Resistance of *Escherichia coli* and *Enterococcus* spp. to selected antimicrobial agents present in municipal wastewater

Aneta Luczkiewicz, Ewa Felis, Aleksandra Ziembinska, Anna Gnida, Ewa Kotlarska, Krystyna Olanczuk-Neyman and Joanna Surmacz-Gorska

ABSTRACT

In this study, the susceptibility to erythromycin (E) and to trimethoprim/sulfamethoxazole (SXT) among isolates of *Enterococcus* spp. and *Escherichia coli* was tested, respectively. Both fecal indicators were detected and isolated from raw (RW) and treated wastewater (TW) as well as from samples of activated sludge (AS) collected in a local wastewater treatment plant (WWTP). Biodiversity of bacterial community in AS was also monitored using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). Additionally, distribution of *sul*1–3 genes conferring sulfonamide resistance was tested among SXT-resistant *E. coli*. Simultaneously, basic physicochemical parameters and concentrations of eight antimicrobial compounds (belonging to folate pathway inhibitors and macrolides class) were analyzed in RW and TW samples. Six of the selected antimicrobial agents, namely: erythromycin, clarithromycin, trimethoprim, roxithromycin, sulfamethoxazole, and *N*-acetyl-sulfamethoxazole were detected in the wastewater samples. Bacterial biodiversity of AS samples were comparable with no relevant differences. Among tested *Enterococcus* spp., E-resistant isolates constituted 41%. SXT resistance was less prevalent in *E. coli* with 11% of isolates. The genes conferring resistance to sulfonamides (*sul*1–3) were detected in SXT-resistant *E. coli* of wastewater origin with similar frequencies as in other environmental compartments, including clinical ones.

Key words | antimicrobial agents, antimicrobial resistance, fecal indicators, sulfonamide resistance genes, wastewater

Aneta Luczkiewicz (corresponding author)
Krystyna Olanczuk-Neyman
Department of Water and Wastewater Technology,
Faculty of Civil and Environmental Engineering,
Gdansk University of Technology,
G. Narutowicza Str., 11/12,
80-952 Gdansk,
Poland
E-mail: *ansob@pg.gda.pl*

Ewa Felis
Aleksandra Ziembinska
Anna Gnida
Joanna Surmacz-Gorska
Environmental Biotechnology Department,
Faculty of Energy and Environmental Engineering,
Silesian University of Technology,
Akademicka 2A Str.,
44-100 Gliwice,
Poland

Ewa Kotlarska
Institute of Oceanology,
Polish Academy of Sciences,
Powstancow Warszawy 55 Str.,
81-712 Sopot,
Poland

INTRODUCTION

The emergence of antimicrobial resistance is a complex problem driven by many factors, which have been considered mainly from the clinical point of view. Antimicrobial agents, used in human and animal therapy, create a selective pressure on microorganisms, and may favor mutant strains in bacterial flora of treated hosts. Such selection may also occur in the different environmental compartments, even if antimicrobial agents and their metabolites are present there in concentrations significantly lower than therapeutic dosages (Ohlsen *et al.* 1998).

Outside hospital units, wastewater is regarded as a significant source of antimicrobial agents and antimicrobial-resistant bacteria in the environment (Al-Ahmad *et al.* 1999; Backhaus & Grimme 1999; Kim & Aga 2007). Thus, there is increasing interest in the environmental risk connected with wastewater impact on receivers, also in terms of dissemination of antimicrobial resistance. Current regulations govern the quality of wastewater treatment plant (WWTP) effluents only in terms of nutrients, organic matter, and suspensions content. Therefore wastewater treatment processes are designed and operated to fulfill those requirements, and hence the important micropollutants present in wastewater can only be removed unintentionally. It is suggested that sorption and/or biodegradation are the

doi: 10.2166/wh.2013.130

main processes involved in antimicrobial agent removal (Kümmerer 2001; Golet *et al.* 2003; Lindberg *et al.* 2006); however, other parameters connected with WWTP operating systems and chemical properties of drug molecules (Golet *et al.* 2003; Batt *et al.* 2007) should also be taken into consideration.

In the case of antimicrobial-resistant bacteria, it is suggested that they persist through wastewater treatment processes (Reinthaler *et al.* 2003), and even that activated sludge (AS) promotes the spread of antimicrobial resistance by horizontal gene transfer due to high density of bacterial cells (Marcinek *et al.* 1998; Soda *et al.* 2008). Thus, WWTP effluents are considered an important reservoir of resistance genes, also of clinical relevance, such as vancomycin resistance genes, extended-spectrum β-lactamase (ESBL) genes, and other genes associated with antimicrobial resistance and virulence.

The aim of the current study was to determine the concentrations of eight antimicrobial compounds (erythromycin, clarithromycin, trimethoprim, roxithromycin and sulfamerazine, sulfamethoxine, sulfamethoxazole and *N*-acetyl-sulfamethoxazole) together with the presence of fecal indicators. Samples were taken from raw (RW) and treated (TW) wastewater of WWTP Gdansk-Wschod, which discharges into the recreationally important coastal waters of Gdansk Bay. Thermotolerant coliforms and enterococci were isolated and then identified at the species level using biochemical methods. Additionally, their susceptibility against folate pathway inhibitor–trimethoprim/sulfamethoxazole (SXT) and macrolide–erythromycin (E) was tested, respectively. Molecular diversity of sulfonamide resistance genes (*sul*1–3) carried by SXT-resistant *Escherichia coli* was also investigated. Simultaneously, samples of AS from bioreactor and recirculation line were also analyzed and additionally tested for biodiversity.

METHODS

Sample collection

The samples were taken from a modified University of Cape Town system WWTP Gdansk-Wschod, which serves a population of about 570,000 people in the area of Gdansk, Sopot, Kolbudy, Pruszcz Gdanski, Zukowo ($Q_{aver.} = 96{,}000\ m^3$ per day). The WWTP also treats wastewater from local industry (5%) and non-disinfected hospital wastewater (0.17%). The population equivalent was estimated as equal to 700,000. The schematic diagram of the WWTP and sampling points are indicated in Figure 1.

The 24 h flow-proportional wastewater samples (5 L) were taken from the raw (RW) and treated (TW) wastewater

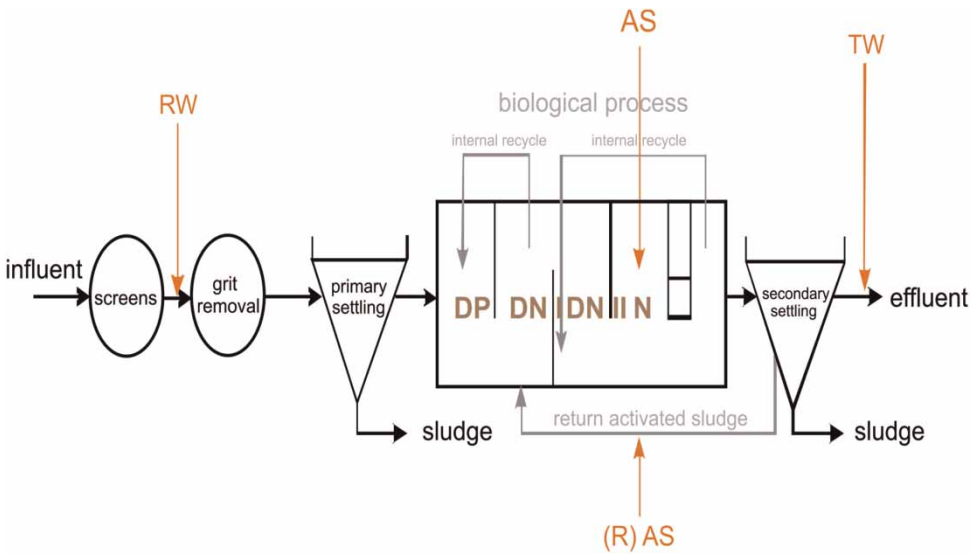

Figure 1 | Scheme of the WWTP (DP, dephosphatation; DN, denitrification; N, nitrification) and the sampling points' location (RW, raw wastewater; AS, activated sludge from aerobic chamber; (R)AS, return AS; TW, treated wastewater).

as well as from the aerobic chamber and return AS in three following days in January and June 2009 (Figure 1). The 24 h retention time in the WWTP was considered during sample collection.

