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Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms

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Abstract

In view of the increasing interest in the possible role played by hospital and municipal wastewater systems in the selection of antibiotic-resistant bacteria, biofilms were investigated using enterococci, staphylococci, Enterobacteriaceae, and heterotrophic bacteria as indicator organisms. In addition to wastewater, biofilms were also investigated in drinking water from river bank filtrate to estimate the occurrence of resistant bacteria and their resistance genes, thus indicating possible transfer from wastewater and surface water to the drinking water distribution network. Vancomycin-resistant enterococci were characterized by antibiograms, and the *van*A resistance gene was detected by molecular biology methods, including PCR. The *van*A gene was found not only in wastewater biofilms but also in drinking water biofilms in the absence of enterococci, indicating possible gene transfer to autochthonous drinking water bacteria. The *mec*A gene encoding methicillin resistance in staphylococci was detected in hospital wastewater biofilms but not in any other compartment. Enterobacterial *amp*C resistance genes encoding β-lactamase activities were amplified by PCR from wastewater, surface water and drinking water biofilms.

Keywords: Biofilm; Antibiotic-resistance genes; Gene transfer; Wastewater; Drinking water bacteria

1. Introduction

The emergence of bacteria resistant to antibiotics is common in areas where antibiotics are used, but antibiotic-resistant bacteria also increasingly occur in aquatic environments [1,2]. The widespread use of antibiotics in medicine and in intensive animal husbandry is indicative of the selection pressure exerted on bacteria [2]. Intensive animal husbandry causes resistant bacteria to enter the environment directly from liquid manure and muck [1]. Several reports have also documented the presence, for example, of vancomycin-resistant enterococci (VRE) in the stools of asymptomatic individuals who have neither recently been in hospital nor received antibiotics [3]. VRE have also been found in sewage, from stools of healthy farm animals and animal products, but also in surface water [4,5]. VRE could cause hospital-acquired infections in debilitated and immunocompromised patients, which are difficult to treat because of the multiresistance of these bacteria.

Bacteria have developed different mechanisms to render ineffective the antibiotics used against them. The genes encoding these defence mechanisms are located on the bacterial chromosome or on extrachromosomal plasmids, and are transmitted to the next generation (vertical gene transfer). Genetic elements, such as plasmids, can also be exchanged among bacteria of different taxonomic affiliation (horizontal gene transfer) [6]. Horizontal gene transfer by conjugation is common in nature, or in technical systems, where the density of bacteria is high and so, accordingly, is the chance of two suitable bacterial cells coming close to each other [7,8].

High bacterial density and diversity are found in biofilms from wastewater systems, especially from activated sludge of sewage treatment plants. Biofilms are also generated in surface water and drinking water distribution systems [9,10]. Most of the studies concerning antibiotic resistance in the aquatic environment have focused on

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bulk water and do not reflect the situation in biofilms, which is the preferred pattern of life of many bacteria.

In this study, the resistance of bacteria from biofilms of different compartments was determined. We investigated biofilms of hospital wastewater because of the potential addition of large amounts of antibiotics and bacteria from patients' feces. In addition, biofilms from a sewage treatment plant, from surface water, and from drinking water from river bank filtrate were investigated to estimate the occurrence of resistant bacteria and their resistance genes. Numbers of enterococci, staphylococci, Enterobacteriaceae and heterotrophic bacteria were determined. Both bacteria cultured from biofilms and total genomic DNA isolated from natural biofilm communities were used to evaluate resistance. The vancomycin-resistance gene, *van*A, from enterococci, the methicillin-resistance gene, *mec*A, from staphylococci, and the β-lactam-resistance gene, *amp*C, specific to some Enterobacteriaceae, were detected by PCR and Southern blot hybridization.

2. Materials and methods

2.1. Sampling of biofilms

A modified Robbins device technique was used to sample biofilms based on the experience gained from previous research projects on drinking water systems [9,10]. Up to 15 stainless steel platelets (450 mm^2) were held in place by stainless steel bolts screwed into a hollow stainless steel cylinder (260 mm long, 150 mm in diameter). Perforated plates right behind the inlet and upstream of the outlet of the device provided a uniform distribution of the water flow. Besides the described device technique, stainless steel platelets (2890 mm^2) were also immersed directly into wastewater and surface water for one month for biofilm formation. At all sampling points, stainless steel platelets were used as the substrate for biofilm formation, whereas both substrate sides were used for the experiments.

2.2. Sampling points

To investigate natural biofilms in drinking water systems, modified Robbins devices were installed at different sampling points within the water distribution system of the city of Mainz. Three devices were installed in house-branch connections of the distribution system. These sampling points were supplied with bank-filtered drinking water from the waterworks and were located at a distance of 1–2 km from the plant. The water flow was approximately 6.0 ± 0.5 m^3 day^{-1}. Disinfection was performed by UV irradiation at the waterworks.

Two sampling points were arranged in the wastewater system of a hospital in the city of Mainz, one downstream of the surgical department and the other at the outlet of the clinical wastewater system, close to the public sewer system.

Two biofilm sampling points were located in the municipal sewage system, one in the biological sludge facilities, and the other at the outlet of the wastewater treatment plant.

To investigate biofilms from surface water, steel platelets were incubated in the river Rhine a short distance upstream of bank filtration. Thus, the river water was not contaminated by the conditioned wastewater from the selected municipal sewage at this sampling point (Fig. 1).

