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Prevalence of Clinically Relevant Antibiotic Resistance Genes in Surface Water Samples Collected from Germany and Australia

C. Stoll,[‡] J. P. S. Sidhu,[†,§,*] A. Tiehm,[‡] and S. Toze[†,∥]

[†]CSIRO Land and Water, Ecosciences Precinct, 41 Boggo Road, Brisbane 4102, Australia

[‡]DVGW-Technologiezentrum Wasser (TZW), Karlsruher Street 84, D-76139 Karlsruhe, Germany,

[§]Faculty of Science, Health and Education, University of the Sunshine Coast, Maroochydore, DC, Queensland 4558, Australia,

[∥]School of Population Health, University of Queensland, Herston Road, Herston, Brisbane, QLD, Australia, 4006.

ABSTRACT: The prevalence and proliferation of antibiotic resistant bacteria is profoundly important to human health, but the extent to which aquatic environments contribute toward the dissemination of antibiotic resistant genes (ARGs) is poorly understood. The prevalence of 24 ARGs active against eight antibiotic classes (*β*-lactams, aminoglycosides, glycopeptides, chloramphenicols, tetracycline, macrolides, trimethoprim, and sulfonamides) was evaluated in surface water samples collected from Germany and Australia with culture independent methods. The ARGs most frequently detected both in Germany and Australia were *sulI*, *sulII* (77−100%), and *dfrA1* (43−55%) which code for resistance to sulfonamide and trimethoprim. Macrolides resistance gene *ermB* was relatively more prevalent in the surface water from Germany (68%) than Australia (18%). In contrast, the chloramphenicol resistance gene *catII* was more frequently detected in Australia (64%) than Germany (9%). Similarly, *β*-lactams resistance gene *amp*C was more prevalent in the samples from Australia (36%) than Germany (19%). This study highlights wide distribution of ARGs for sulfonamide, trimethoprim, macroline, *β*-lactams and chloramphenicol in the aquatic ecosystems. Aquatic ecosystems can therefore be reservoirs of ARGs genes which could potentially be transferred from commensal microorganisms to human pathogens.

INTRODUCTION

The occurrence and spread of antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs) have become an important public health and environmental contamination issue worldwide.[1−5] Hospital settings, wastewater treatment plants and animals raised for food are well recognized sources of ARB.[6−9] However, the significance of aquatic environments as reservoirs of ARGs is comparatively less understood.

Antibiotic resistant bacteria are constantly released into the aquatic environment through the disposal of human and animal waste.[10,11] Furthermore, intensive use of antibiotics in treatment of bacterial infections in humans, animals and for agricultural purposes has led to their constant release into the environment.[12−14] The released antibiotics persist in soil and aquatic environments and apply antibiotic selective pressure on autochthonous bacterial communities.[4] Bacteria acquire antimicrobial resistance as a result of random chromosomal mutation or horizontal gene transfer.[15,16] Horizontal gene transfer enables the exchange of genetic material located on mobile elements (transposons, integrons or plasmids) among related or unrelated bacterial species[17] which is the main mechanism for spreading ARGs.[3,18] Auatic environments provide ideal settings for the horizontal exchange of mobile genetic agents encoding for antibiotic resistance in bacteria.[19,4] There is an evidence of transfer of resistance elements to known human commensal bacteria and pathogens.[20−22] Consequently, aquatic ecosystems are increasingly recognized as sources for antibiotic resistant bacteria (ARB) and reservoirs of ARGs.[4,23,24]

Previous studies have reported the presence of antibiotic resistance among culturable bacteria in the surface water,[25−28] groundwater[29] and drinking water.[30−32] Most of the studies, however, use phenotypic characterization with cultivation of isolates (usually Gram negative) on the agar plates with minimal inhibitory concentration of antibiotics. This approach does not provide information about the overall prevalence of antibiotic resistant genotypes, as only a fraction of microorganisms (<1%) in aquatic environment can be cultured by standard methods,[33] thus culture dependent techniques are likely to provide a significant underestimation of the ARB and antibiotic resistance gene pool in aquatic ecosystems.

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Table 1. Target Genes, Primer Sequences and Amplicon Size for the Detection of Antibiotic Genes in Surface Water[a]

antibiotic class	target gene	primer sequence (5′-3′)	amplicon size (bp)	reference
Beta-lactams	*ampC*	F-TTC TAT CAA MAC TGG CAR CC	550	32
		R- CCY TTT TAT GTA CCC AYG A		
	bla_{SHV}	F- TCG CCT GTG TAT TAT CTC CC	857	43
		R- CGC AGA TAA ATC ACC ACA ATG		
	bla_{PSE-1}	F-TGC TTC GCA ACT ATG ACT AC	438	44
		R- AGC CTG TGT TTG AGC TAG AT		
Glycopeptides	*vanA*	F-TCT GCA ATA GAG ATA GCC GC	377	45
		R- GGA GTA GCT ATC CCA GCA TT		
	vanB	F-GTG ACA AAC CGG AGG CGA GGA	433	46
		R- CCG CCA TCC TCC TGC AAA AAA		
	vanC	F- GAA AGA CAA CAG GAA GAC CGC	796	46
		R- ATC GCA TCA CAA GCA CCA ATC		
Tetracycline	*tet*(A)	F-GTG AAA CCC AAC ATA CCC C	880	43
		R- GAA GGC AAG CAG GAT GTA G		
	tet(B)	F- CCT TAT CAT GCC AGT CTT GC	774	43
		R- ACT GCC GTT TTT TCG CC		
	tet(C)	F-ACT TGG AGC CAC TAT CGA C	881	43
		R- CAT CAA TCC ATG CCA ACC C		
	tet(M)	F-ACA GAA AGC TTA TTA TAT AAC	171	47
		R- TGG CGT GTC TAT GAT GTT CAC		
Sulfonamides	*sulI*	F-TTC GGC ATT CTG AAT CTC AC	822	43
		R- ATG ATC TAA CCC TCG GTC TC		
	sulII	F-CGG CAT CGT CAA CAT AAC C	722	43
		R- GTG TGC GGA TGA AGT CAG		
Chloramphenicols	*catI*	F-GGC ATT TCA GTC AGT TG	585	48
		R- CCG CCC TGC CAC TCA TC		
	catII	F-CCT GGA ACC GCA GAG AAC	495	48
		R- CCT GCT GAA ACT TTG CCA		
	floR	F-CGC CGT CAT TCC TCA CCT TC	215	43
		R- GAT CAC GGG CCA CGC TGT GTC		
Aminoglycosides	*aac(3)-IIa*	F-CGG CCT GCT GAA TCA GTT TC	436	44
		R- AAA GCC CAC GAC ACC TTC TC		
	aac(3)-IV	F-GTG TGC TGC TGG TCC ACA GC	627	43
		R- AGT TGA CCC AGG GCT GTC GC		
	aac(6′)-Ie-aph(2″)-Ia	F-CAG AGC CTT GGG AAG ATG AAG	348	49
		R- CCT CGT GTA ATT CAT GTT CTG GC		
	aph(2″)-Ic	F-CCA CAA TGA TAA TGA CTC AGT TCC C	444	49
		R- CCA CAG CTT CCG ATA GCA AGA G		
Trimethoprim	*dfrA1*	F-AAG AAT GGA GTT ATC GGG AAT G	391	43
		R- GGG TAA AAA CTG GCC TAA AAT TG		
	dfrA12	F-AAA TTC CGG GTG AGC AGA AG	429	44
		R- CCC GTT GAC GGA ATG GTT AG		
	dfrA13	F-GCA GTC GCC CTA AAA CAA AG	294	44
		R- GAT ACG TGT GAC AGC GTT GA		
Macrolides	*ermA*	F-AAG CGG TAA ACC CCT CTG A	190	50
		R- TTC GCA AAT CCC TTC TCA AC		
	ermB	F-CAT TTA ACG ACG AAA CTG GC	405	51
		R- GGA ACA TCT GTG GTA TGG CG		

[a]F: Forward primer; R: Reverse primer.

Alternative molecular techniques such as PCR or molecular gene probe assays allow sensitive and specific detection of ARGs in the environment without the need to culture bacteria. Several recent studies have used PCR based methods to study the occurrence of ARGs in surface water,[34,9] groundwater[7,35,36] and wastewater.[32,37,38] However, the current understanding on

the presence and abundance of ARGs in the aquatic environment is limited.