Determination of physical and chemical parameters

The effectiveness of wastewater processes were controlled in RW and TW by total suspended solids (TSS), chemical and biological oxygen demand (COD and BOD_5) as well as by concentration of total nitrogen (TN) and total phosphorus (TP). Analyses were conducted using procedures based on *Standard Methods* (APHA 1992) and performed within 6 h after sample collection.

Determination of antimicrobial agents

The presence of antimicrobial agents was determined in RW and TW. All samples of the volume of 1 L were frozen and stored in dark-glass bottles at −20 °C until analysis. After defrosting, samples were filtered through glass fiber filters with a pore size of <1 μm and diameter 55 mm (Schleicher & Schuell Bioscience). The 100 mL of influent (RW) and 200 mL of effluent (TW) were spiked with a mixture of internal standards (IS). The pH of all samples was adjusted to 7.5. As the internal standards the following substances were used: sulfamerazine-d4 (IS for sulfonamides and trimethoprim), sulfamethoxazole-d4 (IS for sulfamethoxazole), *N*-acetyl-sulfamethoxazole-d5 (IS for *N*-acetyl-sulfamethoxazole) and (E)-9-[O-(2-methyloxime)]-erythromycin (IS for macrolides). Sulfamerazine-d4, sulfamethoxazole-d4, *N*-acetyl-sulfamethoxazole-d5 were purchased from Sigma-Aldrich and (E)-9-[*O*-(2-methyloxime)]-erythromycin was synthesized according to the procedure described by Schluesener *et al.* (2003). The solid phase extraction of the wastewater samples was performed by means of Oasis HLB (200 mg, 6 mL) cartridges (Waters). Conditioning and drying of the cartridges were performed according to the procedure described by Hijosa-Valsero *et al.* (2011). The analytes were eluted with 4 × 2 mL acetone. The eluates were concentrated to the volume of 200 μL, diluted with 250 μL of methanol and evaporated under a gentle nitrogen stream to the volume of 100 μL. Then the extracts were redissolved in 400 μL Milli-Q water. All the samples were analyzed by reverse-phase liquid chromatography-tandem mass spectrometry (HPLC-MS-MS). The HPLC system consisted of a G1313A autosampler, a G1311A quaternary HPLC pump, a G1379A degasser (all Agilent), a CTO-10A column oven, and a SCL-10A system controller (all Shimadzu). More details concerning chromatographic analysis performance are presented in the publication of Hijosa-Valsero *et al.* (2011).

DNA extraction and polymerase chain reaction (PCR) conditions for AS samples

AS samples (volume of 10 mL) from the bioreactor and return AS of WWTP Gdansk-Wschod were pelleted by centrifugation (5,000 × g, 10 min, 4 °C) and stored at −20 °C. Total genomic DNA was extracted from 0.2 g of the AS samples using a Fast DNA Spin Kit for Soil (MP Biomedicals) according to the manufacturer's instructions. The amount of DNA was measured spectrophotometrically using Qubit (Invitrogen) and stored at −20 °C until PCR amplification.

In this study, partial 16S rRNA gene amplification was performed using primers 338F with GC clamp and 518R, which amplified a partial (ca. 180 bp) 16S rRNA gene fragment of all the bacteria (Muyzer *et al.* 1993). PCR procedure was described previously (Ziembinska *et al.* 2009).

Denaturing gradient gel electrophoresis (DGGE) conditions and estimation of bacterial biodiversity

The DGGE of the PCR products obtained in reactions with 338F-GC and 518R primers underwent electrophoretic separation in the Dcode Universal Mutation Detection System (BioRad). Polyacrylamide gel (8% for 16S rRNA gene, 37:1 acrylamide-bisacrylamide, Fluka) with a gradient of 30–60% denaturant was prepared according to the manufacturer's instructions. The gel was run for 10 h at 70 V in a 1× TAE buffer (Sambrook & Russell 2001) at a constant temperature of 60 °C. The gel was stained with SYBR Gold (1:10,000, Invitrogen) in MiliQ water for 30 min and distained in MiliQ water for 40 min, then visualized under UV light and photographed using Quantity One 1D (BioRad).

The analysis of DGGE fingerprints was performed using Quantity One 1D software (BioRad). Bacterial biodiversity was estimated on the basis of densitometric measurements and Shannon diversity index for the samples was calculated, according to the equation:

$$H' = -\sum P_i \ln P_i$$

with:

$$P_i = n_i/N_i$$

where $P_i =$ relative probability of DNA band appearance in the fingerprint, $N_i =$ amount of DNA bands in the fingerprint, $n_i =$ densitometrically measured intensity of DNA band.

Detection and isolation of fecal indicators

Fecal indicators were detected using membrane filtration method. Appropriate dilutions of analyzed wastewater samples were filtered through 0.45 μm cellulose-acetate filters in triplicate. Next, in order to detect fecal coliforms filters were placed on mFC agar (Merck) and incubated at 44.5 °C for 24 h, while enterococci were cultivated using *Enterococcus* selective agar (Merck) at 37 °C for 48 h (according to ISO 7899–2:2000). Dark red or maroon colonies were regarded as presumptive enterococci, and in the case of fecal coliforms various shades of blue colonies were counted as presumptive *E. coli*. For further investigations, representative isolates were taken from membranes presenting from 20 to 50 typical colonies and kept in nutrient broth supplemented with 15% glycerol at −80 °C.

Species identification (ID) and antimicrobial susceptibility tests (AST)

Altogether 153 isolates of presumptive *E. coli* and 199 isolates of presumptive enterococci were collected. The ID and AST tests were carried out using the Phoenix Automated Microbiology System (Phoenix AMS, BD Diagnostics) as described in detail elsewhere (Luczkiewicz *et al.* 2010). In brief, selected isolates of presumptive enterococci and *E. coli* were cultured overnight. Appropriate commercially available Phoenix panels and procedure according to the manufacturer's recommendations were used. Each isolate was used to inoculate ID broth and concentration was adjusted from 0.5 to 0.6 McFarland using BBLTM Crystal Spec TM Nephelometer (BD Diagnostics). Next, AST broth was inoculated with 25 μL of ID suspension. Then, ID and AST broths were used to fill up corresponding panel sections and panels were placed into the Phoenix ASM and incubated at 35 °C. The quality control was performed according to the manufacturer's instructions. Due to the inability of the Phoenix ASM system to discriminate between *E. casseliflavus* and *E. gallinarum* the classification *E. casseliflavus/gallinarum* was used.