2.3. Cultivation of bacteria

Bacteria were removed from the platelets surfaces using a sterile scraper and pooled in 10 ml of PBS buffer (10 mM Na-phosphate, pH 7.5, 130 mM NaCl). The bacteria were quantified by the pour plate method, i.e. by plating serial dilutions of the bacterial suspension obtained on the double scale. Different nutrient media were used for selective cultivation of bacteria: kanamycin esculin azide (KAA, Merck Eurolab, Darmstadt, Germany) for enterococci, and Chromocult® agar (Merck) as a selective agent for Enterobacteriaceae. For the heterotrophic plate count the bacterial dilutions were plated on R2A agar (Difco) [11].

Staphylococci were identified by means of the ID32 STAPH kit (BioMérieux, Nürtingen, Germany) in addition to the protein A-clumping factor latex test (Staphaurex Plus®, Abbott) to distinguish between *Staphylococcus aureus* and coagulase-negative staphylococci. Enterobacteriaceae isolates were identified by means of the API 20E identification kit (BioMérieux).

Antibiotics were added to the nutrient media to evaluate the percentage of resistant bacteria by comparison with plating experiments without any antibiotics in the media. According to the German DIN 58940 standard [12], the following concentrations of antibiotics in media were used: vancomycin, 32 μg ml^{-1}; ceftazidime, 32 μg ml^{-1}; cefazolin, 32 μg ml^{-1}; penicillin G, 16 μg ml^{-1}; cefotaxime, 16 μg ml^{-1}; imipenem, 8 μg ml^{-1}; methicillin (oxacillin), 2 μg ml^{-1}.

2.4. DNA preparation

To prepare genomic DNA from cultured bacterial strains, the bacteria from 100 ml of liquid media were used for DNA isolation according to the manufacturer's instructions (DNeasy Tissue Kit, Qiagen, Hilden, Germany). Because only a small percentage of environmental bacteria, especially drinking water bacteria [9], can be cultivated on synthetic media, genomic DNA was prepared from biofilm. Unlike cultured planktonic bacteria, biofilms were scraped off the surface of the platelets, suspended in 10–20 ml of sterile water, centrifuged at $8000 \times g$ for 10 min, and the pellet was used for DNA preparation with the test kit mentioned above. To increase the DNA yield,

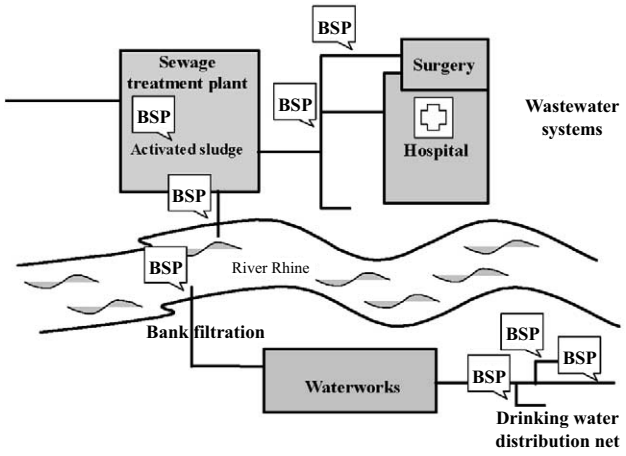

Fig. 1. Indication of the different biofilm sampling points (BSP).

the manufacturer's instructions were modified. Firstly, mechanical disruption of the bacteria was performed using the Ribolyser (Hybaid) technique, and then the lysozyme treatment was extended to 1.5 h. The bacterial densities of the biofilms from wastewater and surface water as measured by DAPI (4′,6-diamidin-2′-phenylindole-dihydrochloride, Merck)-staining according to Schwartz et al. [10] were approx. 10^6–10^7 bacteria cm^{-2} after an incubation period of four weeks. The bacterial densities of drinking water biofilms were found to be one order of magnitude lower, namely, about 10^5 bacteria cm^{-2}. The DNA yields from wastewater biofilms differed between 15.0 μg ml^{-1} and 180 μg ml^{-1}. The amount of DNA prepared from drinking water biofilms was smaller (approx. 2–10 μg ml^{-1} after four weeks of incubation). Here, isolated DNAs were combined to increase the nucleic acid concentration.

The GeneQuant RNA/DNA calculator (Pharmacia Biotech, Freiburg, Germany) was employed to measure optical density at 260 nm and 280 nm, and the quality of the DNA was analyzed by agarose gel electrophoresis.

2.5. PCR and Southern blot hybridization

DNA from natural biofilm populations or cultured bacterial strains was employed for PCR. For screening analyses of cultured bacteria, aliquots of suspended bacteria were centrifuged at $5000 \times g$ for 10 min. The pellets were resuspended in 10 μl of sterile water (Seradest) for PCR. For amplification of the specific DNA sequences, the GeneAmp® PCR System 9700 (Applied Biosystems, Weiterstadt, Germany) was used. In all PCR experiments, HotStarTaq DNA polymerase (Qiagen) was used. It was activated by 15-min incubation at 95°C. Various temperature profiles were used with the specific primers (Table 1) to detect the antibiotic-resistance genes. To PCR amplify the resistance genes from biofilms, approx. 0.5 μg genomic DNA was used.

To amplify the enterococcal *van*A and *van*B resistance genes, PCR systems were employed according to Uhl et al. [13]. To verify the first PCR results, a nested *van*A gene-specific PCR was applied according to Klein et al. [14]. The vancomycin-resistant strain *Enterococcus faecium* B7641 (*van*A; vancomycin minimum inhibitory concentration (MIC) > 256 μg ml^{-1}; teicoplanin MIC > 16 μg ml^{-1}) was used as a positive control. To define the detection limit of this PCR system, genomic DNA from this reference strain was isolated using a commercial extraction kit. Serial dilutions were prepared starting from a 230-μg ml^{-1} genomic DNA solution. The DNA concentrations in the PCR assays ranged between 50 pg and 5 fg (in 10 concentration steps). The lower limit of PCR detection of *van*A genes ranged between 50 and 500 fg of genomic DNA.

