Concentration of facultative pathogenic bacteria and antibiotic resistance genes during sewage treatment and in receiving rivers
Stefanie Heß, Frauke Lüddeke and Claudia Gallert

ABSTRACT
Whereas the hygienic condition of drinking and bathing water by law must be monitored by culture-based methods, for quantification of microbes and antibiotic resistance in soil or the aquatic environment, often molecular genetic assays are used. For comparison of both methods, knowledge of their correlation is necessary. Therefore the population of total bacteria, Escherichia coli, enterococci and staphylococci during sewage treatment and in receiving river water was compared by agar plating and quantitative polymerase chain reaction (qPCR) assays. In parallel, all samples were investigated for clinically relevant antibiotic resistance genes. Whereas plating and qPCR data for total bacteria correlated well in sewage after primary treatment, qPCR data of river water indicated higher cell numbers for E. coli. It is unknown if these cells are ‘only’ not growing under standard conditions or if they are dead. Corresponding to the amount of non-culturable cells, the ‘breakpoints’ for monitoring water quality should be adapted. The abundances of clinically relevant antibiotic resistance genes in river water were in the same order of magnitude or even higher than in treated sewage. For estimation of the health risk it is important to investigate which species carry respective genes and whether these genes are disseminated via gene transfer.

Key words | advanced sewage treatment technologies, antibiotic resistance genes, aquatic environment, fecal indicators, staphylococci, survival and growth

INTRODUCTION
In addition to culture-based investigations, there is an increasing number of studies analyzing bacterial communities and abundances of antibiotic resistance genes in soil or the aqueous environment by molecular genetic approaches such as quantitative polymerase chain reaction (qPCR) or metagenomics (Bengtsson-Palme et al. 2014; Bäumlisberger et al. 2015). Guidelines for monitoring the hygienic quality of drinking water (WHO Drinking Water Directive 2011) or bathing water (EU Bathing Water Directive 2006) are still relying on culture-based approaches. Aside of the clear advantages of culture-based quantification by determining MPN (most probable numbers) or CFU (colony forming units), the apparent shortcomings are the relatively long time required for growth of the bacteria and an under-estimation of cell numbers due to those species that are non-culturable on the chosen media. Hitherto identification and quantification of microbes by molecular assays are, however, not yet standard assays to describe the hygienic state of drinking and bathing water in the European Union, aggravating the comparability of published studies.

Although to date no legal thresholds for the emission of bacteria and antibiotic resistance genes in treated sewage exist, a number of studies address the retention of micropollutants and of bacteria by so-called ‘advanced sewage treatment technologies’ (Alexander et al. 2015). At present water-borne diseases and the spread of antibiotic resistance are considered major threats for human and animal health in the 21st century (WHO 2014). To evaluate the risk potential of the permanent discharge of (treated) sewage into receiving water bodies for human and animal health, culture-based approaches and the results of qPCR assays should be compared. However, little is known about the correlation of these data (Krometis et al. 2013).

To bridge this gap, in this study a microbial characterization of sewage and river water samples by culture-based and
by qPCR assays was undertaken. It was investigated whether the thresholds defined in the EU Bathing Water Directive (2006) for culture-based assays could be adopted for quantitative molecular genetic assays. For this purpose, 44 sewage samples (raw and treated sewage) and 40 samples taken downstream of the respective receiving river water were analyzed for their concentration of total bacteria, *Escherichia coli*, enterococci and staphylococci, as well as for the presence of clinically relevant antibiotic resistance genes.

**MATERIAL AND METHODS**

**Sampling sites and sampling**

Within the project SchussenAktivplus (for details see www.schussenaktivplus.de), from 2012 to 2014, eight sampling campaigns were conducted (03.05.2012, 04.07.2012, 24.10.2012, 12.03.2013, 14.05.2013, 10.06.2013, 19.11.2013, 07.05.2014). At each campaign, sewage from the sewage treatment plant (STP) Eriskirch (overall 44 samples) and water samples from the receiving Rivers Schussen and Argen (overall 40 samples), both of which are tributaries of Lake Constance (Baden-Württemberg, South Germany), were collected. Twenty-four hour composite samples of sewage after primary and secondary treatment in the STP Eriskirch (40,000 population equivalents including sewage from one hospital and two senior residences) as well as from five installed processes for tertiary treatment (Figure S1 in the Supplementary Material, available with the online version of this paper; for details see Lüddeke et al. [2015]) were taken. In addition water samples were taken from the River Schussen upstream of the inlet of the stormwater overflow basin Mariatal (but downstream of the inlets of 18 STPs, sample PN 0), downstream of the inlet of the stormwater overflow basin Mariatal (sample PN 1), downstream of the inlet of the STP Ravensburg-Langwiese (170,000 population equivalents, sample PN 3) and downstream of the inlet of the STP Eriskirch near the river mouth of the Schussen into Lake Constance (sample PN 6). As a reference of a ‘less’ anthropogenic affected river water, a sample of the Argen river (PN 4; ‘only’ downstream of the inlet of the STP Isny (40,000 population equivalents)) was taken (Figure S2, available with the online version of this paper). These samples were withdrawn 20–50 cm below the surface in the middle of the river. All water samples were filled into autoclaved glass bottles and refrigerated at 4 °C until processing. Filtration and centrifugation of the samples was finished at the latest 48 h after sampling.