Antibiotics use varies from country to country. In Europe, countries with higher overall antibiotic use have been shown to have a higher prevalence of ARB.[39] The observed variation in patterns of resistance to various classes of antibiotics between countries has been shown to be due to the variation in selection pressure which results from the community use of these drugs.[39] In Europe, antibiotics use in agriculture has been heavily scrutinized, and use of antibiotics for growth promotion has been banned since 2006.[40] For example, use of virginiamycin as an animal growth promoter has been banned in the EU since 1998. Whereas in Australia, antibiotics such as virginiamycin, tylosin and bacitracin are used as growth promoters for livestock production.[41]

The main goal of this study was an evaluation of the degree to which surface water acts as reservoir of ARGs which could potentially be transferred to human pathogens. The specific aims of this study were (i) to evaluate the frequency of occurrence of 24 ARGs for eight antimicrobial groups in surface water (ii) to determine if the pattern of ARGs prevalence in surface water samples collected from Germany was any different from Australia in response to antibiotic use practices, and (iii) to determine if there is a link between the extent of fecal pollution and the detection of ARGs.

MATERIALS AND METHODS

Study Sites. In Australia, surface water samples (n = 22) were collected from Brisbane River and its tributaries (11 sites). In Germany, surface water samples (n = 43) were collected from Rivers Rhine and Danube and their tributaries. Seventeen water samples were collected from River Danube near the localities of Leipheim (9), Ulm-Wiblingen (3), Ehingen-Gamerschwang (3), and Dettingen (2). In addition, two samples each were collected from the two tributaries of the River Danube (Iller and Schmiech). Further, 18 samples were collected along the River Rhine near Karlsruhe (km 363−386; 16 samples) and Düsseldorf (km 732,1; two samples). Additionally, for the River Rhine and its tributary Alb near the city of Karlsruhe was sampled four different times.

Water Sample Collection and Processing. Duplicate grab samples (500 mL each) were collected from each site in sterile containers and transported to the laboratory on ice. The water samples collected in Brisbane were prefiltered through 47 mm diameter, 8 μm nitrocellulose membrane filters (Millipore, Australia) to remove clay and other suspended material from the samples. The samples were then filtered through 0.22 μm (47 mm), nitrocellulose membrane filters (Millipore, Australia) to capture bacteria. In Germany, due to low water turbidity, collected water samples were directly filtered through 0.2 μm Supor-200 membrane filters (Pall Life Science, Germany). All of the membrane filters were stored at −20 °C for subsequent extraction of nucleic acid in a single batch.

Quantification of Fecal Indicator Bacteria (FIB). A portion of each collected water sample was used for quantification of FIB with standard culture methods within 24 h of collection. For the German samples, *Escherichia coli* and total coliform bacteria were enumerated according to DIN EN ISO 9308-1, the Colilert-18/Quanti-Tray (IDEXX) method or the Chromocult coliform agar (Merck) method. For the Australian samples, 1 and 10 mL of water samples was filtered through 0.45 μm nitrocellulose (Millipore) filters (47 mm) and placed on respective selective agar plates in triplicate. *E. coli* was enumerated on Chromocult coliform agar (Merck) and *Enterococcus* spp. on Chromocult enterococci agar (Merck). Plates were incubated at 37 °C overnight and then typical colonies were counted to determine the average number of colony forming units (cfu) 100 mL^{-1}.

DNA Extraction and Initial Screening. Genomic DNA was extracted directly from the membranes by using UltraClean Soil DNA Kit (Mo Bio Laboratories, USA) as per manufactures instructions. Briefly, each membrane filter was cut into half and both halves were directly added to the sample tubes containing extraction buffer and beads. At the end of the bead beating step the membrane was removed from the tubes and the standard protocol for extraction of nucleic acid was followed as per manufacturer instructions. Extracted nucleic acid was stored at −80 °C prior to analysis. The integrity of the extracted DNA was check by performing PCR with the eubacteria specific primers 2f and 1492r on a 10 and 100 fold dilution of extracted nucleic acid.[42] The presence of amplified DNA was confirmed by electrophoresis on 1% agarose gels.

PCR Detection of Antibiotic Resistant Genes. In this study, collected water samples were screened for the presence of 24 ARGs against eight antibiotic classes. The ARGs include; sulfonamide resistance genes (*sulI* and *sulII*), trimethoprim resistance genes (*dfrA1*, *dfrA12*, *dfrA13*), β-lactam resistance genes (*ampC*, bla_{SHV}, and bla_{PSE-1}), aminoglycosides resistance genes (*aac(3)-IIa*, *aac(3)-IV*, *aac(6′)-Ie-aph(2″)-Ia*, *aph(2″)-Ic*), chloramphenicol resistance genes (*catI*, *catII*, and *floR*), tetracycline resistance genes (*tet*(A), *tet*(B), *tet*(C), and *tet*(M)), macrolide resistance genes (*ermB* and *ermA*) and glycopeptide resistance genes (*vanA*, *vanB*, and *vanC*). These ARGs were selected due to their reported presence in the environment. It was expected that ARGs concentration would be higher in the urban environment impacted by anthropogenic activities than in pristine and less-impacted sites. Therefore, samples were collected from a number of sites which represented both impacted and pristine sites from Germany and Australia. Previously published primer sets were used for the PCR amplification of ARGs (Table 1).

PCR Positive Controls. Antibiotic resistant coliform bacteria isolated from the surface water in Germany were screened for the presence of ARGs *sulI*, *sulII*, *dfrA1*, *dfrA12*, *tet*(A), *tet*(B), bla_{SHV}, and *ampC* genes. For the remaining genes, DNA extracted from the wastewater samples were screened for the presence of appropriate sized band on the electrophoresis gels. The PCR amplified products were purified using the E.Z.N.A. Gel Purification Kit (Omega Bio-Tek Inc., USA) and cloned into a pGEM-T vector system (Promega, Germany), transferred into *E. coli* JM109 competent cells, and plated on LB agar plates containing ampicillin, IPTG, and X-gal as per manufacturer's instructions. Recombinant plasmids with corresponding inserts were purified using the Plasmid Purification Midi Kit (Qiagen, Germany) and DNA sequencing was carried out at Starseq (Germany). The plasmids were then used as PCR positive controls.

PCR Amplification and Gel Electrophoresis. PCR amplification of ARGs was performed in 20 μL reaction mixtures. Each reaction mixture contained 1x buffer with $MgCl_2$ (Molzym, Germany), 200 μM each dNTP (Roth, Germany), 0.5 μM each primer (Invitrogen, Germany), 2U of Taq Polymerase (Molzym, Germany) and 2 μL of DNA template. The PCR amplifications were performed using Tpersonal thermocycler (Biometra, Germany). The thermocycling parameters were: initial denaturing for 3 min at 95 °C,

followed by 35 cycles of 30s at 94 °C, annealing at 55 °C for 30s and extension for 30−120s depending on the product length at 72 °C followed by a final extension for 10 min at 72 °C. All PCR experiments included positive controls (corresponding plasmid DNA or genomic DNA) and a negative control (sterile water). Ten microliters of amplified product was electrophoresed on a 1% Tris-acetate-EDTA agarose gel containing 2 μg of ethidium bromide mL^{-1}. DNA molecular weight marker pBR 328 (Roth, Germany) was used as a standard DNA ladder.

Statistical Analysis. The student's *t test* was performed to compare the significance of difference between the occurrence of VGs between German and Australian sites. The critical *P*-value for the *t* test was set at 0.05 and all tests were considered significant if the *P* value was <0.05. Pearson correlation analysis was carried out on the $\log_{10}$ transformed FIB numbers to determine the existence of correlation between *E. coli* and *Enterococcus* spp. in samples collected from Australia and between *E. coli* and total coliforms from Germany.

RESULTS

Antibiotic Resistant Genes by Antibiotic Class. The frequency of detection of sulfonamide resistance genes was 100% from both Germany and Australia (Figure 1 and 3).