For amplification of the *mec*A gene encoding the methicillin resistance of *S. aureus* and coagulase-negative staphylococci, the temperature profile was employed according to Murakami et al. [15]. The *S. aureus* 348, *S. aureus* 91, and *S. aureus* 170 strains were methicillin-resistant clinical isolates that served as positive controls, producing a PCR product of 533 bp.

Table 1
PCR primers and oligonucleotide probes used in this study

Short name	Target	Sequence (5′-3′)	Position	Reference
vanA-For	*Enterococcus*; *van*A vancomycin-resistance gene	CATGACGTATCGGTAAAATC	49–67[a]	[13]
vanB-For	*Enterococcus*; *van*B vancomycin-resistance gene	CATGATGTGTCGGTAAAATC	49–67[a]	[13]
vanAB-Rev	*Enterococcus*; *van*A and *van*B vancomycin-resistance gene	ACCGGGCAGRGTATTGAC	933–914[a]	[13]
vanA-nestedFor	*Enterococcus*, *van*A vancomycin-resistance gene	CTGCAATAGAGATAGCC	67–86[a]	[14]
vanA-nestedRev	*Enterococcus*, *van*A vancomycin-resistance gene	CCTCATCGATAGGGTCGTAA	442–423[a]	[14]
mecA1-For	*Staphylococcus*; *mec*A methicillin-resistance gene	AAAATCGATGGTAAAGGTTGGC	1282–1304[b]	[15]
mecA2-Rev	*Staphylococcus*; *mec*A methicillin-resistance gene	AGTTCTGCAGTACCGGATTTGC	1814–1792[b]	[15]
mecA3-Probe	*Staphylococcus*; *mec*A methicillin-resistance gene	ATCTGTACTGGGTTAATC	1581–1598[b]	This study
ampC-For	Enterobacteriaceae; *amp*C β-lactam-resistance gene	TTCTATCAAMACTGGCARCC[c]	627–647[d]	This study
ampC-Rev	Enterobacteriaceae; *amp*C β-lactam-resistance gene	CCYTTTTATGTACCCAYGA[c]	1177–1157[d]	This study

[a] *E. faecium* sequence gi155036 [16].
[b] *S. aureus* sequence gi46628 [17].
[c] Wobbles according to IUPAC: M means A or C, R means A or G and Y means C or T
[d] *E. coli* sequence gi4691723 (J. Kim and Y. Kwon, unpublished data).

The temperature profile and the PCR primers (Table 1) for amplification of the enterobacterial *amp*C gene coding for class C β-lactam resistance were newly designed in this study. The PCR profile consisted of 30 s at 94°C, 30 s at 49°C, and 1 min at 72°C. After 35 cycles, a final step at 72°C was performed for a period of 7 min.

In all PCR systems used for the detection of resistance genes, the primer concentrations were 0.3 μM, and the concentration of each dNTP was 200 μM. 2.5 U of HotStarTaq polymerase were added to each PCR mix.

Enterococci were detected by non-specific amplification of a 23S rRNA-related DNA fragment for *Eubacteria*, followed by hybridization with digoxigenin-labelled oligonucleotide probes specific to *E. faecium*, *E. faecalis*, and *E. gallinarum* [10,18].

Aliquots of the PCR solutions were loaded on an agarose gel (1–2%, depending on the DNA-fragment size) and separated by electrophoresis. The DNA bands were then visualized by ethidium bromide staining. The LumiImager workstation (Roche Diagnostics, Mannheim, Germany) was used for gel documentation and DNA fragment characterization. For Southern blot hybridization, the DNA fragments were transferred to nylon membranes (Qiagen) and crosslinked by UV irradiation for 3 min. The filters were hybridized with 15 pmol of specific oligonucleotides. All probes were labelled with digoxigenin at the 3′-end by a terminal transferase reaction (Roche Diagnostics). Oligonucleotide probes hybridized to the specific target DNA molecules were visualized by a digoxigenin-specific antibody conjugated with alkaline phosphatase. The bound probe was detected in a chemiluminescence reaction according to manufacturer's instructions (Roche Diagnostics). The chemiluminescence reaction was analyzed at the LumiImager workstation. Positive and negative controls were included in all experiments to confirm the specificity of detection.

For *van*A-probe labelling, digoxigenin-labelled dUTP was used for PCR with *van*A-specific primers (vanA-For; vanAB-Rev) to increase signal detection with the digoxigenin-specific antibody. In comparison to the 3′-end labelling of oligonucleotides, the number of labelled dUTP within the polymerized oligonucleotide sequence was increased. The PCR amplicon was separated in an agarose gel, extracted, and purified using a commercial extraction kit (Qiagen). The hybridization reaction was performed with different formamide concentrations in the hybridization buffer. The formamide concentration in the buffer was adjusted to 20% by the use of control plasmids isolated from *E. faecium* B7634.

Southern blot hybridization for detection of the methicillin-resistance gene in staphylococci was performed with the digoxigenin-labelled mecA3-probe (Table 1). Here, the target sequence is located within the amplified DNA. Specific hybridization signals were obtained when the formamide concentration was adjusted to 20% in the hybridization and washing buffers.

2.6. Agar diffusion test for sensitivity testing

The agar diffusion test method described in the German standard DIN 58940 was employed for sensitivity testing [12]. Mueller–Hinton agar (BioMérieux) was used as nutrient. *E. faecalis* ATCC 29212 and *E. faecium* ATCC 6057 served as control strains.