**Enumeration of total bacteria, total culturable bacteria, *E. coli*, enterococci and staphylococci, isolation and identification of pure cultures and tests for their antibiotic resistance**

To determine the population density of total bacteria, cells (10 μL sample) were stained with 20 μL of a 0.1 μmol/L 4,6-diamidino-2-phenylindol (DAPI)-solution on ice for 20 minutes in the dark. Blue fluorescent cells were then counted using a Zeiss fluorescence-microscope (Carl Zeiss Microscopy GmbH, Göttingen, Germany). Staining of duplicate sample preparations was performed and blue fluorescent cells of 11 view fields per sample, respectively (overall 22 view fields per sample) were counted. For enumeration of culturable bacteria, 100 μL of three dilution steps of the respective sewage or river water were plated in duplicate on nutrient agar (Merck Millipore 105443, Darmstadt, Germany) and incubated for 1 week at 21 °C. Grown colonies were then counted and bacterial densities calculated.

Selective media were applied for enumeration and isolation of *E. coli* (ECD-agar), enterococci (Slanetz-Bartley-agar and bile-esculin-agar) and staphylococci (Chapman-Stone-agar supplemented with 0.05 g/L sodium azide and Mannit-Salt-agar), as described by Lüddeke et al. [2015]. If necessary bacteria were concentrated by membrane filtration (cellulose nitrate, pore size 0.45 μm, Ø 50 mm; Sartorius, Göttingen, Germany) before plating. Per sample between 15 and 20 colonies were randomly picked and identified using Micronaut-Staph®-microtiter plates for staphylococci and Micronaut-Strep2®-microtiter plates for enterococci, according to manufacturer’s instructions (MERLIN, Gesellschaft für mikrobiologische Diagnostika, Bornheim, Germany). *E. coli* was identified by targeting a specific *tuf*-gene fragment (primers and PCR-conditions as described by Maheux et al. [2009]). Briefly, the DNA amplification was performed in a total volume of 25 μL containing 0.625 units True Start HS Taq DNA Polymerase, 2.5 mmol/L MgCl₂ (Thermo Fisher Scientific, Waltham, MA), 0.25 mM of each dNTP, 10 μmol/L of both primers and 0.5 μL of template DNA (extraction with phenol/chloroform, 40 ng/μL).

Ampicillin-resistant *E. coli* isolates (n = 864) in agar diffusion tests (DIN 58940 2011) were screened for carrying one of the *blaTEM*-genes, using the primers and PCR conditions as described by Monstein et al. [2007]. Susceptibility against erythromycin of 1465 *Staphylococcus* isolates was also tested with the agar-diffusion test according to DIN 58940 (2011). Subsequently, all erythromycin-resistant staphylococci were screened for carrying *erm*(A), *erm*(B), *erm*(C) or *erm*(43) genes (used primers and PCR conditions are listed in...
Table S1, available with the online version of this paper). Numbers of isolates obtained from samples and carrying the respective resistance genes, were correlated to respective plate counts to obtain the concentration of \(E.\) \(coli\) or staphylococci harboring the respective genes (Table 2).