In this study, the confidence level of 90% was required as the lowest limit of acceptability for ID results. In characterization of antibiotic susceptibility, only two categories were used: sensitive (S) and resistant (R) (as 'intermediate resistant' and 'resistant' behavior). Phenotype of multidrug resistance (MDR) was defined as resistance to antimicrobial agents from three or more categories, according to Magiorakos *et al.* (2011). The antimicrobial agents used in this study were as follows: for *E. coli*: amikacin (AN), gentamicin (GM), tobramycin (NN), imipenem (IPM), meropenem (MEM), cefazolin (CZ), cefuroxime (CXM), ceftazidime (CAZ), cefotaxime (CTX), cefepime (FEP), aztreonam (ATM), ampicillin (AM), amoxicillin/clavulanate (AMC), piperacillin (PIP), piperacillin/tazobactam (TZP), trimethoprim/sulfamethoxazole (SXT), ciprofloxacin (CIP), levofloxacin (LVX), tetracycline (TE); and for *Enterococcus* spp.: gentamicin-synergy (GMS), streptomycin-synergy (STS), ampicillin (AM), ciprofloxacin (CIP), levofloxacin (LVX), moxifloxacin (MXF), teicoplanin (TEC), vancomycin (VA), chloramphenicol (C), erythromycin (E), linezolid (LZD), nitrofurantoin (FM), tetracycline (TE), clindamycin (CC), lincomycin (L), pristinamycin (PR). The bacterial susceptibility data were analyzed using the Clinical and Laboratory Standards Institute standards for antimicrobial susceptibility testing (CLSI 2011). Since the susceptibility criteria for CC, L, and PR are not available in CLSI recommendations, the results obtained were analyzed using MIC interpretive standard according to Comité de l'Antibiogramme de la Société Francaise de Microbiologie

recommendations (CASFM 2010). Additionally, tests for the detection of ESBLs among *E. coli* isolates were provided.

In this study, only detailed analysis of *E. coli* isolates resistant to trimethoprim/sulfamethoxazole and enterococci resistant to erythromycin are presented.

Detection of *sul*(1–3) genes in *E. coli* isolated from wastewater samples

DNA was obtained from whole-cell lysates as follows. Isolates were plated on Luria-Bertani agar (Difco, USA), and after incubation several colonies were collected and resuspended in 500 μL of distilled water. After incubation in a boiling water bath, lysate was centrifuged and 0.5 μL of supernatant was used as template for amplification.

The PCR mixtures used to detect *sul* genes had the following composition: 10 μL reaction mixtures contained: 1 × PCR buffer (0.25 mM of each dNTPs, 2 mM $MgCl_2$, and 0.5 U of *Taq* polymerase), 5 pmol of each primer and 0.5 μL of template prepared as indicated above. The temperature profile was as follows: initial denaturation (96 °C for 5 min), then 1 min at 55 °C, and 1 min at 70 °C, followed by 24 cycles of denaturation (96 °C for 15 s), annealing (55 °C for 30 s), and extension (70 °C for 3 min); and a final extension (70 °C for 5 min). All PCR reactions were carried out in a TPersonal thermocycler (Biometra) using *Taq* polymerase, nucleotides, and buffers purchased from A&A Biotechnology (Gdynia, Poland). To amplify *sul* genes previously published primers (Grape *et al.* 2003; Perreten & Boerlin 2003) were used. Strains carrying *sul* genes (kindly provided by Prof. V. Perreten) were used as positive controls. PCR products were analyzed by electrophoresis on a 1% agarose gel in 1× TAE buffer (Sambrook & Russell 2001) and stained with ethidium bromide (0.5 μg mL^{-1}), visualized under UV light and documented using Vilbeur Lourmat image acquisition system.

Data analyses

Significant differences between antibiotic resistance rate in RW and TW wastewater were determined for fecal indicators using a two-sided z-test for two proportions ($p < 0.05$). For statistical computations 'Scilab environment' was used.

RESULTS AND DISCUSSION

The specific location of Gdansk Bay and the nearby multifunctional agglomeration (Gdansk–Sopot–Gdynia–Rumia–Reda–Wejherowo) located on Gdansk Bay implies a variety of action connected with urban, industrial, as well as recreational activity concentrated in this area. Fecal degradation of the gulf costal waters was previously observed (Olanczuk-Neyman & Jankowska 2001) and led to beach closures and the prohibition of bathing. As a main source of fecal contamination of Gdansk Bay, the numerous direct tributaries, as well as the effluents from local WWTPs, have been recognized.

During the study the average values of COD, BOD_5, TN, and TP as well as TSS observed in treated wastewater (Table 1) were lower than those legally established for the examined WWTP Gdansk-Wschod (125 mg O_2 L^{-1}, 15 mg O_2 L^{-1}, 10 mg N L^{-1}, 1 mg P L^{-1}, 35 mg L^{-1}, respectively). In the case of the average number of fecal coliforms and enterococci, their number in RW reached 5.7×10^6 CFU per 100 mL and 3.5×10^6 CFU per 100 mL, respectively. Reduction in fecal indicators was significant (up to 99.9%) and similar to results obtained previously for this system (Olanczuk-Neyman *et al.* 2003) as well as for other wastewater systems working on activated sludge processes (Blanch *et al.* 2003; Ferreira da Silva *et al.* 2006). It should be mentioned, however, that due to the occasional high number of bacterial cells detected in raw wastewater, their number may even reach 3.7×10^4 CFU per 100 mL for enterococci and up to 3.2×10^4 CFU per 100 mL for *E. coli* in treated wastewater.

Biochemical tests of all isolates of thermotolerant coliforms ($n = 153$) identified them as *E. coli*. In the case of enterococci ($n = 199$), 60.8% of isolates were identified as *E. faecium*, 22.1% as *E. faecalis*, while the rest belonged to *E. hirae* (12.1%), *E. casseliflavus/gallinarum* (4.5%),

Table 1 | Basic parameters of raw (RW) and treated (TW) wastewater, (*mean*/σ)/min−max

	TSS	TN	TP	BOD_5	COD
	mg L^{-1}			mg O_2 L^{-1}	
RW	520/91 455–624	72.8/5.5 68.0–78.8	10.6/0.7 10.0–11.4	370/51 320–422	1,136/122 995–1,212
TW	5.9/0.4 5.5–6.2	8.2/0.3 8.0–8.5	0.41/0.06 0.35–0.45	5.6/0.3 5.3–5.8	44.6/3.9 40.2–47.5

and *E. durans* (0.5%) (Figure 2). Similar predominance of *E. faecium* followed by *E. faecalis* was reported in wastewater samples collected in the UK and Spain (Manero *et al.* 2002; Blanch *et al.* 2003); however, in Sweden, *E. faecalis* was the most common (Blanch *et al.* 2003). In Portugal and in the United States, the most abundant was *E. hirae* (Bonilla *et al.* 2006; Ferreira da Silva *et al.* 2006), which is mainly found in the gastrointestinal tract of animals and rarely associated with human intestines and with human enterococcal infections (Devriese *et al.* 2002; Rice *et al.* 2003).

Data concerning enterococcal species composition in wastewater are rare, thus no general comments can be drawn in relation to diet, climate, and others factors. It is also not clear how wastewater processes influence the enterococcal composition. In this study, the ratio of both *E. faecalis* and *E. hirae* was similar in raw wastewater, however, during the treatment an insignificant increase of *E. hirae* numbers was observed in AS, and then a decrease in treated wastewater.

In the case of AS biodiversity, no dominant genotype was detected, however, slight seasonal changes in DGGE patterns were observed in the case of GC-rich genotypes (the bottom of the gel), where more dense DNA bands were present (Figure 3, lower frame). Also, there is one DNA band present in AS from the summer sample presenting higher density than the others from return AS and it differentiates summer from winter DGGE patterns (Figure 3, upper frame), which is probably the result of non-specific PCR amplification.

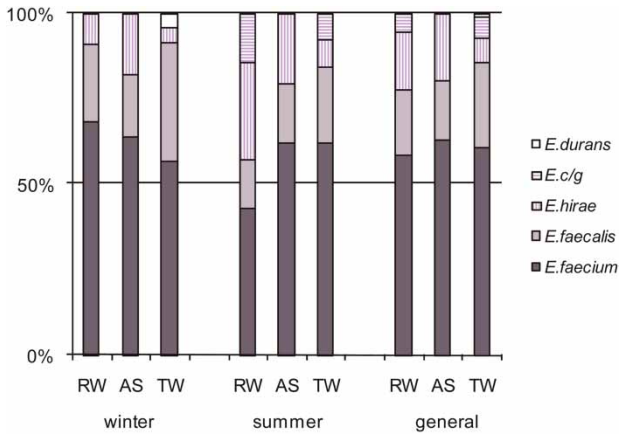

Figure 2 | Composition of enterococci obtained in raw wastewater (RW), AS, and return activated sludge (AS) as well as treated wastewater (TW); *E. c/g - E. casseliflavus/gallinarum*.

