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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
VanC1/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. faecalis*	?	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 $\geq$ 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 ^{0}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 ^{0}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 was 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 x 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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