2.7. DNA sequencing and analysis

Amplified DNA fragments from PCR experiments were used for sequencing studies. The DNA was extracted from an agarose gel and purified with the QIAquick™ gel extraction kit (Qiagen) according to the manufacturer's instructions. Sequencing was performed by GENterprise (Mainz, Germany), using forward and reverse primers specific for resistance-gene amplification [19]. FASTA and BLAST DNA homology searches were performed with the DNA database software of the NIH (USA) obtained from the internet address: http://www.ncbi.nlm.nih.gov.

3. Results

3.1. Enterococci/streptococci

The enterococci/streptococci loads and the presence of resistant bacteria were highest in hospital biofilms (Table 2). Biofilms from activated sludge at the municipal sewage treatment plant showed slightly lower colony counts for enterococci/streptococci. Another reduction in the biofilm load was observed at the outlet end of the conditioned wastewater from the plant. Only minor amounts of enterococci/streptococci were found in biofilms from surface water, and none were isolated from drinking water biofilms.

The amount of vancomycin-resistant enterococci in hospital wastewater biofilms was found to be 25 (±9.4)%; in activated sludge at the municipal sewage treatment plant, 16 (±6.5)%; at the plant outlet, 12.5 (±5.5)%; and in surface water biofilms, 1.0% of the colony counts for enterococci/streptococci on KAA medium without vancomycin (Table 2).

The resistance patterns of 39 vancomycin-resistant enterococci were determined by the agar diffusion test. All of these bacteria were also resistant to tetracycline and erythromycin. High levels of resistance were recorded to ampicillin (62%), amoxicillin/clavulanic acid (33%), imipenem (51%) and gentamicin (59%). On the other hand, resistance to ciprofloxacin (2.5%) and cotrimoxazole (12.8%) was low.

All isolated vancomycin-resistant *E. faecium* strains were tested *van*A-positive by means of the *van*A-specific PCR assays, whereas vancomycin-sensitive strains did not show the 884-bp PCR amplicon. Vancomycin-resistant *E. faecalis* was never found in biofilms.

An additional, nested PCR system was established to upgrade the previous PCR system for more specific and sensitive detection in natural biofilms with low concentrations of *van*A targets. Therefore, the primer sets of vanA-nestedFor and vanA-nestedRev (Table 1) were used to amplify a 377-bp DNA amplicon. Vancomycin-resistant and *van*A-positive *Enterococcus* strains were tested together with sensitive strains to improve the system. The resistant strains exhibited the band of 377 bp throughout.

In contrast to the *van*A detection, no vancomycin-resistant strain carried the *van*B resistance gene.

Aliquots of the different biofilm genomic DNAs were used for PCR experiments aimed at detecting the enterococcal *van*A genes. Specific DNA amplicons were detected after the first PCR in biofilms from hospital wastewater and municipal sewage sampling points (Fig. 2, lanes 5, 6). Weak PCR amplicons were found when biofilms from the municipal treatment plant were investigated (Fig. 2, lane 4). The first PCR experiments with biofilms from surface water and drinking water resulted in weak or hardly visible amplicons. These results indicated that the concentration of *van*A resistance genes in wastewater systems was higher than that at the other sampling points. However, the nested PCR demonstrated that enterococcal *van*A resistance genes were also present in biofilms from surface water, and even in biofilms from the public drinking water distribution system (Fig. 2, lanes 15, 16).

To confirm the occurrence of enterococcal vancomycin-resistance genes in drinking water biofilms, genomic DNAs from the three sampling points of the distribution network were tested. In all cases, the *van*A gene was amplified after nested PCR. Sequencing of the PCR products revealed a high degree of homology (96%) to the *van*A gene of *E. faecium*.

Concerning the origin of the *van*A sequences from drinking water biofilms, different strategies were used to ascertain that no enterococci were present in these biofilm habitats. As indicated before, no enterococci were isolated using KAA medium for the selective cultivation of enterococci directly from the biofilms (Table 2). A molecular biology technique that detects rDNA sequences together with other functional genes like *van*A was employed to verify the absence of enterococci. It was based on an non-specific PCR with 23S rDNA-directed universal primers, and hybridization with a digoxigenin-labelled gene probe specific to *E. facalis*, *E. faecium*, and *E. gallinarum*. In parallel to the *van*A detection experiments, biofilms from the three different sampling points of the municipal drinking water distribution network were directly analyzed. In no case were 23S rDNA-related sequences from enterococci detected in uncultured biofilm bacteria, but *van*A sequences with high homologies to enterococci were amplified (Table 4). To extend the experiments concerning the origin of the *van*A sequence, cultivation analyses with heterotrophic drinking water bacteria from R2A medium were also performed (see below).

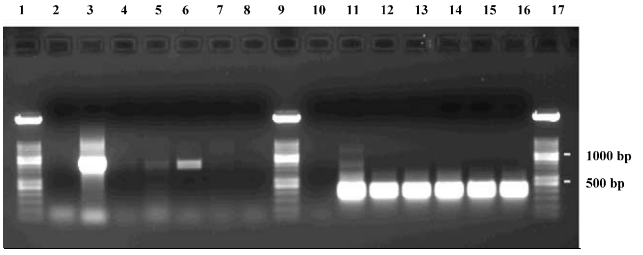

Fig. 2. Detection of the enterococci-specific *van*A gene coding for vancomycin resistance using total DNAs from different biofilms. Lanes 1, 9 and 17: DNA size marker; lanes 2 and 10: negative controls; lanes 3 and 11: positive controls; lane 4: biofilm from the outlet of the municipal treatment plant; lane 5: biofilm from the biological sludge at the municipal treatment plant; lane 6: biofilm from hospital wastewater system; lanes 7 and 8: drinking water biofilms. Nested PCR detection of *van*A (lanes 10–16) was performed with 5-μl aliquots from the first PCR mix (lanes 4 to 8); lanes 12–14: wastewater biofilms; lanes 15, 16: drinking water biofilms.