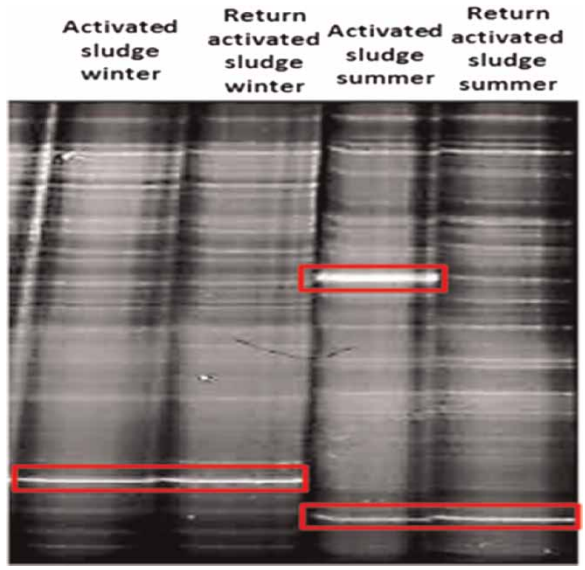

Figure 3 | DGGE fingerprints obtained in the study from AS and return AS in winter and summer. Slight pattern changes are highlighted by the frames.

The level of biodiversity of all bacteria in AS and return AS was estimated with Shannon biodiversity index (H), but biodiversity was comparable for all samples with no relevant differences (Figure 4). Biodiversity for return sludge samples was slightly higher (3.42–3.52) than the AS index (3.26–3.12) in both sampling times. Such a situation seems to be natural due to the fact that return sludge represents total biodiversity of bacteria with all autochthonic and allochthonic bacteria resulting in wastewater in a thicker form, while AS is less dense. It should also be underlined that the level of biodiversity in this case presents the same pattern as DGGE fingerprints with no significant changes. That results in the conclusion that in the tested period at WWTP

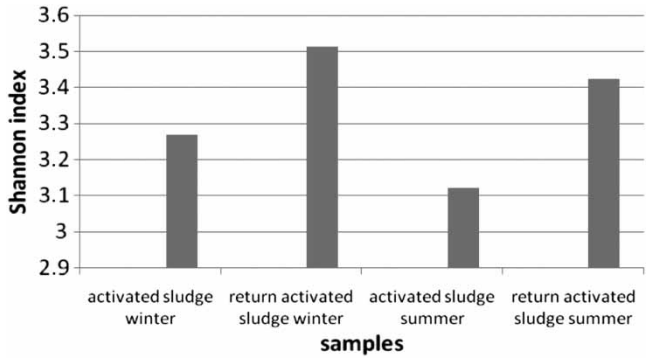

Figure 4 | Shannon biodiversity index for AS and return AS samples collected in winter and summer from WWTP Gdansk-Wschod.

Gdansk-Wschod AS contained relatively stable biocenosis without significant fluctuations.

In the study, raw and treated wastewater samples collected from WWTP Gdansk-Wschod were also monitored for the presence of eight antimicrobial agents belonging to the two classes of folic acid antagonists and macrolides, namely: clarithromycin, roxithromycin, erythromycin, trimethoprim, sulfamethoxazole, *N*-acetyl-sulfamethoxazole, sulfamerazine, and sulfamethoxine. Two of them, sulfamerazine and sulfamethoxine (antimicrobial agents used mainly in veterinary medicine), were not detected in municipal wastewater samples. It indirectly confirms that the occurrence and concentration of antimicrobial agents in raw municipal wastewater, although related to several factors, mostly depends on the local human consumption of antibiotics and their excretion. In the case of other tested folic acid antagonists, trimethoprim and sulfamethoxazole, in medical practice they have been commonly used together (as co-trimoxazole) to treat a broad spectrum of aerobic bacteria since the late 1960s. From both components, which are taken orally in a standard ratio 1 to 5, about 30% of sulfamethoxazole and up to 90% of trimethoprim are excreted unchanged in the urine. In the case of macrolides, up to 15% of orally administered erythromycin and up to 40% of clarithromycin is, however, excreted in active form in the urine (Zuckerman 2004).

Among tested macrolides, the highest concentrations in raw wastewater were obtained for clarithromycin (up to 1,817 ng L^{-1}). Erythromycin was not detected in the raw wastewater samples but its occurrence in wastewater confirmed treated wastewater analysis. During recent years in Poland, and other European countries, an increase in clarithromycin and azithromycin consumption has been observed at the expense of erythromycin (Ferech *et al.* 2006). The seasonal fluctuations of macrolides concentrations observed in raw wastewater (Table 2) may be related to their intended use to treat respiratory tract infections.

In the case of tested folic acid antagonists, components such as trimethoprim, sulfamethoxazole, and *N*-acetyl-sulfamethoxazole were detected in raw and treated wastewater samples. Sulfamethoxazole and its main derivative *N*-acetyl-sulfamethoxazole, were detected in all wastewater samples. Trimethoprim, a drug used in combination with sulfamethoxazole in various antimicrobial preparations, was present in raw wastewater at the average concentrations of 482 ± 116 ng L^{-1} and 441 ± 81 ng L^{-1} in winter and summer periods, respectively. For sulfamethoxazole mass balance, the concentration of its main derivative, *N*-acetyl-sulfamethoxazole, generated in the human body in reaction to sulfamethoxazole acetylation, should always be taken into account. Under environmental conditions, the cleaving back of the acetyl derivate to sulfamethoxazole has been reported (Göbel *et al.* 2005; Kümmerer 2009). In this study, *N*-acetyl-sulfamethoxazole concentration in raw wastewater was slightly higher then observed for sulfamethoxazole

Table 2 | Occurrence and removal of selected antimicrobial agents from WWTP Gdansk-Wschod during the wastewater treatment process

Compound	Temperature	Raw wastewater (ng L^{-1})	Treated wastewater (ng L^{-1})	Removal (%)
Clarithromycin	$T = 10\ ^{\circ}C$	$1{,}416 \pm 401$	761 ± 106	43
	$T = 20\ ^{\circ}C$	904 ± 433	185 ± 32	76
Roxithromycin	$T = 10\ ^{\circ}C$	161 ± 0	132 ± 14	18
	$T = 20\ ^{\circ}C$	105 ± 17	55 ± 6	47
Erythromycin	$T = 10\ ^{\circ}C$	n.d.	17	n.r.
	$T = 20\ ^{\circ}C$	n.d.	14	n.r.
Sulfamethoxazole	$T = 10\ ^{\circ}C$	$1{,}464 \pm 203$	508 ± 25	65
	$T = 20\ ^{\circ}C$	$1{,}225 \pm 173$	642 ± 114	47
N-acetyl-sulfamethoxazole	$T = 10\ ^{\circ}C$	$1{,}763 \pm 470$	16	98
	$T = 20\ ^{\circ}C$	$1{,}358 \pm 224$	0	100
Trimethoprim	$T = 10\ ^{\circ}C$	482 ± 116	445 ± 72	7
	$T = 20\ ^{\circ}C$	441 ± 81	269 ± 14	38

n.d., not detected; n.r., not removed.

(Table 2). The obtained results as well as results of other studies showed the importance of considering retransformable metabolites when the fate of antimicrobial agents is investigated (Göbel *et al.* 2005).