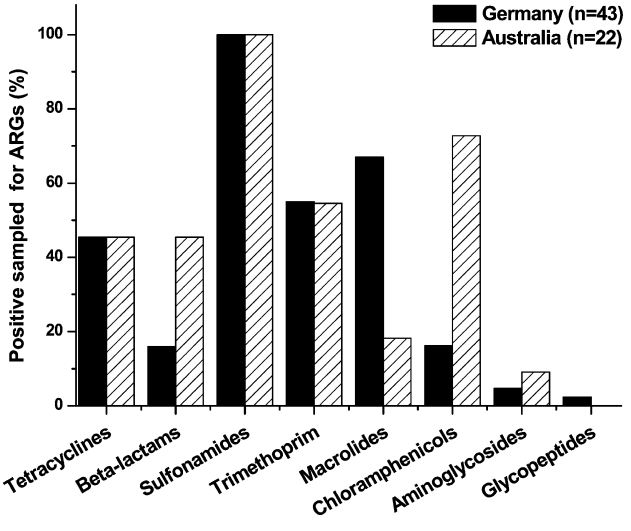

Figure 1. Percent samples tested positive for eight groups of antibiotic tested.

Similarly, while not detected in as many samples, the frequency of detection of trimethoprim and tetracycline resistance genes was similar ($p > 0.05$) in Germany and Australia at 55 and 45%, respectively. β-lactam and chloramphenicol resistance genes were more frequently detected in Australia (45 and 72% samples, respectively) compared to Germany, where the frequency of detection was only 16% for both groups. Conversely, prevalence of macrolide resistance genes was significantly higher ($p < 0.05$) in Germany (67%) than Australia (18%). Aminoglycoside and glycopeptide resistance genes were detected in less than 10% of samples in both Germany and Australia. A comparative analysis of ARGs from two German rivers revealed higher prevalence of trimethoprim resistance *dfrA1* and β-lactamas resistance *amp*C genes in River Rhine samples compared to River Danube (Figure 4). In contrast, the chloramphenicol resistance gene *catII* and aminoglycoside resistance gene *aac(6′)-Ie-aph(2″)-Ia* were less frequently detected and only in water samples from River Danube.

β-Lactamase Resistance Genes. The genes bla_{SHV} and *ampC* were detected in the surface water samples collected from both Australia and Germany (Figure 3). The gene bla_{SHV} was more frequently detected in the samples from Germany (16%) than Australia (9%). Whereas gene *amp*C was more frequently detected in the samples collected from Australia (36%) than Germany (19%). The bla_{PSE-1} gene was not detected in any of the samples collected from Australia or Germany.

Tetracycline Resistance Genes. In general, 45% of samples from Germany and Australia tested positive to one or more tetracycline resistance genes (Figure 2). Three efflux

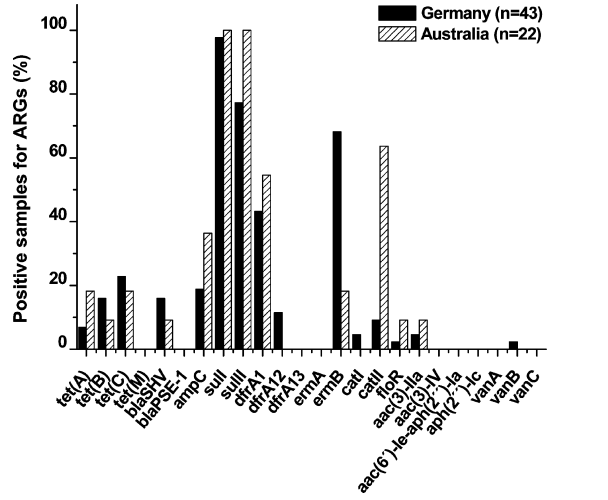

Figure 2. Relative abundance of ARGs in the surface water samples collected in Germany and Australia.

pump encoding tetracycline resistance genes *tet*(A) *tet*(B) and *tet*(C) were detected in the water samples collected from both Australia and Germany (Figure 3). The *tet*(A) gene was found to be more prevalent in Australia (18%) than Germany (7%). In comparison, *tet*(B) was found more frequently in samples from Germany (16%) than Australia (9%). Similarly *tet*(C) was found to be only slightly more prevalent in Germany (22%) than Australia (18%). However, prevalence of *tet*(C) gene was significantly higher ($p < 0.05$) in the samples from River Rhine as (49%) compared to River Danube (18%). The *tet*(M) gene, which belongs to the group of ribosomal protection proteins was not detected in any of the samples analyzed.

Sulfonamide and Trimethoprim Resistance Genes. Sulfonamide resistance genes *sulI* and *sulII* were the most frequently detected of this gene class in the surface water samples collected from both Germany and Australia (100%) (Figure 2). The *sulI* gene was more prevalent (98%) than *sulII* gene (77%) in the surface water samples collected from Germany. The trimethoprim resistance gene *dfrA1* was detected in 55 and 43% of the samples collected from Australia and Germany, respectively. The second trimethoprim resistance gene *dfrA12* was detected less frequently (11%) and only in the water samples collected from Germany (Figure 3). The *dfrA13* gene was not detected in any of the samples collected from either Australia or Germany.

Aminoglycoside Resistance Genes. In this study, we tested for the genes encoding for three enzymes that are

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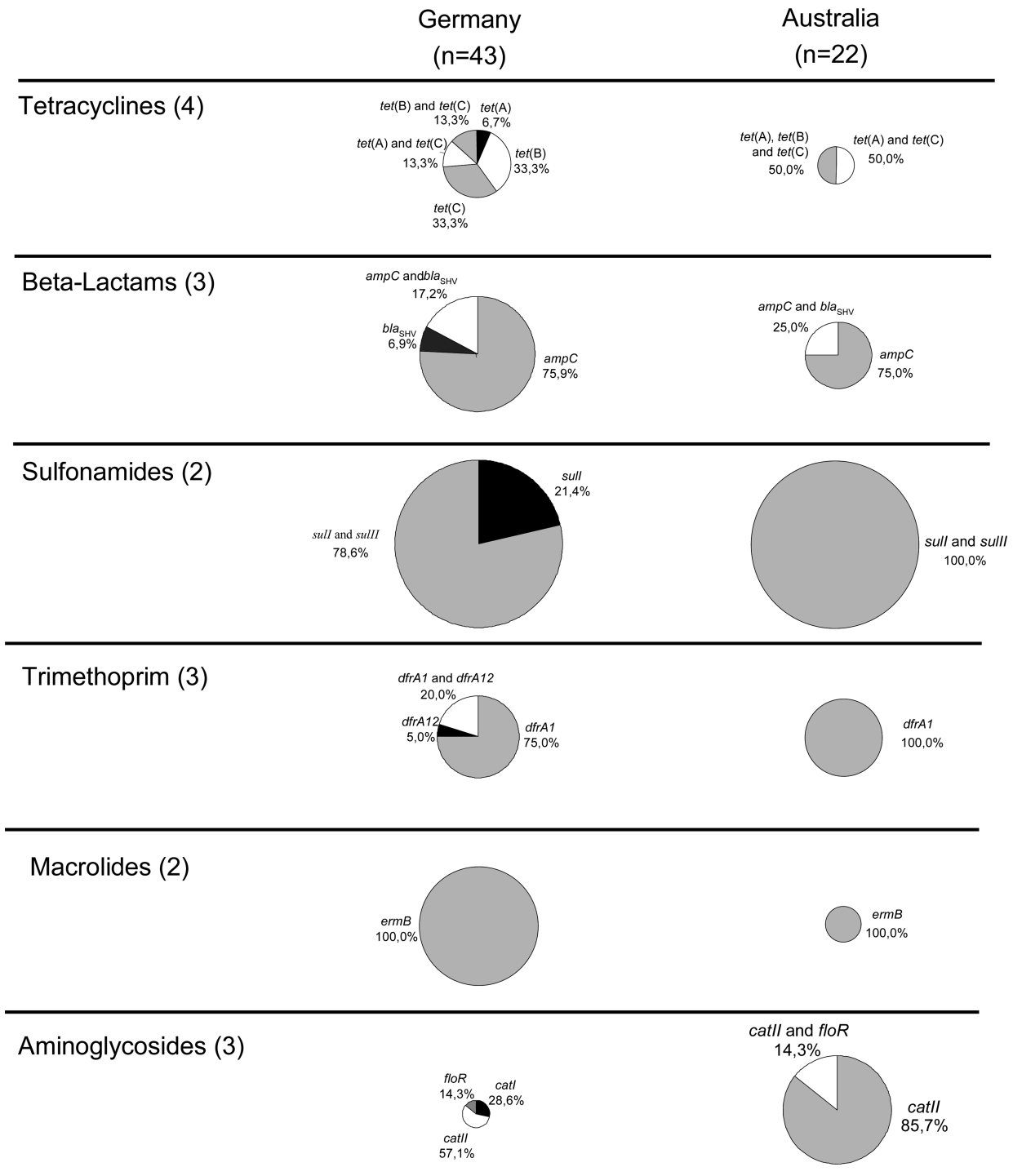