3.2. Enterobacteriaceae

Similar to the results obtained for enterococci/streptococci, the highest load of Enterobacteriaceae was observed in hospital biofilms, followed by biofilms from activated sludge of the municipal sewage-treatment plant (Table 2). The bacterial cell count at the outlet of the plant was 72% lower than the cell count at the activated sludge-sampling point. Another decrease in the Enterobacteriaceae load was measured in surface water biofilms, and no Enterobacteriaceae were detected in drinking water biofilms. The

Table 2
Bacteria from biofilms cultivated on different media

	Hospital ($n=6$)	Activated sludge at municipal sewage ($n=5$)	Efflux of municipal sewage ($n=5$)	Surface water ($n=5$–6)	Drinking water ($n=6$)
Enterococci and streptococci on KAA medium					
CFU cm^{-2} without antibiotics	666 (±36)	433.8 (±25)	26 (±4.4)	4	0
Enterobacteriaceae on Chromocult medium					
CFU cm^{-2} without antibiotics	6.7×10^{6} ($\pm1.4\times10^{6}$)	1.6×10^{3} ($\pm0.5\times10^{3}$)	445 (±195)	80 (±25)	0
Heterotrophic bacteria on R2A medium					
CFU cm^{-2} without antibiotics	5.8×10^{5} ($\pm1.7\times10^{5}$)	2.8×10^{4} ($\pm3.2\times10^{3}$)	1.2×10^{4} ($\pm4.4\times10^{3}$)	9.4×10^{3} ($\pm1.8\times10^{3}$)	3.0×10^{3} (±750)

largest number of cefazolin-resistant Enterobacteriaceae was measured in biofilms from hospital wastewater (54 ± 15%), followed by activated sludge biofilms (19 ± 12%) and the plant discharge with 11 ± 4.1%. The percentage resistance in surface water biofilms was 27 ± 10%. The highest percentage of cefotaxime resistance was found in hospital wastewater biofilms (17 ± 7.5%), followed by activated sludge biofilms (5.3 ± 0.6%), and the plant discharge (1.9 ± 0.4%). No cefotaxime-resistant Enterobacteriaceae were isolated from surface water biofilms.

Regarding the *amp*C resistance gene, a high degree of homology (71% by BLAST) was found among *Enterobacter cloacae* (GenBank AB016611), *Escherichia coli* (GenBank AF124205), and *Citrobacter freundii* (GenBank X76636). Primers were determined within the consensus regions of the coding sequence of *amp*C. The newly designed PCR primers ampC-For and ampC-Rev are presented in Table 1.

To verify the specificity of the molecular biology detection system, Enterobacteriaceae were isolated from surface water biofilms upstream, downstream, and directly at the influx of conditioned wastewater into the sewage treatment plant. Ceftazidime and penicillin G were added to the Chromocult medium to support growth of resistant Enterobacteriaceae. Isolates were tested by *amp*C-specific PCR. All of the 23 *amp*C-positive bacteria were identified as *Citrobacter*, *Enterobacter* and *E. coli*.

This result obtained from environmental samples correlated with the molecular biology prediction from the sequence data.

For PCR detection, genomic DNA was extracted from the biofilm populations from the different sampling points. Specific PCR amplicons of 550 bp were amplified from DNA fractions of hospital wastewater biofilms with strong DNA bands (Fig. 3, lanes 8–10). PCR experiments with

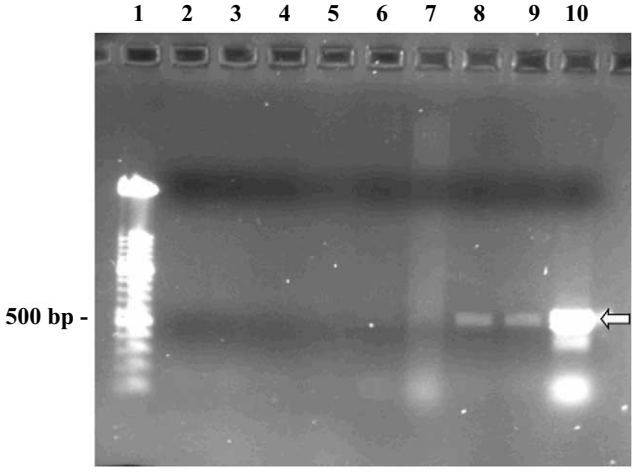

Fig. 3. Detection of the enterobacterial *amp*C resistance gene coding for β-lactamase in total DNAs isolated from different biofilms. Lane 1: DNA size marker; lane 2: negative control; lanes 3, 4: drinking water biofilms; lanes 5–7: biofilms from sewage treatment plant; lanes 8–10: biofilms from hospital wastewater. Position of *amp*C PCR product is indicated by arrow.

