten suggereren dat in een omgeving van rottend plantenmateriaal *E. moraviensis* een minder betrouwbare voorspeller kan zijn van een recente fecale besmetting dan *E. faecium* (Hoofdstuk 3 en 4).

The fecale bron van de meest voorkomende *Enterococcus* soort in onttrokken water van het infiltratiegebied Castricum, *E. moraviensis*, was geïdentificeerd. Ganzen zijn gebleken dragers te zijn van *E. moraviensis* en *E. haemoperoxidus*. Dat is de eerste melding

van fecale bronnen van deze soorten in de literatuur. Het feit dat *E. moraviensis* vaak werd aangetroffen in de uitwerpselen van ganzen, dat ganzen en hun uitwerpselen regelmatig werden gezien in directe omgeving van de winputten en dat ganzen hiermee hebben bijgedragen aan de substantiële fecale belasting in bepaalde delen van de infiltratiegebied (voornamelijk in warmere periode van het jaar), wijzen erop dat uitwerpselen van ganzen een belangrijke bron zijn van de *Enterococcus* soorten gevonden in onttrokken water. Deze conclusie leidde tot de preventieve maatregelen, er werden netten geplaatst om de ganzen uit de directe omgeving van de winmiddelen te weren. Omdat ganzen dragers kunnen zijn van voor mensen pathogene micro-organismen, kan de aanwezigheid van *E. moraviensis* in drinkwatermonsters een gezondheidsrisico voor de consumenten van dat drinkwater indiceren. Dit nader uitgewerkte voorbeeld laat zien dat ook enterokokken waarvan aanvankelijk geen gastheer bekend is, wel degelijk een fecale bron kunnen hebben en dus als een indicator voor fecale besmetting gebruikt moeten worden. De aanwezigheid van *E. moraviensis* in water kan wijzen op de mogelijke aanwezigheid van voor mensen pathogene organismen en de functie van *E. moraviensis* als fecale indicator kan niet worden genegeerd (Hoofdstuk 5).

De groei van enterokokken in het milieu is gelimiteerd door de competitie met de van nature aanwezige microbiële populatie voor de beschikbare voedingsstoffen alsook door de predatie van protozoa. Deze resultaten, samen met de experimenten die aantoonden dat *E. moraviensis* zich niet vermenigvuldigde in de habitats aanwezig bij de duinpassage (sediment uit de winput, biofilm gevormd door onttrokken water en grond), wijzen erop dat bacteriën vanaf het maaiveld het grondwater kunnen bereiken via de relatief ondiepe onverzadigde en verzadigde zone. De barrièrefunctie van de onverzadigde en verzadigde zone is dus beperkt. Daarom is het van groot belang om de aanwezigheid van uitwerpselen van dieren (of zelfs mensen) in de directe omgeving van winputten te vermijden (Hoofdstuk 3, 5 en 6).

In Hoofdstuk 7 staat een observatie vermeld dat antibioticaresistente enterokokken via de effluenten van de RWZI's in het oppervlaktewater terecht kunnen komen. In deze effluenten zijn relatief hoge aantallen van *Enterococcus faecium* aangetroffen die ampicillineresistent zijn en tegelijkertijd resistent zijn voor lage concentraties vancomycine. Ampicilline- en vancomycineresistente enterokokken (indien aanwezig in het oppervlakte water) worden tijdens het proces van het bereiden van drinkwater in Nederland verwijderd of geïnactiveerd. Toch is het een aanbeveling om klinisch relevante *Enterococcus* stammen in oppervlaktewater te monitoren, vooral als dat water gebruikt wordt voor recreatie of irrigatie.

Onderzoekbehoeften

- Vergelijkend onderzoek naar de overleving van enterokokken en mens pathogene organismen zoals *Salmonella*, *Shigella*, *Campylobacter*, *Giardia* en *Cryptosporidium* in een plantmateriaal-rijke suspensies.

- Met gebruik van moleculaire technieken vaststellen of de enterokokken populaties gevonden in grond van dezelfde stam afkomstig (klonaal) zijn. Waarmee men de bron van een microbiële verontreiniging zou kunnen vaststellen.

- Onderzoek naar de transportroutes van de micro-organismen vanaf het maaiveld richting grondwater en de verwijdering van micro-organismen tijdens deze verticale routes.

- Uitbreiding van MALDI-TOF MS database met de omgevingsstammen van fecale indicator organismen en nieuw beschreven soorten.

- Het plaatsen van netten (preventieve maatregelen om ganzen te weren) op bepaalde plekken in het Castricum infiltratiegebied te continueren voor minstens 5 jaar. Dat is van belang om de effectiviteit van deze preventieve maatregelen (statistisch) te kunnen evalueren.

- Met gebruik van de Whole genome sequencing techniek te onderzoeken of een onderscheid gemaakt kan worden gemaakt tussen de stammen afkomstig uit een recente fecale besmetting en stammen die resultaat zijn van groei onder andere omstandigheden dan in de darm.

- Monitoring van lage vancomycine resistentie (2-8 µg ml^{-1}) in ampicilline resistente *E. faecium* (ARE*fm*) geïsoleerd uit de effluenten van de RWZI's, recreatiewater en oppervlaktewater gebruikt voor de productie van drinkwater (inname locaties).

- Onderzoek naar de mechanismen die lage vancomycine resistentie reguleren in ARE*fm*.

- Onderzoek naar de horizontale gen-overdracht tussen bacteriën in water.

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Maja Taučer-Kapteijn

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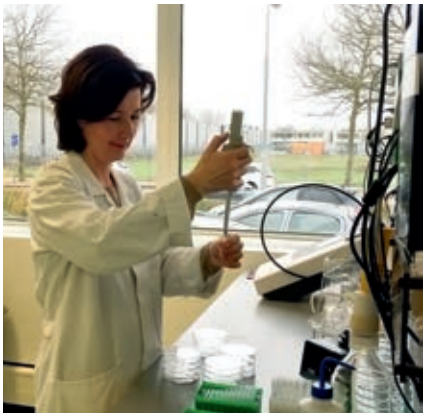
Curriculum vitae



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List of Publications

Taučer-Kapteijn M, Medema GJ, Hoogenboezem W. 2013. Comparison between Rapid ID 32 Strep System, Matrix Assisted Desorption Ionisation- Time of Flight Mass Spectrometry and 16S gene sequence analysis for the species identification of enterococcus spp. isolated from water. *Water Science & Technology: Water supply* 13(5): 1383-1389.

Taučer-Kapteijn M, Hoogenboezem W, Medema GJ. 2016. Environmental growth of the faecal indicator *Enterococcus moraviensis. Water Science and Technology: Water Supply* 16(4), 971-979.

Taučer-Kapteijn M, Hoogenboezem W, Medema GJ. 2017. Source tracking of *E. moraviensis* and *E. haemoperoxidus*. *Journal of Water and Health*. 15(1):41-49.

Taučer-Kapteijn M, Hoogenboezem W, Heiliegers L, de Bolster D, Medema GJ. 2016. Screening municipal wastewater effluent and surface water used for drinking water production for the presence of ampicillin and vancomycin resistant enterococci. *International Journal of Hygiene and Environmental Health* 219(4-5):437-42.