**Enumeration of total bacteria, \(E.\) \(coli\), enterococci and staphylococci as well as of antibiotic resistance genes in sewage and river water samples by qPCR**

In addition to the culture-based approach, total bacteria, \(E.\) \(coli\), enterococci and staphylococci as well as antibiotic resistance genes \(bla_{TEM}\), \(erm(A)\), \(erm(B)\), \(erm(C)\) and \(erm(43)\) were determined by qPCR using the MiniOpticon™ CFX96 (BioRad; Hercules, CA). From 200 to 400 mL of the respective samples were centrifuged (20 minutes, 1,860 g; ROTIXA/K, Hettich, Tuttinglen, Germany) and the washed pellets frozen before DNA was extracted using the PowerViral™ Environmental RNA/DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA). To control the extraction efficiency, duplicate samples were spiked with \(3.0 \times 10^8\) cells of \(Staphylococcus\) \(aureus\) DSM 2569. DNA was extracted, the concentration of the \(Staphylococcus\)-specific \(tuf\) gene fragment (Matsuda et al. 2007) determined by qPCR and subsequently compared to that of the ‘natural’ sample: almost 100% of the number of added \(Staphylococcus\) cells were recovered. To exclude that inhibitors of the PCR affected quantification, extracts were spiked with a defined gene copy number of e.g. \(erm(A)\). The recovery rate was checked. The qPCR assays (total volume: 20 \(\mu\)L) were performed with SsoAdvanced Universal Sybr Green Supermix (10 \(\mu\)L; BioRad), 1 \(\mu\)L of the respective 10 \(\mu\)mol/L primer-solution (used primers are listed in Table S1) and 8 \(\mu\)L of the RNA-free DNA extract (5 ng/\(\mu\)L), respectively diluted with DNase-free water. Each sample was analyzed two times in duplicate (standard deviation \(<0.2\)). As a quantification-standard, either a plasmid (pEX A2) with the respective amplicon (16S rDNA) as an insert (purchased from MWG Eurofins Genomics, Ebersberg, Germany) or the amplicon itself, quantified with a NanoDrop spectrometer (NANO DROP 2000 Spectrophotometer, Thermo Scientific, Waltham, Massachusetts, USA), was used. Comparison of the purchased standard and the use of the amplicon as standard revealed a deviation of \(<0.1\) log-units. Results (melting curve data (Table S1), \(Ct\)- and \(Cq\)-values – base line subtracted quantification) were obtained using the Data Analysis Module (CFX Manager™ software, BioRad). To compare qPCR-data with respective CFU-values from the culture-based approach, average gene copy numbers of the targets were used: according to Stoddard et al. (2015), average copy numbers of the 16S rDNA-gene in bacterial cells were three and in enterococci or staphylococci five, whereas the \(uidA\)-gene copy number of \(E.\) \(coli\) was one (Chern et al. 2011).

**Statistical analysis**

Abundances of respective species and resistance genes in sewage and river water samples in Tables 1 and 2 represent average values (\(\pm\) represents standard deviations). Changes in bacterial concentrations during sewage treatment and in river waters determined with qPCR and the culture based approach were tested in respect to statistical significance using bilateral t-test. Levels of significance (\(p\)-values) were added in brackets. For comparison of qPCR data with culture-based data, correlation diagrams and box plots were created with Microsoft Excel (Figure 1(a) and 1(b)). The line dividing the rectangle formed by the first and third quartile displays the median of the respective data. Extreme values are indicated by the whiskers (Figure 1(b)). Differences between the concentration determined by the molecular genetic approach and the concentration determined by the culture-based approach were calculated and expressed as ‘non-culturable’ bacteria which were either dead or in a ‘viable but not culturable (VBNC)’-status.

**RESULTS AND DISCUSSION**

**Quantification of total and culturable bacteria during sewage treatment and in receiving river water of the Schussen and Argen**

**Changes of the total population during sewage treatment**

The numbers of total bacteria in effluents from secondary and several pilot-scale tertiary treatment processes in the STP of Eriskirch were in the same order of magnitude \((p \leq 0.001;\) Table 1). Whereas the results of Seo et al. (2010) revealed an underestimation of total cells using DAPI for staining, there were no significant differences of the concentration of total bacteria determined by microscopy after DAPI-staining or by qPCR of the 16S rRNA gene after correction for the average number of respective gene copies \((p \leq 0.001)\). Within the confidence limits, the bacterial densities in sewage were identical for both counting methods. Although only little more than 90% of the total bacteria were eliminated during conventional and advanced sewage treatment (Figure 1(b), Table 1), the remaining bacterial concentration (approximately \(5 \times 10^8\) per mL) was not
Table 1 | Abundances of total bacteria, *E. coli*, enterococci and staphylococci from sewage during treatment and in the receiving river water