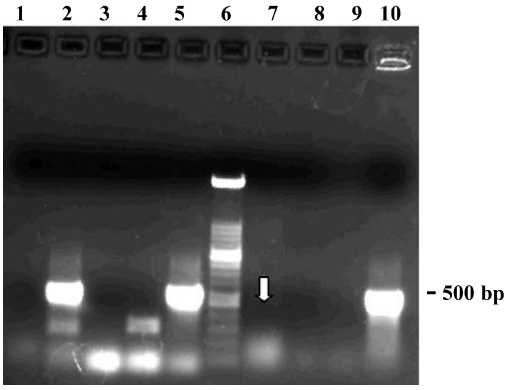

Fig. 4. Detection of the *mec*A gene coding for methicillin resistance in staphylococci. For PCR, total DNAs from hospital wastewater biofilms were used. Lane 1: negative control; lane 10: positive control (MRSA strain 170); lanes 7–9: PCR using DNAs from three different biofilm samples; a weak signal is indicated by the arrow; lanes 2–5: amplicons of a second PCR using aliquots (2 μl) from the first PCR (lanes 7–10); lane 6: DNA size marker.

biofilms from municipal sewage resulted in weak amplicons only (Fig. 3, lanes 5–7), indicating a lower target concentration. In order to detect low concentrations of amplified PCR products, an additional PCR experiment with 2-μl aliquots of the first PCR was performed. It was demonstrated that the *amp*C gene was also present in surface water biofilms and in biofilms from the drinking water distribution system (Table 4). In spite of the presence of the *amp*C resistance gene, no *E. coli* or coliform bacteria were cultivated from drinking water biofilms on synthetic media as mentioned above.

To confirm specificity with the PCR amplicons obtained, double-stranded sequence analysis of the *amp*C amplicons from drinking water biofilms revealed a high degree of homology with the *amp*C β-lactamase gene of *E. cloacae* (96%) and *Klebsiella pneumoniae* (86%), and a lower degree of homology for *C. freundii* and *E. coli*.

3.3. Staphylococci

Genomic DNA from biofilms was used for PCR detection of the *mec*A gene (Table 1). Specific amplicons were detected only in the biofilm from the hospital wastewaters; the other sampling points were negative in terms of the *mec*A resistance gene (Table 4).

In hospital biofilms only one of three samples produced a weak signal after a first PCR. In a second PCR assay with 2-μl aliquots of the first PCR solution, a stronger signal was detected (Fig. 4). The agarose gel was blotted and hybridized with the labelled mecA3-probe. The hybridization experiment confirmed the presence of low concentrations of the staphylococcal *mec*A resistance gene in biofilms from hospital wastewater. In addition, the PCR amplicon was sequenced in a double-stranded manner. A BLAST nucleotide search identified the *mec*A gene in *S. aureus*, *S. epidermidis*, and *S. sciuri* (each 96%).

Table 3
Percentage of antibiotic-resistant heterotrophic bacteria in biofilms

	Hospital ($n=5$)	Activated sludge at municipal sewage ($n=4$)	Efflux of municipal sewage ($n=5$)	Surface water ($n=3$)	Drinking water ($n=8$)
Vancomycin[a]	6.8(±5.0)	11(±3.8)	15(±10)	2.3(±0.5)	20(±10)
Ceftazidime[a]	45(±21)	44(±17)	27(±17)	11(±1.6)	5.1(±2.4)
Cefazolin[a]	58(±23)	39(±16)	39(±20)	8.1(0)	48(±27)
Penicillin G[a]	71(±25)	30(±8.0)	20(±6.7)	31(±3.3)	43(±26)
Imipenem[a]	8.1(±3.5)	2.8(±0.2)	0.6(±0.4)	0.4(±0.1)	0

[a]The values were relative to those without antibiotics. Each value of the independent analysis was used as 100% value for determination of the percentage of resistance.

Parallel cultivation experiments were performed to assess the persistence of staphylococci in hospital biofilms. Following enrichment with a synthetic medium [20], *S. aureus*, *S. chromogenes*, and *S. epidermidis* were cultivated. Of all staphylococci isolated, only the *S. epidermidis* isolate was resistant to methicillin. PCR and hybridization experiments confirmed these results.

3.4. Heterotrophic bacteria

Heterotrophic bacteria were analyzed and characterized to obtain information about culturable bacteria in biofilms and to investigate whether *van*A and *amp*C resistance genes were present in culturable or non-culturable drinking water bacteria. The counts of heterotrophic bacteria were highest in hospital biofilms (Table 2). Lower cell counts were found in surface and drinking water biofilms. The heterotrophic plate counts in wastewater systems from the hospital were found to be lower than the enterobacterial colony counts due to composition of the R2A medium, which is commonly used for the enumeration and subculture of oligotrophic bacteria from drinking water [11], but discriminated the growth of Enterobacteriaceae.

Heterotrophic bacteria resistant to vancomycin, ceftazidime, cefazolin and penicillin G were cultivated from all biofilms, including drinking water biofilms (Table 3). Imipenem-resistant heterotrophic bacteria were not cultivated from drinking water biofilms. In order to decide whether any of these resistance mechanisms were based on the presence of the *van*A gene for vancomycin or the *amp*C gene for β-lactam, PCR experiments were performed with the drinking water bacteria. The resistant bacteria were pooled for this purpose, and genomic DNA was extracted. *Van*A and *amp*C-specific PCR resulted in no distinct amplicons. In addition, heterotrophic drinking water bacteria from biofilms that were cultivated in the absence of antibiotics showed no *van*A or *amp*C-specific amplicons, indicating that no culturable bacteria with *van*A or *amp*C resistance genes were present in drinking water biofilms. Furthermore, no enterococcal-specific 23S rDNA-related sequences were detected in cultivated drinking water bacteria from biofilms in the presence or absence of antibiotics by PCR and hybridization experiments.