Figure 3. Comparison of the distribution of distribution of "ARGs" in surface water samples from Germany and Australia. Percentage of positive samples for specific ARG with regard to the positive samples for an antibiotic group. Size of the circle correlates with percentage of positive samples for an antibiotic group.

responsible for the inactivation of aminoglycosides, which include acetyltransferases (AAC), nucleotidyltransferases (ANT) and phosphotransferases (APH). Out of four gentamicin resistance genes tested of in this study (*aac(3)-IIa*, *aac(3)-IV*, *aph(2″)-Ic* and *aac(6′)-Ie-aph(2″)-Ia*) only the *aac(3)-IIa* gene was detected in the surface water samples in relatively low frequency of 5 and 9% from Germany and Australia, respectively.

Chloramphenicol Resistance Genes. In general, chloramphenicol resistance genes were detected in significantly higher ($p < 0.05$) number of samples in Australia (72%) compared to Germany where the frequency of detection was only 16% (Figure 3). Furthermore, chloramphenicol acetyltransferases (*catII*) gene was most frequently detected (64%), whereas *catI* was not detected in any of the samples. All three genes were detected in the water samples collected from Germany with a low frequency of 5, 9, and 3% for *catI*, *catII* and *floR*, respectively (Figure 3). In Australia, the *catI* gene was not detected in any of the water samples from but the *catII* gene was highly prevalent (64%). The Florfenicol resistance gene

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floR was more prevalent in the water samples from Australia (9%) than Germany (3%).

Macrolide Resistance Genes. The *ermA* gene was not detected in any of the samples collected from both Australia and Germany (Figure 2). In contrast, the *ermB* gene was detected in significantly higher ($p < 0.05$) frequency (68%) in Germany compared to Australia (18%). Also, the prevalence of *ermB* gene was found to be higher in the samples collected from River Rhine (69%) than River Danube (47%) (Figure 4).

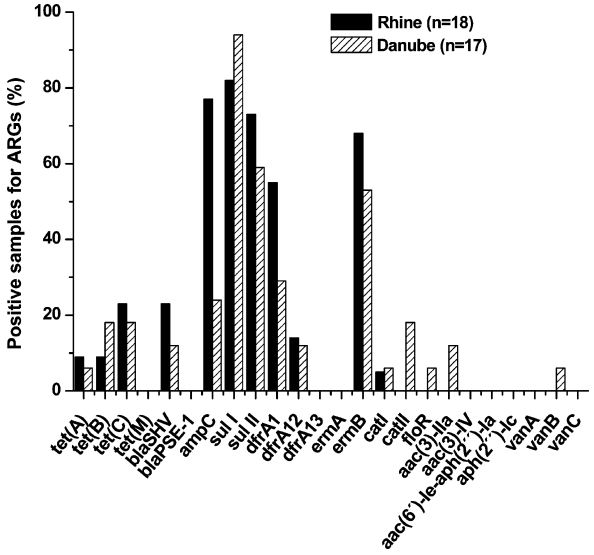

Figure 4. Relative abundance of ARGs in surface water samples collected from River Rhine and Danube in Germany.

Glycopeptide Resistance Genes. The genes *vanA* and *vanC*, were not detected in any of the surface water samples collected from both Australia and Germany. Glycopeptide resistance gene *vanB* had very low prevalence (2.3%) in the water samples collected from only Germany (Figure 3).

FIB Numbers and Occurrence of Multiple ARGs in Samples. Selected water samples ($n = 8$) from Germany were analyzed for *E. coli* and total coliforms and results were plotted after $\log_{10}$ transformation. Similarly, samples ($n = 11$) collected from Australia were plotted after $\log_{10}$ transformation for *E. coli* and *Enterococcus* spp. numbers. The numbers of FIB in surface water samples collected in Australia varied from 1.72 to 2.86 $\log_{10}$ and 1.91 to 3.01 $\log_{10}$ 100 mL^{-1} for *E. coli* and *Enterococcus* spp., respectively. There was a significant correlation between *E. coli* and *Enterococcus* spp. ($r_p = 0.90$; $P < 0.0001$). The numbers of FIB in surface water samples collected in Germany varied from 1.72 to 3.46 $\log_{10}$ and 2.76 to 4.16 $\log_{10}$ 100 mL^{-1} for *E. coli* and total coliforms, respectively. There was also a significant correlation between *E. coli* and total coliform bacteria from samples collected in Germany ($r_p = 0.85$; $P < 0.0001$). A graph was plotted between FIB numbers and ARGs numbers for samples collected from Germany and Australia to check if samples with high FIB counts carried higher number of ARGs (Figure 5). In water samples from Australia, samples with higher FIB numbers had more ARGs present. Whereas higher prevalence of ARGs was not always associated with the presence of high FIB numbers in samples from Germany.

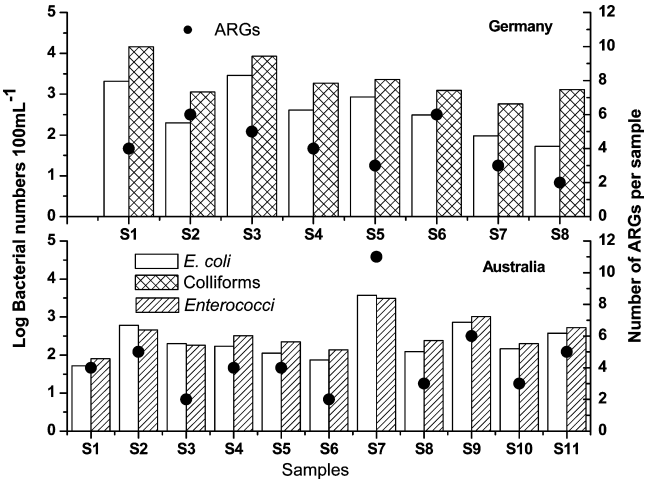

Figure 5. Distribution of ARGs and FIB numbers in surface water samples from Australia and Germany.

DISCUSSION

Occurrence and proliferation of antibiotic resistance in pathogenic and zoonotic bacteria has profound implications for human health as these bacteria can be transmitted to humans via direct or indirect contact.[52,22] An improved understanding of the abundance, distribution and diversity of ARGs in the aquatic environment is essential to manage the spread of antibiotic resistant bacteria and protect human health.

Sulfonamides act as competitive inhibitors of enzyme dihydropteroate synthase (DHPS) in the folic acid pathway of bacterial cells. The widespread prevalence of *sulI* and *sulII* genes in the aquatic ecosystems in Germany and Australia (77–100%) is most likely due to easy dissemination of these genes via highly mobile genetic elements. This is because these two genes (*sulI* and *sulII*) encoding for alternative sulfonamide-resistant DHPS in gram-negative bacteria are normally located on mobile genetic elements like class 1 integrons or plasmids.[53] As observed in our study, both genes have been reported to occur with roughly the same frequency in the clinical isolates of sulfisoxazole-resistant bacteria.[54] A previous study from Australia, also reported high levels of sulfamethoxazole resistance in *E. coli* isolates (63%) from the Brisbane River.[25] Extensive dissemination of sulfonamide resistance genes in the aquatic environment has also been reported from other parts of the world.[55,56,34,57,3] Wide spread distribution of sulfonamides resistant genes in aquatic environment could also be due to its extensive use in both humans and animals.[8,40] Sulfonamides are frequently detected in wastewater in significantly higher concentrations than macrolides and quinolones.[81] The extremely high prevalence of *sulI* and *sulII* genes in both Germany and Australia suggests that aquatic ecosystems are a major reservoir of sulfonamide resistant bacteria which is a cause of concern as sulfonamides are highly important broad spectrum antimicrobial agents.[8,59] Furthermore, both these genes have been detected in known human pathogens such as *Salmonella typhimurium*, *Streptococcus pneumonia* and *E. coli*.[57]

Trimethoprim alone or in combination with sulfonamide is extensively used as inexpensive antibiotic in humans and animals. Almost 20 plasmid mediated *dfr* genes are known to be responsible for imparting trimethoprim resistance which code for trimethoprim resistant dihydrofolate reductase (DHFR).[54] The relatively high prevalence of *dfrA1 gene* in 55% and 43% of the samples collected from Australia and

Germany, respectively, along with detection of the *dfrA12* gene in 11% samples from Germany suggests the aquatic environment is a significant reservoir of these genes. Trimethoprim resistance genes have been detected in *E. coli* isolates from surface water.[34] However, to date, very little is known about the occurrence and distribution of trimethoprim resistance genes in the other bacterial genera found in the aquatic ecosystem.