<table>
<thead>
<tr>
<th>Sample source (in parentheses number of samples)</th>
<th>Bacteria*</th>
<th><em>E. coli</em>†</th>
<th>Enterococci*</th>
<th>Staphylococci*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cells/mL (DAPI)</td>
<td>16S rDNA copies/ mL (Divisor: 3)</td>
<td>Heterotrophic plate count/mL</td>
<td>CFU/ 100 mL</td>
</tr>
<tr>
<td>STP Eriskirch</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>cf PT (6)</td>
<td>7.8 ± 0.2</td>
<td>8.3 ± 0.2</td>
<td>7.2 ± 0.6</td>
<td>6.4 ± 0.1</td>
</tr>
<tr>
<td>cf SS (7)</td>
<td>7.0 ± 0.2</td>
<td>6.7 ± 0.6</td>
<td>4.5 ± 0.3</td>
<td>4.2 ± 0.5</td>
</tr>
<tr>
<td>cf FF (8)</td>
<td>6.7 ± 0.3</td>
<td>6.5 ± 0.8</td>
<td>4.3 ± 0.4</td>
<td>3.7 ± 0.4</td>
</tr>
<tr>
<td>cf Ak (2)</td>
<td>6.7 ± 0.1</td>
<td>6.3 ± 1.3</td>
<td>4.4 ± 0.3</td>
<td>3.2 ± 0.1</td>
</tr>
<tr>
<td>cf Oz (5)</td>
<td>6.8 ± 0.1</td>
<td>6.4 ± 0.2</td>
<td>4.4 ± 0.1</td>
<td>2.8 ± 0.6</td>
</tr>
<tr>
<td>cf OzSa (6)</td>
<td>6.7 ± 0.2</td>
<td>6.9 ± 0.7</td>
<td>4.5 ± 0.2</td>
<td>3.0 ± 0.6</td>
</tr>
<tr>
<td>cf OzAk (6)</td>
<td>6.7 ± 0.2</td>
<td>6.7 ± 0.7</td>
<td>4.2 ± 0.3</td>
<td>2.7 ± 0.5</td>
</tr>
<tr>
<td>cf OzSaAk (4)</td>
<td>6.7 ± 0.2</td>
<td>5.5 ± 0.2</td>
<td>4.5 ± 0.1</td>
<td>2.9 ± 0.5</td>
</tr>
<tr>
<td>Receiving river water</td>
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<tr>
<td>of the Schussen and Argen as a much</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>PN 0 (Schussen) (8)</td>
<td>6.6 ± 0.1</td>
<td>6.7 ± 0.5</td>
<td>4.3 ± 0.2</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td>PN 1 (Schussen) (8)</td>
<td>6.6 ± 0.1</td>
<td>6.9 ± 0.4</td>
<td>4.2 ± 0.2</td>
<td>3.0 ± 0.5</td>
</tr>
<tr>
<td>PN 3 (Schussen) (8)</td>
<td>6.7 ± 0.1</td>
<td>7.1 ± 0.5</td>
<td>4.5 ± 0.2</td>
<td>2.9 ± 0.6</td>
</tr>
<tr>
<td>PN 6 (Schussen) (8)</td>
<td>6.6 ± 0.2</td>
<td>6.6 ± 0.5</td>
<td>4.6 ± 0.2</td>
<td>3.1 ± 0.7</td>
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<tr>
<td>reference river water</td>
<td></td>
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<tr>
<td>PN 4 (Argen) (8)</td>
<td>6.5 ± 0.2</td>
<td>6.5 ± 0.6</td>
<td>4.0 ± 0.4</td>
<td>2.2 ± 0.5</td>
</tr>
</tbody>
</table>

*Average values derived from eight sampling campaigns during a period of 3 years, represented in log_{10} units per volume.
†According to Stoddard et al. 2015. <DL, below detection limit (<10^1 gene-copies per reaction); ± represents standard deviations; numbers in brackets indicate that the respective concentration was close to the detection limit including standard deviation; full-scale effluents after primary treatment (cf PT), biological treatment and secondary settling (cf SS) and flocculation filtration (cf FF), as well as pilot-scale effluents of granulated charcoal filtration (cf Ak), ozonation (cf Oz) and after ozonation followed by sand filtration (cf OzSa), granulated charcoal filtration (cf OzAk) and a combination of a sand and granulated activated charcoal filtration (cf OzSaAk).
Table 2 | Abundances of the antibiotic resistance genes *bla*TEM, *erm*(A), *erm*(B), *erm*(C), *erm*(43) during sewage treatment and in receiving river water