4. Discussion

The role of the environment in the emergence and

Table 4
Detection of resistance genes in bacteria and DNA prepared from biofilms

Biofilm sampling point	Cultivated resistant bacteria	Total DNA from biofilms
Vancomycin resistance for enterococci; detected resistance gene: *van*A		
Wastewater systems:		
•hospital ($n=4$)	positive	positive
•municipal sewage ($n=6$)	positive	positive
River water ($n=4$)	positive	positive
Drinking water ($n=6$)	negative	positive
Methicillin resistance for staphylococci; detected resistance gene: *mec*A		
Wastewater systems:		
•hospital ($n=4$)	positive	positive
• municipal sewage ($n=4$)	not tested	negative
River water ($n=4$)	not tested	negative
Drinking water ($n=6$)	negative	negative
β-Lactam resistance for Enterobacteriaceae; detected resistance gene: *amp*C		
Wastewater systems:		
• hospital ($n=5$)	not tested	positive
• municipal sewage ($n=6$)	positive	positive
River water ($n=4$)	positive	positive
Drinking water ($n=5$)	negative	positive

spread of antibiotic-resistant bacteria, their possible pathways, and the way in which environmental bacteria contribute to the spread of resistance genes are not yet clear. In this study, both conventional cultivation methods for the detection and identification of resistant bacteria, and genetic tests to detect bacterial resistance genes in biofilms were used to study various aquatic compartments, including hospital and municipal wastewater, surface water and drinking water systems.

Enterococci and Enterobacteriaceae were found in all biofilms except those in drinking water. Enterococci and Enterobacteriaceae are naturally occurring microorganisms from human and animal intestines. They are found in many aquatic compartments where enterococci have an advantage in terms of persistence and multiplication because of their tolerance to various environmental factors, such as alkaline pH, increased temperature and sodium chloride concentrations [21,22].

However, the incidence of nosocomial infections caused by members of the Enterobacteriaceae family which produce extended-spectrum β-lactamases and other enzymes capable of hydrolysing β-lactam antibiotics is increasing in the United States and Europe [23]. β-Lactamases, such as the *amp*C-lactamases, have been characterized from strains such as *E. cloacae*, *Pseudomonas aeruginosa*, *C. freundii*, *Serratia marcescens*, *Shigella flexneri*, *Shigella sonnei*, and *Proteus mirabilis* [24–28].

Enterococci are also nosocomial pathogens able to cause urinary tract infections, surgical wound infections, endocarditis and bacteremia [29]. Resistance to antibiotics, such as glycopeptides, is a problem in the therapy of these infections. In addition to their use in humans, glycopeptides such as Avoparcin have also been applied as growth promoters in livestock fattening. Studies showed that Avoparcin-selected glycopeptide-resistant enterococci are cross-resistant to vancomycin and teicoplanin, two antibiotics used in the therapy of humans. Glycopeptide-resistant *E. faecium* strains were isolated from animal feces from fattening farms using growth promoter-supplemented nutrients, but also from commercially available meat [1,3,30]. Since the EU-wide ban of Avoparcin for use as an agricultural antibiotic in 1997, the incidence of VRE in food and in the community appears to be decreasing, thereby demonstrating the effectiveness of the ban [31]. Within the framework of a European study, samples from urban raw sewage, treated sewage, surface water and hospital sewage in Sweden were screened for VRE. VRE were still isolated from 60% of untreated sewage samples, 35% of hospital sewage samples, 19% of treated sewage samples, and in low amounts from surface water [5]. Such studies consider the detection and spread of resistant bacteria in different compartments, such as communities, food and wastewater, but do not specifically address the occurrence of antibiotic-resistance genes in a spectrum of bacteria within different adjoining aquatic compartments.

In the present study, different aquatic compartments (wastewater, surface water and drinking water) within one municipal area were analyzed for resistant bacteria and their resistance genes. The utilization of resources by embankment filtration of surface water for drinking water conditioning has to be seen in connection with the data from these other adjoining compartments. The study of fecal indicators has dominated many studies of antibiotic-resistant bacteria in the aquatic environment, but little work has been done to assess the prevalence of drug-resistant bacteria in treated drinking water and their relationships to antibiotic-resistant microorganisms in untreated source waters [32,33]. They have found increased rates of resistant bacteria in drinking water within the distribution net by standard plate-count experiments, and have concluded that the treatment of raw water and its subsequent distribution select for antibiotic-resistant bacteria.

In agreement with these data, increased phenotypic resistance rates were also detected at the drinking water sampling points in the present study. Additionally, genotypical investigations concerning the underlying resistance mechanisms were performed to distinguish between intrinsic and acquired antibiotic resistance. The occurrence of the *van*A and *amp*C resistance genes in heterotrophic bacteria confirmed the influence of the water sources on the genotype of the drinking water bacteria.

Furthermore, most of the available literature data concerning resistant bacteria and their genotypes in the environment are from such as bulk water analysis, food samples and patient isolates. In the present study, biofilms were analyzed for resistant bacteria and their genotypes.

Different genotypes of glycopeptide resistance exist in enterococci. Of these the *van*A genotype is the most frequent in Central Europe [5,30,34]. The expression of glycopeptide resistance in enterococci can be induced by glycopeptides but not by other antibiotics [35].

In this study, high cell densities of enterococci and Enterobacteriaceae were cultivated from hospital wastewater biofilms, whereas densities from activated sludge were lower but still substantial. The lowest amounts were found in biofilms from conditioned wastewater of the plant and from surface water. These data affirm that the wastewater treatment at the plant reduced the release of bacteria into the aquatic environment. Vancomycin-resistant enterococci and β-lactam-hydrolysing Enterobacteriaceae were cultivated from all wastewater biofilms and were found in lower frequencies in biofilms from the surface water environment.