Previous studies dealing with the presence and removal of antimicrobial agents reported variable results (Carballa *et al.* 2004; Pérez *et al.* 2005; Junker *et al.* 2006; Batt *et al.* 2007; Göbel *et al.* 2007). In AS processes, the removal of tested macrolides and folic acid antagonists was found to be incomplete, except for *N*-acetyl-sulfamethoxazole. This metabolite was removed from wastewater with an efficiency reaching 100%. The removal efficiency observed for sulfamethoxazole and clarithromycin was also relatively high (up to 65 and 76%, respectively), while for trimethoprim it did not exceed 38%. Similar high resistance of trimethoprim and susceptibility of sulfamethoxazole to biodegradation was reported by Pérez *et al.* (2005) in wastewater treatment based on AS processes. Additionally, according to the obtained data, the removal efficiency depended on the temperature, which increased (except for sulfamethoxazole) during the summer season (Table 2).

Biological degradation and interactions with AS, suspected to be involved in antimicrobial agents' removal from wastewater, are not yet fully understood (Heise *et al.* 2006; Junker *et al.* 2006). Several other parameters should also be investigated in antimicrobial agents' removal, such as AS age, retention time in the system (Göbel *et al.* 2007), together with development and standardization of the analytical techniques.

Simultaneously with detection of macrolides and folic acid antagonists, susceptibility of *Enterococcus* spp. to erythromycin and *E. coli* to trimethoprim/sulfamethoxazole was determined. In the study, the results were obtained for all tested *E. coli* ($n = 152$) and for 93% of tested enterococci ($n = 185$), mainly due to the grow limitation observed among isolates of *E. casseliflavus/gallinarum*, which was discussed in detail in Luczkiewicz *et al.* (2010).

Since trimethoprim/sulfamethoxazole is active against many *Enterobacteriaceae* and extracted by urine it is also commonly used to treat uncomplicated *E. coli* infections of the urinary tract (UTI). Worldwide, *E. coli* was found to be the most prevalent uropathogen in both the community and hospital-acquired UTIs, and in Poland accounted for 84 and 38% infections, respectively (Hryniewicz *et al.* 2001). Due to several advantages, also economic ones, SXT is often used as first-line therapy for acute uncomplicated UTI. In this study, among *E. coli* ($n = 153$) of wastewater origin, resistance to SXT reached 11% and was almost three times lower than reported for uropathogenic *E. coli* in Poland (up to 31%) (Hryniewicz *et al.* 2001). It should also be noted that among SXT-resistant *E. coli*, 25% were simultaneously resistant to fluoroquinolones (CIP and LVX), antimicrobial agents, which are also prescribed for UTIs (Figure 5). Higher prevalence was,

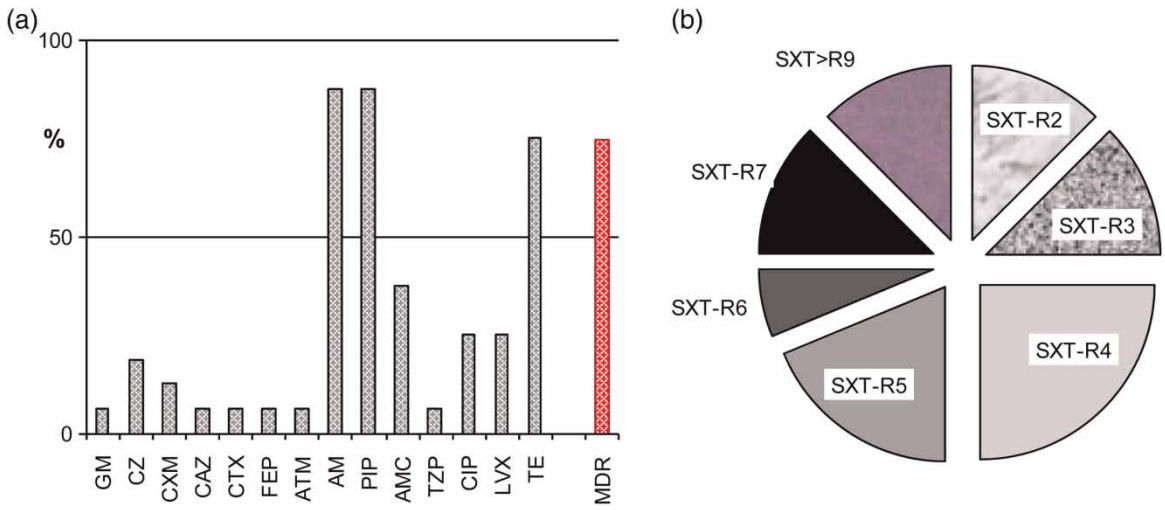

Figure 5 | Susceptibility of SXT-resistant *E. coli* (a) to a single antimicrobial agent together with presence of multidrug-resistant (MDR) isolates and (b) to all tested antimicrobial agents (R-X – resistant to X antimicrobial agents); resistance to AN, NN, IPM, and MEM was not found among SXT-resistant *E. coli*.

however, observed for resistance to penicillins (AM and PIP), as well as to tetracycline (88 and 75%, respectively). Among SXT-resistant *E. coli*, 75% showed clinically relevant MDR phenotypes. In some countries, it is suggested that trimethoprim/sulfamethoxazole should be abandoned as a first-line therapy for UTIs, if the resistance rate to SXT exceeds 20% (Brown *et al.* 2002).

Molecular analysis of genes conferring resistance to sulfonamides among SXT-resistant *E. coli* isolates showed a prevalence of *sul*2 (81%) and *sul*1 (50%) genes (Figures 6 and 7). Five isolates (31%) simultaneously carried both genes. The *sul*3 gene was found once (6%), in the WWTP effluent. A minor incidence of *sul*3 gene is also suggested by the literature data. For the first time the *sul*3 gene was detected in *E. coli* from pigs (Perreten & Boerlin 2003). So far, the incidental presence of the *sul*3 gene has also been reported for *E. coli* of UTI (Grape *et al.* 2003), poultry meat (Soufi *et al.* 2011), and wastewater origin (Szczepanowski *et al.* 2009). The data obtained indicate that the molecular diversity of resistance genes in isolates of wastewater origin may be similar to those obtained in other environments, including clinical ones.

Resistance level to the tested macrolide, erythromycin, in general reached 44% and varied among enterococcal species. *E. durans* and *E. hirae* presented the lowest prevalence of erythromycin resistance (0 and 4%, respectively), while 61% of *E. faecalis* and 45% of *E. faecium* and all isolates of *E. casseliflavus/gallinarum* were E-resistant (results obtained for *E. durans* and *E. casseliflavus/gallinarum* may not be, however, reliable due to the small number of isolates tested). *E. faecalis* was also the most common species among E-resistant isolates of wastewater origin in Sweden and Spain (Blanch *et al.* 2003). Resistance to erythromycin reported for *E. faecium* and *E. faecalis* isolated from raw

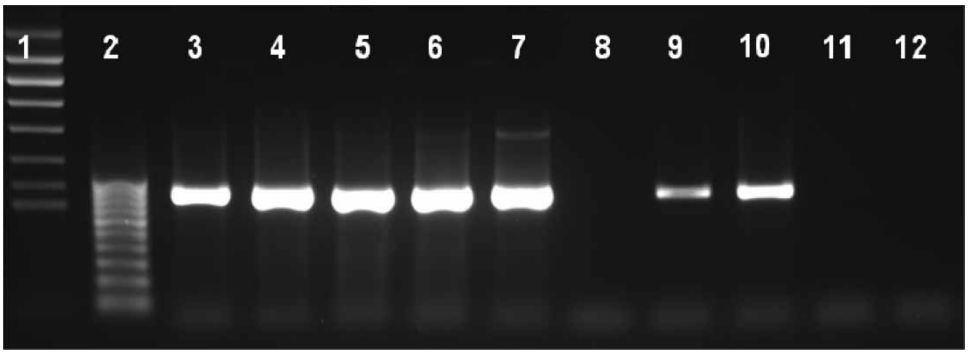

Figure 6 | Identification of *sul*1 gene in *Escherichia coli* strains. DNA molecular size marker Ideal (DNA Gdansk) (Lane 1), DNA molecular size marker 1,000–100 bp (DNA Gdansk) (Lane 2), isolate GC 27 (Lane 3), isolate GC 141 (Lane 4), isolate GC 149 (Lane 5), isolate GC 150 (Lane 6), isolate GC 164 (Lane 7), isolate GC 176 sensitive to SXT (Lane 8), isolate GC 175 (Lane 9), positive control – *E. coli* strain rl0040 (with *sul*1 and *sul*2 genes) (Lane 10), negative control – laboratory strain *E. coli* DH5α SXT-sensitive (Lane 11), negative control (distilled water as a template for PCR reaction) (Lane 12).