Approximately two-thirds of antibiotics administered to humans are β-lactams.[38] Plasmid-mediated $bla_{\text{PSE-1}}$ and bla_{SHV} genes coding for β-lactamase are known to provide resistance against third-generation cephalosporins and monobactam.[58] In this study, $bla_{\text{PSE-1}}$ gene was not detected, whereas bla_{SHV} gene was detected in samples from both Germany and Australia with 16 and 9% frequency, respectively. This suggests a low distribution of these genes in these aquatic environments. A variety of *bla* genes have been reported in the aquatic environment and sediments from other places in the world.[32,60−62] Another β-lactamase gene *amp*C was detected more frequently in water samples from Australia than Germany with 36 and 19% frequency, respectively. Presence of *amp*C gene has been reported previously from wastewater and drinking water biofilms.[32] High prevalence of *amp*C gene (78%) in wastewater has been reported from Germany.[37] However, there is a paucity of information on the frequency of occurrence of *amp*C gene in the surface water.

The mechanisms responsible for the chloramphenicol and florfenicol resistance include chloramphenicol acetyltransferases (encoded by *cat* genes) and chloramphenicol efflux pumps (encoded by *cml* genes) and multidrug transporters.[63] In this study, the *catII* gene was frequently detected in Australia (64%), whereas only *catI* gene was detected in samples from Germany in relatively low frequency (5%). The relatively high prevalence of chloramphenicol resistance genes in Australia (72%) as compared to Germany (16%) could potentially be due to more stringent control of its use in animals in the EU. The application of chloramphenicol in animals used as food in EU has been banned since 1994 and its use in veterinary medicine is limited to pets and nonfood producing animals.[63] In this study, *floR* genes were detected only infrequently both in Germany (2%) and Australia (9%). Florfenicol is solely used in veterinary medicine and high prevalence (66%) of *floR* gene has been reported in *Listeria monocytogenes* isolates from dairy environment.[64] However, no information on the prevalence of *floR* gene in surface water is available.

The most common mechanism mediating macrolide resistance is a post-transcriptional modification of the rRNA by methyltransferases.[65] Macrolide resistance (encoded by *erm* genes) can be easily transferred among bacteria as these genes are associated with plasmids and transposons.[66,67] Collected surface water samples were tested for the presence of methyltransferase genes *ermA* and *ermB*. The *ermA* gene was not detected in any of the samples collected from Germany and Australia. In contrast, the *ermB* gene was more frequently detected in Germany (68%) as compared to Australia (18%). Furthermore, the percentage of *ermB* positive sample from River Rhine water was higher (69%) than from the Danube River (47%). However little is known about the presence of *erm* genes in the aquatic environment. There is only one report on the detection of *ermB* gene, it was detected frequently in reclaimed water from two aquifer recharge systems in Germany.[7]

Tetracycline is extensively used for the treatment of bacterial infections in humans and animals.[68] Bacterial resistance to tetracycline is mediated mainly by energy dependent efflux pump (efflux proteins) and ribosomal protection proteins.[69] Out of the reported 38 different tetracycline resistance (*tet*) genes[70] this study only tested for the prevalence of four of these genes. The presence of one or more *tet* genes in 44 and 45% of samples collected from Germany and Australia, respectively, suggests a wide occurrence of *tet* genes in these aquatic ecosystems. Similarly, 88% of gram negative bacterial isolates from estuarine waters have been reported to have at least one of six *tet* genes.[71] Three efflux proteins encoding genes (*tet*(A), *tet*(B) and *tet*(C)) were detected in the surface water samples collected from Australia and Germany with <23% frequency (Figure 3). In China, *Enterobacteriaceae* isolates from surface water have been reported to have higher frequency of *tet*(A) and *tet*(B) genes (40−43%) as compared to *tet*(C) and *tet*(D) genes (7−9%).[5] The *tet*(M) gene, which is most commonly found in clinical *Enterococcus* spp. and terrestrial bacteria[72,73] was not detected from surface water samples collected from either Australia or Germany. Wide prevalence of *tet*(M) gene in bacterial isolated (60%) from marine sediments has been previously reported.[72]

Gentamicin and apramycin were introduced in veterinary medicine in the early 1980s in several European countries. Among the aminoglycosides, only gentamicin and apramycin (the latter because of cross-resistance to gentamicin) are relevant for human health. There has been evidence of gentamicin resistant *Enterococci* spread from animal meat to humans.[74] In humans, gentamicin is used (in combination with β-lactams) for treatment of severe infection, such as sepsis and endocarditis.[8] In this study, we detected only *aac(3)-Ia* gene in the surface water samples with 5% and 9% prevalence in Germany and Australia, respectively, which suggests a low prevalence of these genes in the aquatic environment in both countries. Gentamicin resistance genes have been detected however, in a number of bacterial genera (*Acinetobacter*, *Pseudomonas*, *Enterobacteriaceae*), and also in other phylogenetically distant bacteria isolated from coastal water polluted with sewage effluent. [75]

Glycopeptides inhibit the synthesis of the cell wall in Gram positive bacteria.[20] There are six recognized phenotypes of the glycopeptide resistance: *vanA*, *vanB*, *vanD*, *vanE*, and *vanG* (transferable resistances) and *vanC-1*, *vanC-2*, *vanC-3* (intrinsic resistances of specific *Enterococci*)[76] Glycopeptide resistance genes *vanA* and *vanB* are generally found in the clinical settings in central Europe.[76] In this study, we tested for the presence of *vanA*, *vanB*, and *vanC* genes in the total DNA extracted from surface water samples. These genes seem to be of minor relevance in both Australia and Germany as in surface water samples only *vanA* was detected in very low frequency (2%) in Germany. However, previous studies have reported the presence of *vanA* gene not only in municipal wastewater but also in drinking water biofilms in Germany.[37,32]

Fecal indicator bacteria such as *E. coli* and *Enterococci* are routinely used as indicators of surface water quality.[77,82] In both Australia and Germany we found very good correlation between FIB bacteria which is agreement with previously reported literature.[77,82] In Australia, water samples with higher FIB counts generally had higher number of ARGs which suggests low quality water presents higher risk of infection. However, this was not always the case with samples collected from German rivers. Further, investigation with larger sample sizes is required to determine if samples with higher FIB numbers always have high prevalence of ARGs.