<table>
<thead>
<tr>
<th>Sample source (in parentheses number of samples)</th>
<th><em>bla</em>TEM CFU/100 mL (E. coli)a</th>
<th><em>erm</em> (B) Copies/100 mL*</th>
<th><em>erm</em> (A) CFU/100 mL* (staphylococci)</th>
<th><em>erm</em> (C) Copies/100 mL* (staphylococci)</th>
<th><em>erm</em> (43) CFU/100 mL* (staphylococci)</th>
<th><em>erm</em> (43) Copies/100 mL*</th>
</tr>
</thead>
<tbody>
<tr>
<td>STP Eriskirch</td>
<td>ef PT (6) 5.6 ± 0.2 6.0 ± 0.4 9.7 ± 0.5 Not isolated</td>
<td>&lt;DL</td>
<td>3.3 ± 0.3 6.0 ± 0.4 (3.0) 4.7 ± 0.3</td>
<td>&gt;DL</td>
<td>Not isolated</td>
<td>&lt;DL</td>
</tr>
<tr>
<td></td>
<td>ef SS (7) 3.6 ± 0.3 4.5 ± 0.3 5.8 ± 0.6 Not isolated</td>
<td>&lt;DL</td>
<td>0.6 ± 0.2 3.3 ± 0.1 Not isolated</td>
<td>&lt;DL</td>
<td>Not isolated</td>
<td>&lt;DL</td>
</tr>
<tr>
<td></td>
<td>ef FF (8) 3.0 ± 0.4 4.0 ± 0.3 4.5 ± 0.4 Not isolated</td>
<td>&lt;DL</td>
<td>–0.1 ± 0.4 4.0 ± 0.3 Not isolated</td>
<td>&lt;DL</td>
<td>Not isolated</td>
<td>&lt;DL</td>
</tr>
<tr>
<td></td>
<td>ef Ak (2) 2.3 ± 0.1 &lt;DL &lt;DL Not isolated</td>
<td>&lt;DL</td>
<td>–0.2 ± (not isolated) Not isolated</td>
<td>&lt;DL</td>
<td>Not isolated</td>
<td>&lt;DL</td>
</tr>
<tr>
<td></td>
<td>ef Oz (5) 0.8 ± 0.1 3.9 ± 0.1 4.3 ± 0.4 (–1.4) Not isolated</td>
<td>&lt;DL</td>
<td>–0.2 ± 0.4 3.4 ± 0.1 Not isolated</td>
<td>&lt;DL</td>
<td>Not isolated</td>
<td>&lt;DL</td>
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<tr>
<td></td>
<td>ef OzSa (6) 1.2 (not isolated) 3.6</td>
<td>4.5 ± 0.2 Not isolated</td>
<td>3.4 ± 0.1 (3.3) Not isolated</td>
<td>&lt;DL</td>
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<tr>
<td></td>
<td>ef OzAk (6) 2.2 ± 0.1 4.0 ± 0.1 3.8 ± 0.2 Not isolated</td>
<td>&lt;DL</td>
<td>–1.5 (not isolated) &lt;DL Not isolated</td>
<td>&lt;DL</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>ef OzSaAk (4) 2.3 (not isolated) 3.2 ± 0.1 (4.0) Not isolated</td>
<td>&lt;DL</td>
<td>–1.1 ± 0.5 (3.7) Not isolated</td>
<td>&lt;DL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Receiving water samples</td>
<td>PN 0 (Schussen) 2.2 ± 0.3 4.2 ± 0.5 6.4 ± 0.8 Not isolated</td>
<td>4.9 ± 0.1 (4.5) 0.2 ± 0.2 4.4 ± 0.2</td>
<td></td>
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<tr>
<td></td>
<td>PN 1 (Schussen) 2.3 ± 0.5 4.3 ± 0.7 6.0 ± 0.6 Not isolated</td>
<td>4.7 ± 0.3 0.3 ± 0.1 4.6 ± 0.4 0.4 ± 0.1 5.5 ± 0.7</td>
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<tr>
<td></td>
<td>PN 3 (Schussen) 2.2 ± 0.4 4.4 ± 0.9 6.2 ± 0.8 Not isolated</td>
<td>3.9 ± 0.6 –0.2 (not isolated) 4.1 ± 0.3 0.5 ± 0.1 6.2 ± 0.6</td>
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<tr>
<td></td>
<td>PN 6 (Schussen) 2.5 ± 0.7 3.8 ± 0.3 5.3 ± 0.6 Not isolated</td>
<td>4.7 ± 0.3 0.4 ± 0.3 3.7 ± 0.3 0.4 ± 0.3 4.0 ± 0.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PN 4 (Argen) 1.2 ± 0.3 3.4 ± 0.6 4.4 ± 0.6 Not isolated</td>
<td>4.6 ± 0.5 0.5 ± 0.3 3.9 ± 0.4 Not isolated</td>
<td>3.6 ± 0.2</td>
<td></td>
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</tr>
</tbody>
</table>