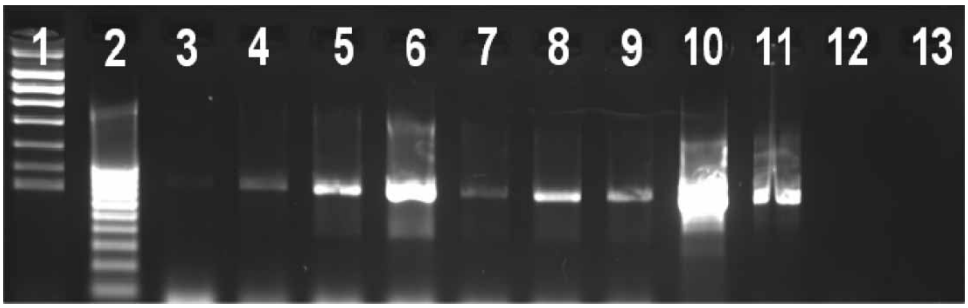

Figure 7 | Identification of *sul*2 and *sul*3 genes in *Escherichia coli* strains. DNA molecular size marker Ideal (DNA Gdansk) (Lane 1), DNA molecular size marker 1,000–100 bp (Lane 2), identification of *sul*2 gene in: isolate GC 27 (Lane 3), isolate GC 54 (Lane 4), isolate GC 57 (Lane 5), isolate GC 141 (Lane 6), isolate GC 143 (Lane 7), isolate GC 145 (Lane 8), identification of *sul*3 gene: isolate GC 57 (Lane 9), positive control – *E. coli* strain rl0040 (with *sul*1 and *sul*2 genes) (Lane 10), positive control – *E. coli* strain DH5α/pUVP4401 (with *sul*3 gene) (Lane 11), negative control – laboratory strain *E. coli* DH5α SXT-sensitive (Lane 12), negative control (distilled water as a template for PCR reaction) (Lane 13).

wastewater (including hospital wastewater) varied from 14 to 84% (Blanch *et al*. 2003; Ferreira da Silva *et al*. 2006).

In this study among E-resistant enterococci ($n = 83$), resistance to nitrofurantoin and to ciprofloxacin was common (59 and 41% of E-resistant isolates) (Figure 8). Also, clinically relevant resistance to vancomycin and high-level aminoglycosides were correlated with resistance to erythromycin. Among all enterococci ($n = 185$), resistance to high-level gentamicin (HLGR, MIC > 500 μg mL^{-1}) and streptomycin (HLSR, MIC > 1,000 μg mL^{-1}) were reported six and ten times, respectively, and among them, all HLGR and eight HLSR were also E-resistant. Among six isolates resistant to vancomycin resistance to erythromycin was simultaneously reported four times. The presence of MDR patterns among 54% of E-resistant enterococci is worth noting (Figure 8).

Macrolides usually share the resistance mechanisms with lincosamides and streptogramin B (MLS_B phenotype) due to the production of ribosomal methylases, encoded by *erm*A-B genes that confer constitutive or inducible resistance to these compounds (Leclercq *et al.* 2013). The production of the MefA efflux pump results in resistance to erythromycin but not to clindamycin and/or streptogramins (M phenotype) (Leclercq *et al.* 2013). Among enterococci, *E. faecalis* is often intrinsically resistant to clindamycin (lincosamide) as well as to streptogramins A and B due to the expression of the chromosomal, species-specific *lsa* gene, encoding an efflux pump, while the *msr*C gene confers low-level resistance (MIC 1–2 mg/mL) to streptogramin B in *E. faecium* also by efflux mechanism (Leclercq 2002; Hollenbeck & Rice 2012). In this study, together with erythromycin resistance, susceptibility to lincosamides: lincomycin (L) and clindamycin (CC) as well as streptogramin B–pristinamycin (PR) – was analyzed. Among E-resistant *E. faecalis,* resistance rates to both tested lincosamides (CC and L) reached 93%, while to streptogramin B (PR) it was equal to 46%. In the case of *E. faecium,* resistance to CC, L, and PR was observed among 34, 40, and 2% of the E-resistant isolates, respectively. For both species, resistance to pristinamycin occurred together with resistance to lincomycin and clindamycin (except one isolate of *E. faecalis*). Thus, among *E. faecalis* constitutive MLS_B phenotype was detected in 43% of E-resistant isolates, while for *E. faecium* 2% were E-resistant isolates. It should be noted that however intrinsic resistance to clindamycin (CC) is postulated for *E. faecalis*, not all of the tested environmental isolates expressed such resistance phenotype. Susceptibility to clindamycin and lincomycin was noted also among clinical isolates of *E. faecalis* (Dina *et al*. 2003), suggesting that the mechanism of resistance to lincosamides and streptogramins remains incompletely elucidated.

Additionally, treatment processes applied in the tested WWTP favored E-resistant *E. faecalis* and *E. faecium* as well as SXT-resistant *E. coli* (Figure 9), which is in an agreement with other scientific evidence, that wastewater

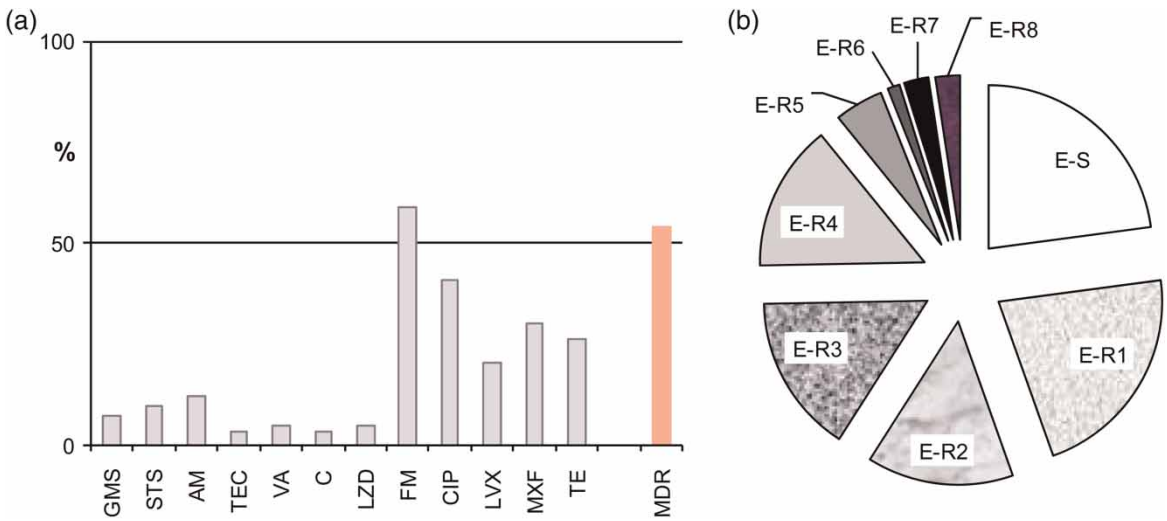

Figure 8 | Susceptibility of E-resistant *Enterococcus* spp. (a) to a single antimicrobial agent together with presence of multidrug-resistant (MDR) isolates and (b) to all tested antimicrobial agents (S, sensitive, R-X, resistant to X antimicrobial agents); AST data of CC, L, and PR were not taken into consideration.