The extremely high prevalence of sulfonamide (100%) and trimethoprim (55%) ARGs both in Germany and Australia suggest that there is a need for intervention in sulfonamide and trimethoprim usage in humans and animals. One of the potential reasons for this could be high use of both antibiotics in human and veterinary medicine along with it's high prevalence in the aquatic environment in both Australia and Germany. In a previous study from southeast Queensland, sulfamethoxazole which is authorized for use in only humans was frequently detected (83%) and in relative high concentrations in surface water samples.[78] Similar results were obtained in a German study, in which sulfamethoxazole was detected in 34 out of 40 water samples collected from different surface waters in northwestern Germany. [79]

In addition, it is worth noting that ARGs to macrolides were more frequently detected in Germany, whereas chloramphenicol and with β-lactams ARGs were more frequently detected in Australia. The reasons for this variation are not clear, but one possible reason could be the difference in antibiotic usage patterns in the two countries. The difference in antibiotic use between the two countries is hard to compare due to lack of availability of antibiotic use data outside the hospital setting in Australia. Raw products for antibiotic production are imported in Australia and it has been suggested that antibiotics used for humans and in agriculture can be calculated from the import volumes.[78] Whereas more detailed information is available from Germany, on the use of antibiotic in human and veterinary medicine.[68]

In Australia, β-lactams were the most commonly used group of antibiotic (between 1992 and 2003) for humans and in animal farming.[80] A similar situation was observed in Germany, where in 2005, amoxicillin was the single most commonly prescribed antibiotic in the human medicine with nearly 80 million defined daily doses per year which corresponds to 80 tonnes per year.[68] In addition, β-lactams constitute the largest group of antibiotics used in veterinary medicine in Germany.[68] In Australia, an analytical study on the concentration of antibiotic prevalence in environmental samples showed that trace concentrations of β-lactams occur in hospital and municipal wastewaters and in surface waters.[78] For example, amoxicillin was detected in 30% of the analyzed surface water samples with maximum concentration of 200 ng L^{-1}.[78] In contrast, Christian et al.[79] detected β-lactam antibiotics in just a few surface water samples collected in Germany. Only 5 of the 12 analyzed β-lactams were detected at concentrations up to 48 ng L^{-1}, but on average the concentrations were <10 ng L^{-1} .[79] These findings were explained with the poor stability of the β-lactam ring.[79] However, the β-lactam concentrations in the aquatic environment seem to be much higher in Australia than in Germany, which could potentially be one of the reasons of more prevalence of β-lactam resistance genes in Australia.

Macrolides are one of the most commonly used antibiotic groups in Australia particularly in human medicine with close to 650 tonnes of erythromycin, roxithromycin, oleandomycin, and tylosin imported into Australia from 1992 to 2003.[80] In Germany, macrolides are third and fourth frequently used antibiotic group for human and animal application, respectively.[68] In Australia as well as in Germany the presence of macrolides in the surface waters has been previously detected.[78,79] In the German study, erythromycin and dehydrato-erythromycin was the most prevalent antibiotic, which occurred in a wide concentration range (above 50 ng L^{-1} with peak concentrations up to 130 ng L^{-1} and 300 ng L^{-1})[79] In Australia, erythromycin was detected in the surface waters but the concentrations detected were below quantifiable limits.[78] The lower prevalence of macrolides in the surface waters in Australia compared to Germany could potentially be the reason of higher frequency of detection of ARGs to macrolides in Germany. The *erm* genes also mediate resistance against other antibiotics like lincosamides and streptogramin. However, from these two studies data is available for the lincosamide antibiotic lincomycin only. In both studies the average concentration of lincomycin in the surface waters was reported to be quite low (1−10 ng L^{-1}) for Australia and Germany, respectively.[78,79]

It is important to note that in this study the method used was the analysis of total DNA isolated from the water samples, hence this technique does not provide information on the actual expression of the ARGs. However, the main goal of this study was to evaluate the degree to which aquatic ecosystems serve as reservoir of ARGs which could potentially be transferred to human pathogens. This study clearly demonstrates that surface water is an important reservoir of ARGs for a number of antibiotic classes such as sulfonamide, trimethoprim, tetracycline, macrolides, and chloramphenicols in both Australia and Germany. Further, studies are required to unravel the pathways involved in the spread of ARGs into the environment. This information will facilitate improved risk assessment from antibiotic use in humans and animals and may also assist in developing strategies to limit spread of antibiotic resistant bacteria.

AUTHOR INFORMATION

Corresponding Author

*Phone: +617 3833 5576; fax: +617 3833 5503; e-mail: Jatinder.Sidhu@csiro.au.

Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Enne, V.; Livermore, D. M.; Stephens, P.; Hall, L. M. C. Persistence of sulphonamide resistance in Escherichia coli in the UK despite national prescribing restriction. *Lancet.* **2001**, *357*, 1325−1328.

(2) Kümmerer, K. Resistance in the environment. *J. Antimicrob. Chemother.* **2004**, *54*, 311−320.

(3) Pruden, A.; Pei, R.; Storteboom, H.; Carlson, K. H. Antibiotic resistance genes as emerging contaminants: Studies in Northern Colorado. *Environ. Sci. Technol.* **2006**, *40*, 7445−7450.

(4) Baquero, F.; Martinez, J. L.; Canton, R. Antibiotics and antibiotic resistance in water environments. *Curr. Opin. Biotechnol.* **2008**, *19*, 260−265.

(5) Tao, R.; Ying, G. G.; Su, H. C.; Zhou, H. W.; Sidhu, J. P. S. Detection of antibiotic resistance and tetracycline resistance genes in *Enterobacteriaceae* isolated from the Pearl rivers in South China. *Environ. Pollut.* **2010**, *158*, 2101−2109.