*aAverage values derived from eight sampling campaigns during a period of 3 years, represented in log10 units per volume, <DL, below detection limit (<10^3 gene copies per reaction); ± represents standard deviations; numbers in brackets indicate that the respective concentration was close to the detection limit including standard deviation; full-scale effluents after primary treatment (ef PT), biological treatment and secondary settling (ef SS) and flocculation filtration (ef FF), as well as pilot-scale effluents of granulated charcoal filtration (ef Ak), ozonation (ef Oz) and after ozonation followed by sand filtration (ef OzSa), granulated charcoal filtration (ef OzAk) and a combination of a sand and granulated activated charcoal filtration (ef OzSaAk).
significantly \((p \leq 0.001)\) higher than the total bacterial concentration present in the receiving water of the River Schussen (Table 1). Concentrations of total bacteria per millilitre in anthropogenically highly influenced river water of the Schussen were the same upstream and downstream of storm water or sewage inlets \((p \leq 0.001;\) Table 1, Figure S2). The differences in the concentrations determined by qPCR of the 16S rRNA gene and the concentrations determined by plating were highest in river water (Table 1). Either the percentage of cells being in the VBNC-status is higher than in sewage after primary treatment or a minor percentage of the species in rivers is able to grow on nutrient rich agar.

A comparison of total cell numbers with those of culturable cell numbers (plating for CFU determination) revealed that the percentage of non-culturable cells increased during sewage treatment (Table 1). While after primary treatment the concentration of culturable bacteria (heterotrophic plate count) in sewage was within the range of inaccuracy of the methods (no significant differences were detectable; \(p \leq 0.001\)), after biological treatment of sewage and separation of the sludge by secondary settling (ef SS, Figure S1) the concentration of culturable bacteria was up to 2.5 log-units lower than the concentration of total bacteria, as determined by qPCR and corrected for gene copy numbers (Table 1).

**Changes of E. coli, enterococci and staphylococci concentrations during sewage treatment**

In sewage and during sewage treatment 5% or less of the total bacteria belonged to *E. coli*, enterococci and
staphylococci (Table 1). Whereas culture-based and qPCR-based analyses indicated a decreasing number of enterococci and of staphylococci during progressing full-scale and pilot-scale sewage treatment, the number of living cells of E. coli decreased similarly, but total E. coli cell numbers, based on qPCR of the uidA gene, remained much higher. Living staphylococci disappeared almost completely during classical sewage treatment and especially during ozonation as a tertiary treatment process (Table 1).

Differences between culture-based E. coli numbers and those derived from qPCR were smallest after primary treatment (Table 1 and Figure 1(b)), indicating ‘healthy’ well growing cells. The differences between culture-based and qPCR data were increasing during mechanical (Table 1) and biological treatment of the sewage. The amounts of non-culturable E. coli were highest after ozonation (Table 1) implicating that the cells were still intact (extracellular DNA was not analyzed), but were not able to grow under standard conditions. Based on these data, it is not possible to conclude if these cells were dead or if they were in a reversible VBNC-status. Whereas the amount of culturable E. coli cells in the influent and effluent of STPs with similar treatment technology as in Eriskirch were in the same order of magnitude (Lüddeke et al. 2015), reported concentrations of E. coli that were determined by qPCR in raw and treated sewage vary widely. About 10^8 E. coli cells per 100 mL were found in sewage after primary treatment and between 10^7 and 10^6 per 100 mL in treated sewage by Wéry et al. (2008), representing, respectively, a 1–2 or 3 log-units higher population density in treated sewage than found in sewage of the STP Eriskirch (Table 1). The concentrations of Enterobacteriaceae in raw and treated sewage, reported by Alexander et al. (2015), were even lower than the concentrations of culturable E. coli in the sewage samples taken in the STP of Eriskirch (2.5–4.2 log_{10} cell equivalents per 100 mL influents of municipal STPs and 0.9–2.1 log_{10} cell equivalents per 100 mL in effluents).