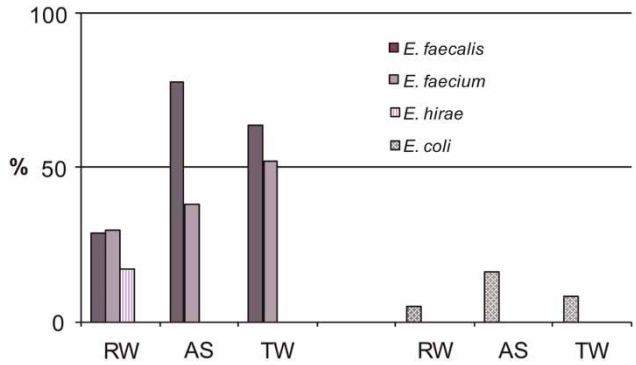

Figure 9 | Presence of E-resistant enterococci and SXT-resistant *E. coli* in raw wastewater (RW), AS and return activated sludge (AS) as well as in treated wastewater (TW); due to low numbers, *E. casseliflavus/gallinarum* ($n = 4$) and *E. durans* ($n = 1$) were not taken into consideration.

treatment processes may cause positive selection of antimicrobial-resistant bacteria (Ferreira da Silva *et al.* 2006; Kim & Aga 2007; Zhang *et al.* 2009). The prevalence of resistant strains over sensitive ones should also be discussed in terms of gene transfer. In this process, commensal bacteria of human origin can be regarded as vectors for the gene transmission to environmental populations.

CONCLUSION

In the analysis of antimicrobial resistance phenomena, *Enterococcus* spp. and *E. coli* are of particular importance. They are not only standard fecal indicators or commensal bacteria of human intestines, but also important potential pathogens. Data obtained in this study indicated that conventional wastewater processes do not prevent the receiver from dissemination of fecal indicators with clinically relevant resistance patterns. It should also be stated, that in the environmental risk assessment, the importance of continuous input of resistant bacteria versus continuous input of sub-inhibitory concentration of antimicrobial agents present in treated wastewater should be reported.

ACKNOWLEDGMENTS

The authors wish to thank Prof. Perreten from the Institute of Veterinary Bacteriology, University of Bern, Switzerland for providing control strains with *sul* genes as well as Dr T. A. Ternes, Dr M. Schlüsener, G. Fink from the Federal Institute of Hydrology (Germany) for the opportunity and assistance during the analyses of selected antimicrobial substances in the environmental samples. *Escherichia coli* and enterococci isolates came from the collection previously described by Luczkiewicz *et al.* (2010) – the general resistance rates to clinically relevant antimicrobial agents were reported there. This research was supported by the Polish Ministry of Science and Higher Education, grants: NN 523 493 134 and NN 305 461 139.

REFERENCES

Al-Ahmad, A., Daschner, F. D. & Kümmerer, K. 1999 Biodegradability of cefotiam, ciprofloxacin, meropenem, penicillin G, and sulfamethoxazole and inhibition of waste water bacteria. *Arch. Environ. Contam. Toxicol.* **37**, 158–163.

APHA 1992 *Standard Methods for the Examination of Water and Wastewater*, 18th edn. American Public Health Association APHA-AWWA-WEF, Washington, DC.

Backhaus, T. & Grimme, L. H. 1999 The toxicity of antibiotic agents to the luminescent bacterium *Vibrio fischeri*. *Chemosphere* **38**, 3291–3301.

Batt, A. L., Kim, S. & Aga, D. S. 2007 Comparison of the occurrence of antibiotics in four full-scale wastewater treatment plants with varying designs and operations. *Chemosphere* **68**, 428–435.

Blanch, A. R., Caplin, J. L., Iversen, A., Kühn, I., Manero, A., Taylor, H. D. & Vilanova, X. 2003 Comparison of enterococcal populations related to urban and hospital wastewater in various climatic and geographic European regions. *J. Appl. Microbiol.* **94**, 994–1002.

Bonilla, T. D., Nowosielski, K., Esiobu, N., McCorquodale, D. S. & Rogerson, A. 2006 Species assemblages of *Enterococcus* indicate potential sources of fecal bacteria at a south Florida recreational beach. *Mar. Pollut. Bull.* **52**, 807–810.

Brown, P. D., Freeman, A. & Foxman, B. 2002 Prevalence and predictors of trimethoprim-sulfamethoxazole resistance among uropathogenic *Escherichia coli* isolates in Michigan. *Clin. Infect. Dis.* **34**, 1061–1066.

Carballa, M., Omil, F., Lema, J. M., Llompart, M., García-Jares, C., Rodríguez, I., Gómez, M. & Ternes, T. 2004 Behavior of pharmaceuticals, cosmetics and hormones in a sewage treatment plant. *Water Res.* **38** (12), 2918–2926.

CASFM 2010 Comite de l'Antibiogramme de la Societe Francaise de Microbiologie recommandations. Available at: http://www.sfm-microbiologie.org/UserFiles/file/CASFM/casfm_2010.pdf (accessed 30 April 2013).

CLSI 2011 Clinical and Laboratory Standards Institute Performance standards for antimicrobial susceptibility testing. *Twenty First Informational Supplement*, M100-S21, 21 (1).

Devriese, L. A., Vancanneyt, M., Descheemaeker, P., Baele, M., van Landuyt, H. W., Gordts, B., Butaye, P., Swings, J. & Haesebrouck, F. 2002 Differentiation and identification of *Enterococcus durans*, *E. hirae* and *E. villorum*. *J. Appl. Microbiol.* **92**, 821–827.

Dina, J., Malbruny, B. & Leclercq, R. 2003 Nonsense mutations in the lsa-like gene in Enterococcus faecalis isolates susceptible to Lincosamides and Streptogramins A. *Antimicrob. Agents Chemother.* **47**, 2307–2309.

Ferech, M., Coenen, S., Malhotra-Kumar, S., Dvorakova, K., Hendrickx, E., Suetens, C., Goossens, H. & ESAC Project Group 2006 European Surveillance of Antimicrobial Consumption (ESAC): outpatient macrolide, lincosamide and streptogramin (MLS) use in Europe. *J. Antimicrob. Chemother.* **58**, 401–407.

Ferreira da Silva, M., Tiago, I., Veríssimo, A., Boaventura, R. A., Nunes, O. C. & Manaia, C. M. 2006 Antibiotic resistance of enterococci and related bacteria in an urban wastewater treatment plant. *FEMS Microbiol. Ecol.* **55**, 322–329.

Göbel, A., McArdell, C. S., Joss, A., Siegrist, H. & Giger, W. 2007 Fate of sulfonamides, macrolides, and trimethoprim in different wastewater treatment technologies. *Sci. Total Environ.* **372**, 361–371.

Göbel, A., Thomsen, A., McArdell, C. S., Joss, A. & Giger, W. 2005 Occurrence and sorption behavior of sulfonamides, macrolides, and trimethoprim in activated sludge treatment. *Environ. Sci. Technol.* **39**, 3981–3989.

Golet, E. M., Xifra, I., Siegrist, H., Alder, A. C. & Giger, W. 2003 Environmental exposure assessment of fluoroquinolone antibacterial agents from sewage to soil. *Environ. Sci. Technol.* **37**, 3243–3249.