(6) Furuya, F. Y.; Lowy, F. D. Antimicrobial-resistant bacteria in the community setting. *Nat. Rev. Microbiol.* **2006**, *4*, 36−45.
(7) Böckelmann, U.; Dörries, H. H.; Ayuso-Gabella, M. N.; Salgot de Marçay, M.; Tandoi, V.; Levantesi, C.; Masciopinto, C.; van Houtte, E.; Szewzyk, U.; Wintgens, T.; Grohmann, E. Quantitative PCR monitoring of antibiotic resistance genes and bacterial pathogens in three European artificial groundwater recharge systems. *Appl. Environ. Microbiol.* **2009**, *75*, 154−163.
(8) Hammerum, A. M.; Heuer, O. E. Human health hazards from antimicrobial-resistant *Escherichia coli* of animal origin. *Clin. Infect. Dis.* **2009**, *48*, 916−21.
(9) LaPara, T. M.; Burch, T. R.; McNamara, P. J.; Tan, D. T.; Mi Yan, M.; Eichmiller, J. J. Tertiary-treated municipal wastewater is a significant point source of antibiotic resistance genes into Duluth-Superior Harbor. *Environ. Sci. Technol.* **2011**, *45*, 9543−9549.
(10) Boon, P. I.; Cattanach, M.. Antibiotic resistance of native and faecal bacteria isolated from rivers, reservoirs and sewage treatment facilities in Victoria, south-eastern Australia. *Lett. Appl. Microbiol.* **1999**, *28*, 164−168.
(11) Sommer, M. O. A.; Dantas, G.; Church, G. M. Functional characterization of the antibiotic resistance reservoir in the human microflora. *Science* **2009**, *325*, 1128−1131.
(12) Batt, A. L.; Snow, D. D.; Aga, D. S. Occurrence of sulphonamide antimicrobials in private water wells in Washington County, Idaho, USA. *Chemosphere* **2006**, *64*, 1963−1971.
(13) Brown, K. D.; Kulis, J.; Thomson, B.; Chapman, T. H.; Mawhinney, D. B. Occurrence of antibiotics in hospital, residential, and dairy effluent, municipal wastewater, and the Rio Grande in New Mexico. *Sci. Total Environ.* **2006**, *366*, 772−783.
(14) Kümmerer, K. Antibiotics in the aquatic environment - A review − Part II. *Chemosphere* **2009**, *75*, 435−441.
(15) Griggs, D. J.; Gensberg, K.; Piddock, L. J. Mutations in gyrA gene of quinolone-resistant *Salmonella* serotypes isolated from humans and animals. *Antimicrob. Agents Chemother.* **1996**, *40*, 1009−1013.
(16) Jorgensen, J. H.; Weigel, L. M.; Ferraro, M. J.; Swenson, J. M.; Tenover, F. C. Activities of newer fluoroquinolones against *Streptococcus pneumoniae* clinical isolates including those with mutations in the *gyrA*, *parC*, and *parE* loci. *Antimicrob. Agents Chemother.* **1999**, *43*, 329−334.
(17) Davison, J. Genetic exchange between bacteria in the environment. *Plasmid* **1999**, *42*, 73−91.
(18) Moore, P. C. L.; Lindsay, J. A. Genetic variation among hospital isolates of methicillin-sensitive *Staphylococcus aureus*: Evidence for horizontal transfer of virulence genes. *J. Clinic. Microbiol.* **2001**, *39*, 2760−2767.
(19) O'Brien, T. F. Emergence, spread, and environmental effect of antimicrobial resistance: How use of an antimicrobial anywhere can increase resistance to any antimicrobial anywhere else. *Clin. Infect. Dis.* **2002**, *34*, S78−S84.
(20) Witte, W. Medical consequences of antibiotic use in agriculture. *Science* **1998**, *279*, 996−997.
(21) Aarestrup, F. M. Veterinary drug usage and antimicrobial resistance in bacteria of animal origin. *Basic Clin. Pharmacol. Toxicol.* **2005**, *96*, 271−281.
(22) Hunter, P.; Wilkinson, D. C.; Catling, L. A.; Barker, G. C. Meta-analysis of experimental data concerning antimicrobial resistance gene transfer rates during conjugation. *Appl. Environ. Microbiol.* **2008**, *74*, 6085−6090.
(23) Martinez, J. L. Antibiotics and antibiotic resistance genes in natural environments. *Science* **2008**, *321*, 365−367.
(24) Zhang, T.; Fang, H. H. P. Antibiotic resistance genes in water environment. *Appl. Microbiol. Biotechnol.* **2009**, *82*, 397−414.
(25) Watkinson, A. J.; Micalizzi, G. R.; Bates, J. R.; Costanzo, S .D. Novel method for rapid assessment of antibiotic resistance in *Escherichia coli* isolates from environmental waters by use of a modified chromogenic agar. *Appl. Environ. Microbiol.* **2007**, *73*, 2224−2229.
(26) Servias, P.; Passerat, J. Antimicrobial resistance of fecal bacteria in waters of the Seine river watershed (France). *Sci. Total Environ.* **2009**, *408*, 365−372.
(27) Edge, T. A.; Hill., S. Occurrence of antibiotic resistance in *Escherichia coli* from surface waters and fecal pollution sources near Hamilton, Ontario. *Can. J. Microbiol.* **2005**, *51*, 501−505.
(28) Harwood, V. J.; Whitlock, J.; Withington, V. Classification of antibiotic resistance patterns of indicator bacteria by discriminant analysis: Use in predicting the source of fecal contamination in subtropical waters. *Appl. Environ. Microbiol.* **2000**, *66*, 3698−3704.
(29) McKeon, D. M.; Calabrese, J. P.; Bissonnette, G. K. Antibiotic resistant Gram-negative bacteria in rural groundwater supplies. *Wat. Res.* **1995**, *29*, 1902−1908.
(30) Armstrong, J.; Shigeno, D.; Calomiris, J.; Seidler, R. J. Antibiotic-resistant bacteria in drinking water. *Appl. Environ. Microbiol.* **1981**, *42*, 277−83.
(31) Kolwzan, B.; Traczewska, T.; Pawlaczyk-Szipilowa, M. Examination of resistance of bacteria isolated from drinking water to antibacterial agents. *Environ. Protection Engineering* **1991**, *17*, 53−60.
(32) Schwartz, T.; Kohnen, W.; Hansen, B.; Obst, U. Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. *FEMS Microbiol. Ecol.* **2003**, *43*, 325−335.
(33) Amann, R. I.; Ludwig, W.; Schleifer, K. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Rev.* **1995**, *59*, 143−169.
(34) Hamelin, K.; Bruant, G.; Shaarawi, A. E.; Hill, S.; Edge, T. A.; Bekal, S.; Fairbrother, J. M.; Harel, J.; Maynard, C.; Masson, L.; Brousseau, R. Occurrence of virulence and antimicrobial resistance genes in *Escherichia coli* isolates from different aquatic ecosystems within the St. Clair River and Detroit River Areas. *Appl. Environ. Microbiol.* **2007**, *73*, 477−484.
(35) Chee-Sanford, J. C.; Aminov, R. I.; Krapac, I. J.; Garrigues-Jeanjean, N; Mackie, R. I. Occurrence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. *Appl. Environ. Microbiol.* **2001**, *67*, 1494−1502.
(36) Koike, S.; Krapac, I. G.; Oliver, H. D.; Yannarell, A. C.; Chee-Sanford, J. C.; Aminov, R. I.; Mackie, R. I. Monitoring and source tracking of tetracycline resistance genes in lagoons and groundwater adjacent to swine production facilities over a 3-year Period. *Appl. Environ. Microbiol.* **2007**, *73*, 4813−4823.
(37) Volkmann, H.; Schwartz, T.; Bischoff, P.; Kirchen, S.; Obst, U. Detection of clinically relevant antibiotic-resistance genes in municipal wastewater using real-time PCR (TaqMan). *J. Microbiol. Meth.* **2004**, *56*, 277−286.
(38) Lachmayr, K. L.; Kerkhof, L. J.; Dirienzo, A. G.; Cavanaugh, C. M.; Ford, T. E. Quantifying nonspecific TEM beta-lactamase (bla_{TEM}) genes in wastewater streams. *Appl. Environ. Microbiol.* **2009**, *75*, 203−211.
(39) Goossens, H.; Ferech, M.; Stichele, R. V.; Elseviers, M. Outpatient antibiotic use in Europe and association with resistance: A cross-national database study. *Lancet.* **2005**, *365*, 579−587.
(40) Kools, S. A. E.; Moltmann, J. F.; Knacker, T. Estimating the use of veterinary medicines in the European Union. *Regul. Toxicol. Pharmacol.* **2008**, *50*, 59−65.
(41) Page, S. W. *The Role of Enteric Antibiotics in Livestock Production*; Avcare Limited: Canberra, Australia, 2003; ISBN 0-9750845-0-X . http://www.animalhealthalliance.org.au/files/animalhealth/information/The%20Role%20of%20enteric%20antibiotics%20in%20livestock%20production.pdf.
(42) Weisburg, W. G.; Barns, S. M.; Pelletier, D. A.; Lane, D. J. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* **1991**, *173*, 697−703.
(43) Maynard, C.; Fairbrother, J. M.; Bekal, S.; Sanschagrin, F.; Levesque, R. C.; Brousseau, R.; Masson, L.; Larivière, S.; Harel, J. Antimicrobial resistance genes in enterotoxigenic *Escherichia coli* O149:K91 isolates obtained over a 23-year period from pigs. *Antimicrob. Agents Chemother.* **2003**, *47*, 3214−3221.