Contrary to the data for E. coli, the differences between culturable enterococci and respective qPCR data for the total population were disappearingly small, varying between 0 log-units in sewage after primary treatment (ef PT) and 0.2 log-units after ozonation (ef Oz; Table 1). Apparently, the time span for survival in a culturable state was longer for the isolated Enterococcus species than for E. coli isolates. In contrast to E. coli, ozone seemed to have a lysogenic effect on enterococci: the concentration of culturable enterococci, determined by plating and of total enterococci gene copies determined by qPCR, respectively, decreased in parallel during ozonation (Table 1). Further molecular genetic experiments are necessary to unravel the effect of ozone on Gram-positive and Gram-negative cells.

Concentrations of culturable staphylococci decreased most during classical sewage treatment and were, especially after secondary settling (ef SS), lower than 10^1 cells per 100 mL. Thus, staphylococci display only a minor group of bacteria that contribute to the flora of sewage or treated sewage (Table 1).

Bacteria in the receiving water of the Schussen river

The differences in concentrations of culturable bacteria, E. coli, enterococci and staphylococci (CFU values) and the respective concentrations of total bacteria, E. coli, enterococci and staphylococci (determined by qPCR of marker genes and corrected for gene numbers per cell) in receiving river samples were higher than those in sewage taken during standard sewage treatment in the STP Eriskirch. Since qPCR detects also dead cells and cells being in the VBNC-status, the concentrations of E. coli, Enterococcus species and Staphylococcus species determined with qPCR were, as expected, higher than those determined with the culture-based approach (Figure 1(a) and 1(b)). Nevertheless, the much higher differences of E. coli concentrations (up to 3.5 log-units) determined by qPCR and by the culture-based approach than of Enterococcus and Staphylococcus species (up to 1.7 log-units) were not expected (Table 1 and Figure 1(b)). Some species of the genera living naturally in an aquatic environment might not be able to grow on chosen media or might be overgrown by others due to very slow growth under respective conditions. A comparison of the numbers of colonies of staphylococci after 48, 72 and 90 h of incubation revealed that some cells needed more time to grow and form colonies on agar plates (Figure 2). Whereas the concentration of staphylococci from sewage after primary treatment increased only about 0.1 log-units, when agar plates were incubated for 72 h instead of 48 h, the concentration of staphylococci from river water increased about 0.7 log-units after incubation for 72 h instead of 48 h (Figure 2). Whereas human pathogens and commensals are comparatively well investigated, there is still little knowledge about the diversity and function of these under standard conditions ‘non-culturable’ bacteria living in aquatic ecosystems. For detection of such species by species-specific genes it is necessary to design gene probes for quantification of abundances in the respective ecosystem. However, species and strain identification by qPCR and calculation of abundances is not enough to
understand their role in ecosystems. Physiological or/and epidemiological data are required in addition, which could either be obtained by culture-dependent assays or by molecular assays, referring to transcriptomics or metabolic enzymes.

The concentrations of total bacteria, *E. coli*, enterococci and staphylococci along the River Schussen (from PN 0 to PN 6; Figure S2) were in the same order as in treated sewage (ef FF; Table 1; \( p \leq 0.001 \)). The reason for that may be that there were already several inlets of treated sewage and of stormwater from overflow basins upstream of, e.g. the sampling point PN 0 (Auerbach et al. 2013). Furthermore run-off water from agriculture might have an overlapping effect. An influence of treated sewage from STPs on microbial communities and bacterial concentrations was observed by several studies (e.g. Czekalski et al. 2015). The concentrations of total bacteria, *E. coli*, enterococci and staphylococci in the ‘reference river’ Argen (sample PN 4) were lower than those in the river water of the Schussen. Although the Argen river is less anthropogenically affected, an almost double runoff (mean discharge of the Schussen: 11.8 m³/s; mean discharge of the Argen: 20.1 m³/s; Auerbach et al. 2013) leads to a discharge of *E. coli*, enterococci and staphylococci into Lake Constance in the same order of magnitude (*E. coli* and enterococci: \( \sim 4 \times 10^7 \) CFU/s; staphylococci: \( 3 \times 10^6 \) CFU/s) as by the River Schussen.