Grape, M., Sundström, L. & Kronvall, G. 2003 Sulphonamide resistance gene *sul3* found in *Escherichia coli* isolates from human sources. *J. Antimicrob. Chemother.* **52**, 1022–1024.

Heise, J., Höltge, S., Schrader, S. & Kreuzig, R. 2006 Chemical and biological characterization of non-extractable sulfonamide residues in soil. *Chemosphere* **65**, 2352–2357.

Hijosa-Valsero, M., Fink, G., Schluesener, M. P., Sidrach-Cardona, R., Martín-Villacorta, J., Ternes, T. & Bécares, E. 2011 Removal of antibiotics from urban wastewater by constructed wetland optimization. *Chemosphere* **83**, 713–719.

Hollenbeck, B. L. & Rice, L. B. 2012 Intrinsic and acquired resistance mechanisms in enterococcus. *Virulence* **5**, 421–433.

Hryniewicz, K., Szczypa, K., Sulilowska, A., Jankowski, K., Betlejewska, K. & Hryniewicz, W. 2001 Antibiotic susceptibility of bacterial strains isolated from urinary tract infections in Poland. *J. Antimicrob. Chemother.* **47**, 773–780.

Junker, T., Alexy, R., Knacker, T. & Kümmerer, K. 2006 Biodegradability of 14C-labeled antibiotics in a modified laboratory scale sewage treatment plant at environmentally relevant concentrations. *Environ. Sci. Technol.* **40**, 318–324.

Kim, S. & Aga, D. S. 2007 Potential ecological and human health impacts of antibiotics and antibiotic-resistant bacteria from wastewater treatment plants. *J. Toxicol. Environ. Health. B. Crit. Rev.* **10**, 559–573.

Kümmerer, K. 2001 Drugs in the environment: emission of drugs, diagnostic aids, and disinfectants into wastewater by hospitals in relation to other sources – a review. *Chemosphere* **45**, 957–969.

Kümmerer, K. 2009 Antibiotics in the aquatic environment – A review – Part I. *Chemosphere* **75**, 417–434.

Leclercq, R. 2002 Mechanisms of resistance to macrolides and lincosamides: nature of the resistance elements and their clinical implications. *Clin. Infect. Dis.* **34**, 482–492.

Leclercq, R., Cantón, R., Brown, D. F. J., Giske, C. G., Heisig, P., MacGowan, A. P., Mouton, J. W., Nordmann, P., Rodloff, A. C., Rossolini, G. M., Soussy, C.-J., Steinbakk, M., Winstanley, T. G. & Kahlmeter, G. 2013 EUCAST expert rules in antimicrobial susceptibility testing. *Clin. Microbiol. Infect.* **19**, 141–160.

Lindberg, R. H., Olofsson, U., Rendahl, P., Johansson, M. I., Tysklind, M. & Andersson, B. A. 2006 Behavior of fluoroquinolones and trimethoprim during mechanical, chemical, and active sludge treatment of sewage water and digestion of sludge. *Environ. Sci. Technol.* **40**, 1042–1048.

Luczkiewicz, A., Fudala-Książek, S., Jankowska, K. & Olańczuk-Neyman, K. 2010 Antimicrobial resistance of fecal indicators in municipal wastewater treatment plant. *Water Res.* **44**, 5089–5097.

Magiorakos, A. P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., Harbarth, S., Hindler, J. F., Kahlmeter, G., Olsson-Liljequist, B., Paterson, D. L., Rice, L. B., Stelling, J., Struelens, M. J., Vatopoulos, A., Weber, J. T. & Monnet, D. L. 2011 Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol. Infect.* **18**, 268–281.

Manero, A., Vilanova, X., Cerdà-Cuéllar, M. & Blanch, A. 2002 Characterization of sewage waters by biochemical fingerprinting of Enterococci. *Water Res.* **36**, 2831–2835.

Marcinek, H., Wirth, R. & Muscholl-Silberhorn, A. 1998 *Enterococcus faecalis* gene transfer under natural conditions in municipal sewage water treatment plants. *Appl. Environ. Microbiol.* **64**, 626–632.

Muyzer, G., De Waal, E. C. & Uitierlnden, A. G. 1993 Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* **59**, 695–700.

Ohlsen, K., Ziebuhr, W., Koller, K. P., Hell, W., Wichelhaus, T. A. & Hacker, J. 1998 Effects of subinhibitory concentrations of antibiotics on alpha-toxin (*hla*) gene expression of methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* isolates. *Antimicrob. Agents Chemother.* **42**, 2817–2823.

Olanczuk-Neyman, K. & Jankowska, K. 2001 Bacteriological quality of the sandy beach in Sopot (Gdansk Bay, Southern Baltic). *Pol. J. Environ. Stud.* **10**, 451–455.

Olanczuk-Neyman, K., Geneja, M., Quant, B., Dembinska, M., Kruczalak, K., Kulbat, E., Kulik-Kuziemska, I., Mikołajski, S.

& Gielert, M. 2003 Microbiological and biological aspects of the wastewater treatment plant 'Wschod' in Gdansk. *Pol. J. Environ. Stud*. **12**, 747–757.

Pérez, S., Eichhorn, P. & Aga, D. S. 2005 Evaluating the biodegradability of sulfamethazine, sulfamethoxazole, sulfathiazole, and trimethoprim at different stages of sewage treatment. *Environ. Toxicol. Chem*. **24**, 1361–1367.

Perreten, V. & Boerlin, P. 2003 A new sulfonamide resistance gene (*sul*3) is widespread in the pig population of Switzerland. *Antimicrob. Agents Chemother.* **47**, 1169–1172.

Reinthaler, F. F., Posch, J., Feierl, G., Wust, G., Haas, D., Ruckenbauer, G., Mascher, F. & Marth, E. 2003 Antibiotic resistance of *E. coli* in sewage and sludge. *Water Res.* **37**, 1685–1690.

Rice, E. W., Boczek, L. A., Johnson, C. H. & Messer, J. W. 2003 Detection of intrinsic vancomycin resistant enterococci in animal and human feces. *Diagn. Microbiol. Infect. Dis*. **46**, 155–158.

Sambrook, J. & Russell, D. W. 2001 *Molecular Cloning: A Laboratory Manual*, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schluesener, M. P., Spiteller, M. & Bester, K. 2003 Determination of antibiotics from soil by pressurized liquid extraction and liquid chromatography–tandem mass spectrometry. *J. Chromatogr*. **1003**, 21–28.

Soda, S., Otsuki, H., Inoue, D., Tsutsui, H., Sei, K. & Ike, M. 2008 Transfer of antibiotic multiresistant plasmid RP4 from *Escherichia coli* to activated sludge bacteria. *J. Biosci. Bioeng*. **106**, 292–296.

Soufi, L., Sáenz, Y., Vinué, L., Abbassi, M. S., Ruiz, E., Zarazaga, M., Ben Hassen, A., Hammami, S. & Torres, C. 2011 *Escherichia coli* of poultry food origin as reservoir of sulphonamide resistance genes and integrons. *Int. J. Food. Microbiol.* **144**, 497–502.

Szczepanowski, R., Linke, B., Krahn, I., Gartemann, K. H., Gützkow, T., Eichler, W., Pühler, A. & Schlüter, A. 2009 Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. *Microbiology* **155**, 2306–2319.

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

Ziembińska, A., Ciesielski, S. & Miksch, K. 2009 Ammonia oxidizing bacteria community in activated sludge monitored by denaturing gradient gel electrophoresis (DGGE). *J. Gen. Appl. Microbiol*. **55** (5), 373–380.

Zuckerman, J. M. 2004 Macrolides and ketolides: azithromycin, clarithromycin, telithromycin. *Infect. Dis. Clin. North. Am*. **18**, 621–649.

First received 11 June 2012; accepted in revised form 28 June 2013. Available online 12 August 2013