Genetic techniques were established to detect resistance genes directly in biofilm populations without cultivation, based on the assumption of gene carriage equalling resistance.

Strong amplicon DNA bands specific to *van*A and *amp*C, respectively, were found in hospital biofilms and in the sludge of the municipal sewage treatment plant.

Resistance genes were rarely detected in the biofilm from the effluent of the sewage treatment plant and from surface water, which correlates with the results of the cultivation experiments.

It was also shown that the vancomycin-resistance gene, *van*A, and the β-lactam-resistance gene, *amp*C, were amplified from the genomic DNA of various drinking water biofilms of a public distribution system, whereas no enterococci or Enterobacteriaceae were cultivated. Also, rRNA- related sequences, characteristic for enterococci, could not be found, which also indicates the absence of these bacteria. The molecular biological analysis based on the detection of rDNA-specific sequences confirmed the result that, despite the presence of *van*A, no enterococcal ribosomal amplicons were found in parallel PCR assays. It is clear that both the *van*A resistance gene and ribosomal DNA have to be amplified from drinking water biofilms if enterococci are present in these biofilms independent of their physiological state.

In light of these findings, the origin of the resistance gene sequences must be questioned. It is known that gene clusters homologous to *van*A and *van*B exist in microorganisms other then enterococci, such as *Paenibacillus popilliae*, *Amycolatopsis orientalis*, and *Streptomyces toyocaensi* [36]. Therefore, heterotrophic bacteria from drinking water biofilms were cultivated and tested for the occurrence of the resistance genes *van*A and *amp*C. No such genes were detected, and it must be concluded that these specific resistance genes are not present in these cultivable microorganisms. The possibility cannot be excluded, however, that related resistance genes of lower homology impart resistance to these bacteria and that these resistance genes cannot be detected with the enterococcal and enterobacterial-specific primers used for *van*A and *amp*C.

It is commonly known that only a small percentage of the microorganisms present in conditioned drinking water can be cultivated on synthetic media [9,10]. Therefore, the amplified genes may have been part of the genome of viable but non-cultivable aquatic bacteria.

Although the emergence of vancomycin-resistant enterococci can be attributed in part to the increasing use of vancomycin in clinical practice and to glycopeptide use in animal husbandry, the origins of enterococcal vancomycin resistance in the aquatic environment are not clear. Besides the transfer of resistant bacteria to the aquatic system, antibiotics in the aquatic environment could have an influence on the resistance situation. Recent studies demonstrated the occurrence of various antimicrobial compounds in wastewaters and in water treatment plants [37–40]. Although the antimicrobial concentrations found in wastewater are generally lower than the concentrations necessary to inhibit the growth of resistant bacteria, they are likely to affect susceptible bacteria and determine selection in favor of resistant bacteria, as is indicated by toxicity tests of aquatic bacteria [41,42].

Great diversity of microorganisms coupled with a high density of immobilized biomass support the exchange of resistance genes by horizontal gene transfer. Both aspects are found especially in biofilms from aquatic systems. About 60–90% of Gram-negative bacteria are able to exchange plasmids carrying resistance genes (R plasmids) via horizontal gene transfer [43]. Pike [44] found that the quantity of coliform bacteria excreted with feces and carrying resistance plasmids was large, amounting, for instance, to 67% in children treated with antibiotics and 46% in adults, in relation to the total number of bacteria. Plasmids could be transferred to sensitive strains of the same species and even to different species. Interspecies transfer of R plasmids has been shown to occur under natural environmental conditions [6,22,45], and the potential for plasmid transfer can be associated with higher rates of survival in an aquatic environment [46,47]. Metabolic activities, shifts of growth influenced by temperature or nutrient limitation, ions such as calcium or magnesium, and increased phosphate levels are natural factors supporting transfer of a genetic element in an aquatic environment [6]. Consequently, it has to be considered whether apathogenic ubiquitous bacterial species, such as heterotrophic bacteria with a vancomycin-resistance gene, serve as carriers of genetically determined resistance, passing these resistance genes on to pathogenic bacteria by horizontal gene transfer.

Another important pathogenic agent in intensive care units is *S. aureus* [23]. In infections with methicillin-resistant *S. aureus* strains, only a few antibiotics such as vancomycin or teicoplanin are available, due to the resistance against all other β-lactam antibiotics [48]. Since 1990, the methicillin resistance of *S. aureus* has increased significantly [49], and is of special concern because of the limited therapeutic possibilities it entails. In this study, staphylococci were only found in hospital wastewater biofilms. The *mec*A gene was detected, but cultivation experiments showed the occurrence of methicillin-resistant coagulase-negative strains only, and not of methicillin-resistant *S. aureus* strains.

The main results of this study can be summarized as follows:

- Vancomycin-resistant enterococci and β-lactam-hydrolysing Enterobacteriaceae were cultivated from all wastewater biofilms and were found less frequently in biofilms from the surface water environment.
- The *van*A vancomycin-resistance gene from enterococci, the *mec*A methicillin-resistance gene from staphylococci, and the *amp*C β-lactam-resistance gene from Enterobacteriaceae were amplified predominantly from hospital wastewater biofilms. *Van*A genes and *amp*C genes were also detected in all other wastewater and environmental biofilms.
- The *van*A and *amp*C antibiotic-resistance genes were detected in drinking water biofilms, whereas enterococci and Enterobacteriaceae, which are said to carry these genes originally, were never found in these biofilms. It

seems possible that the amplified genes were part of the genome of viable but non-cultivable aquatic bacteria.

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