By correlation of *E. coli*, *Enterococcus* sp. and *Staphylococcus* sp. concentrations from culture-based and qPCR analyses, the highest differences were observed in surface water (Figure 1(a)). The data reveal that a classification of the sanitation state of river water should not be based alone on culture-based ‘breakpoints’ as for instance fixed in the EU Bathing Water Directive (2006). Further studies are necessary to estimate the risk potential for human and animal health resulting from a significant proportion of ‘non-culturable’ bacteria in pristine or anthropogenic influenced ecosystems.

**Abundance of antibiotic resistance genes in sewage and receiving river water**

Neither in sewage, nor in river water are the concentration of *bla*\(_{\text{TEM}}\)-harboring *E. coli*, *erm*(B)-harboring enterococci/staphylococci and *erm*(A), *erm*(C)- or *erm*(43)-harboring staphylococci correlated with the respective concentration of these antibiotic resistance genes in the sample (Table 2). This observation was expected since the copy number of the respective genes per cell is not exactly known and may slightly differ with respect to the growth phase and species. Furthermore, the dissemination of the respective antibiotic resistance genes is not restricted to the mentioned genera. Whereas abundances of copy numbers of *bla*\(_{\text{TEM}}\) in relation to the isolates carrying *bla*\(_{\text{TEM}}\) were rather small (Table 2), indicating a recently ‘limited’ distribution among Gram-negative bacteria, numbers of *erm*(A), *erm*(B), *erm*(C) and *erm*(43) were much higher than numbers of enterococci and staphylococci. The detected copy numbers of *erm*(B) exceeded the concentration of enterococci by more than 3 log-units (Tables 1 and 2). Rodriguez-Mozaz et al. (2015) detected similar high *erm*(B) copy numbers in the influent and effluent of a municipal STP and the receiving river. A possible explanation could be that the macrolide, lincosamide and streptogramin B resistance gene *erm*(B) is not only harbored by enterococci but was hitherto also detected in species of 16 other genera (http://ardb.cbcb.umd.edu/).
Moreover, for estimation of the risk potential for human and animal health, it is essential to know which species carry these genes, whether they are located on transferable elements, transfer rates and the conditions of gene transfer events.

The comparison of the data obtained with the culture-based approach and with molecular genetic assays complement each other and provide new information about the status and survival of bacterial cells during sewage treatment and after discharge into receiving rivers. Further investigations are necessary for a comprehensive knowledge of the effect of the chronic discharges of bacteria and antibiotic resistance genes into aquatic environments and to calculate the resultant health risk. Simultaneously, sewage treatment processes must be optimized in order to reduce bacteria and antibiotic resistance gene emission.

CONCLUSIONS

- Whereas the concentrations of total bacteria, E. coli, enterococci and staphylococci calculated from qPCR of specific genes and determined by plating (cfu) correlated well in sewage after primary treatment, qPCR data of treated sewage and of river water revealed much higher copy numbers for E. coli. In consequence, the culture-based breakpoints for monitoring water quality may underestimate the real abundance of E. coli, if gene copy numbers do not change significantly in different ecosystems.

- The abundance of clinically relevant antibiotic resistance genes in river water were in the same order of magnitude or higher than in treated sewage. For estimation of the resultant health risk, it is necessary to know which species carry respective genes and if these genes are disseminated via gene transfer.

- The effect of ozone seemed to have a different effect on cell integrity of Gram-positive and Gram-negative bacteria: whereas the concentrations of enterococci detected with the culture-based and qPCR approach decreased about 1.6 log-units respectively, the concentration of culturable E. coli decreased about 1.4 log-units but the E. coli concentration detectable by qPCR did not significantly change.

ACKNOWLEDGEMENTS

SchussenAktivplus was funded by the Federal Ministry for Education and Research BMBF (02WRS1281M) and co-funded by the Ministry of Environment Baden-Württemberg. In addition, Jedele & Partner GmbH, Ökonsult GbR,
the City of Ravensburg, the AZV Mariatal and the AV Unteres Schussental financially contributed to this research project.

We want to thank the team at the University of Tübingen under the guidance of Prof. Dr. R. Triebkorn and the SchussenAktivPlus project team for organizing sampling campaigns and for supplying sewage and river water samples. We thank Dr. H. Güde and Dr. H. Löfler, Petra Obad, Kathrin Lehner and Simone Eckenfels, of the Institute for Lake Research, State Institute for the Environment, Measurements and Conservation Baden-Württemberg, for their support. Furthermore, we thank Prof. Dr. J. Winter, KIT, for providing laboratory space and helpful discussions during experimentation and preparation of the manuscript.

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First received 14 April 2016; accepted in revised form 3 June 2016. Available online 18 June 2016