(44) Chen, S.; Zhao, S.; White, D. G.; Schroeder, C. M.; Lu, R.; Yang, H.; McDermott, P. F.; Ayers, S.; Meng, J. Characterization of multiple-antimicrobial-resistant *Salmonella* serovars isolated from retail meats. *Appl. Environ. Microbiol.* **2004**, *71*, 1−7.
(45) Klein, G.; Pack, A.; Reuter, G. Antibiotic resistance patterns of *Enterococci* and occurrence of vancomycin-resistant *Enterococci* in raw minced beef and pork in Germany. *Appl. Environ. Microbiol.* **1998**, *64*, 1825−1830.
(46) Clark, N. C.; Cooksey, R. C.; Hill, B. C.; Swenson, J. M.; Tenover, F. C. Characterization of glycopeptide-resistant *Enterococci* from U.S. hospitals. *Antimicrob. Agents Chemother.* **1993**, *37*, 3211−3217.
(47) Aminov, R. I.; Garrigues-Jeanjean, N.; Mackie, R. I. Molecular ecology of tetracycline resistance: Development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. *Appl. Environ. Microbiol.* **2001**, *67*, 22−32.
(48) Vassort-Bruneau, C.; Lesage-Descauses, M. C.; Martel, J. L.; Lafont, J. P.; Chaslus-Dancla, E. CAT III chloramphenicol resistance in *Pasteurella haemolytic*a and *Pasteurella multocida* isolated from calves. *J. Antimicrob. Chemother.* **1996**, *38*, 205−213.
(49) Vakulenko, S. B.; Donabedian, S. M.; Voskresenskiy, A. M.; Zervos, M. G.; Lerner, S. A.; Chow, J. W. Multiplex PCR for detection of aminoglycoside resistance genes in *Enterococci*. *Antimicrob. Agents Chemother.* **2003**, *47*, 1423−1426.
(50) Strommenger, B.; Kettlitz, C.; Werner, G.; Witte, W. Multiplex PCR assay for simultaneous detection of nine clinically relevant antibiotic resistance genes in *Staphylococcus aureus*. *J. Clin. Microbiol.* **2003**, *41*, 4089−4094.
(51) Gevers, D.; Danielsen, M.; Huys, G.; Swings, J. Molecular characterization of *tet*(M) genes in Lactobacillus isolates from different types of fermented dry sausage. *Appl. Environ. Microbiol.* **2003**, *69*, 1270−1275.
(52) Iversen, A; Kühn, I.; Rahman, M.; Franklin, A.; Burman, L. G.; Olsson- Liljequist, B.; Torell, E.; Möllby, R. Evidence for transmission between humans and the environment of a nosocomial strain of *Enterococcus faecium*. *Environ. Microbiol.* **2004**, *6*, 55−59.
(53) Antunes, P.; Machado, J.; Sousa, J. C.; Peixe, L. Dissemination of sulfonamide resistance genes (*sul1*, *sul2*, and *sul3*) in Portuguese *Salmonella* enterica strains and relation with integrons. *Antimicrob. Agents Chemother.* **2005**, *49*, 836−839.
(54) Skold, O. Resistance to trimethoprim and sulphonamides. *Vet. Res.* **2001**, *32*, 261−273.
(55) Hu, J.; Shi, J.; Chang, H.; Li, D.; Yang, M.; Kamagata, Y. Phenotyping and genotyping of antibiotic-resistant *Escherichia coli* isolated from a natural river basin. *Environ. Sci. Technol.* **2008**, *42*, 3415−3420.
(56) Hamelin, K.; Bruant, G.; Shaarawi, A. E.; Hill, S.; Edge, T. A.; Bekal, S.; Fairbrother, J. M.; Harel, J.; Maynard, C.; Masson, L.; Brousseau, R. A virulence and antimicrobial resistance DNA microarray detects a high frequency of virulence genes in *Escherichia coli* isolates from Great Lakes recreational water. *Appl. Environ. Microbiol.* **2006**, *72*, 4220−4206.
(57) Pei, R.; Kim, S. C.; Carlson, K. H.; Pruden, A. Effect of river landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). *Water Res.* **2006**, *40*, 2427−2435.
(58) Poole, K. Resistance to beta-lactam antibiotics. *Cell. Mol. Life Sci.* **2004**, *61*, 2200−2223.
(59) Barana, W.; Adamek, E.; Ziemianska, J.; Sobczak, A. Effects of the presence of sulfonamides in the environment and their influence on human health. *J. Hazard. Mater.* **2011**, *196*, 1−15.
(60) Henriques, I. S.; Fonseca, F.; Alves, A.; Saavedra, M. J.; Correia, A. Occurrence and diversity of integrons and β-lactamase genes among ampicillin-resistant isolates from estuarine waters. *Res. Microbiol.* **2006**, *157*, 938−947.
(61) Xi, C.; Zhang., Y.; Marrs, C. F.; Ye, W.; Simon, C.; Foxman, B.; Nriagu, J. Prevalence of antibiotic resistance in drinking water treatment and distribution systems. *Appl. Environ. Microbiol.* **2009**, *75*, 5714−5718.
(62) Lu, S. Y.; Zang, Y.; Geng, S.; Li, T.; Ye, Z.; Zang, D.; Zou, F.; Zhou, H. High diversity of extended-spectrum-lactamase producing bacteria in an urban river sediment habitat. *Appl. Environ. Microbiol.* **2010**, *76*, 5972−5976.
(63) Schwarz, S.; Kehrenberg, C.; Doublet, B.; Cloeckaert, A. Molecular basis of bacterial resistance to chloramphenicol and florfenicol. *FEMS Microbiol. Rev.* **2004**, *28*, 519−542.
(64) Srinivasan, V.; Nam, H. M.; Nguyen, L. T.; Tamilselvam, B.; Murinda, S. E.; Oliver, S. P. Prevalence of antimicrobial resistance genes in *Listeria monocytogenes* isolated from dairy farms. *Foodborne Pathog. Dis.* **2005**, *2*, 201−211.
(65) Vester, B.; Douthwaite, S. Macrolide resistance conferred by substitution in 23S rRNA. *Antimicrob. Agents Chemother.* **2001**, *45*, 1−12.
(66) Liu, Y. F.; Wang, C. H.; Janapatla, R. P.; Fu, H. M.; Wu, H. M.; Wu, J. J. Presence of plasmid pA15 correlates with prevalence of constitutive MLSB resistance in group A streptococcal isolates at a university hospital in southern Taiwan. *J. Antimicrob. Chemother.* **2007**, *59*, 1167−1170.
(67) Okitsu, N.; Kaieda, S.; Yano, H.; Nakano, R.; Hosaka, Y.; Okamoto, R.; Kobayashi, T.; Inoue, M. Characterization of *ermB* gene transposition by Tn1545 and Tn917 in macrolide-resistant *Streptococcus pneumoniae* isolates. *J. Clin. Microbiol.* **2005**, *43*, 168−173.
(68) Kresken, M. (GERMAP) 2008 − Resistance monitoring in Germany: Antibiotic use and occurrence of resistance in human medicine. *J. Vet. Pharmacol. Therapeutics*. **2008**, 32, 51-51.
(69) Chopra, I.; Roberts, M. Tetracycline antibiotics: Mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol. Mol. Biol. Rev.* **2001**, *65*, 232−240.
(70) Roberts, M. C. Update on acquired tetracycline resistance genes. *FEMS Microbiol. Lett.* **2005**, *245*, 195−203.
(71) Henriques, I. S.; Fonseca, F.; Alves, A.; Saavedra, M. J.; Correia, A. Tetracycline-resistance genes in gram-negative isolates from estuarine waters. *Lett. Appl. Microbiol.* **2008**, *47*, 526−533.
(72) Rahman, M. H.; Nonaka, L.; Tago, R.; Suzuki, S. Occurrence of two genotypes of tetracycline (TC) resistance gene *tet*(M) in the TC resistant bacteria in marine sediments of Japan. *Environ. Sci. Technol.* **2008**, *42*, 5055−5061.
(73) Aarestrup, F. M.; Agerso, Y.; Gerner-Smidt, P.; Madsen, M.; Jensen, L. B. Comparison of antimicrobial resistance phenotypes and resistance genes in *Enterococcus faecalis* and *Enterococcus faecium* from humans in the community, broilers, and pigs in Denmark. *Diagn. Microbiol. Infect. Dis.* **2000**, *37*, 127−137.
(74) Donabedian, S. M.; Thal, L. A.; Hershberger, E.; Perri, M. B.; Chow, J. W.; Bartlett, P; Jones, R.; Joyce, K.; Rossiter, S.; Gay, K.; Johnson, J.; Mackinson, C.; DeBess, E.; Madden, J.; Angulo, F.; Zervos, M. J. Molecular characterization of gentamicin-resistant enterococci in the United States: Evidence of spread from animals to humans through food. *J. Clin. Microbiol.* **2003**, *41*, 1109−1113.
(75) Heuer, H.; Krogerrecklenfort, E.; Wellington, E. M. H.; Egan, S.; van Elsas, J. D.; van Overbeek, L.; Collard, J. M.; Guillaume, G.; Karagouni, A. D.; Nikolakopoulon, T. L.; Smalla, K. Gentamicin resistance genes in environmental bacteria: Prevalence and transfer. *FEMS Microbiol. Ecol.* **2002**, *42*, 289−302.
(76) Werner, G.; Klare, I.; Hübner, J.; Kern, W. V.; Witte, W. Vancomycin-resistente Enterokokken. *Chemother. J.* **2008**, *5*, 183−193.
(77) Sidhu, J. P. S.; Hodgers, L.; Ahmed, W.; Chong, N. M.; Toze, S. Prevalence of human pathogens and indicators in stormwater runoff in Brisbane. *Aust. Water Res.* **2012**, DOI: doi info.10.1016/j.watres.2012.03.012.
(78) Watkinson, A. J.; Murby, E. J.; Kolpin, D. W.; Costanzo, S. D. The occurrence of antibiotics in an urban watershed: From wastewater to drinking water. *Sci. Total Environ.* **2009**, *407*, 2711−2723.
(79) Christian, T.; Schneider, R.; Farber, H. A.; Skutlarek, D.; Meyer, M. T.; Goldbach, H. E. Determination of antibiotic residues in manure, soil, and surface waters. *Acta Hydrochim. Hydrobiol.* **2003**, *31*, 36−44.

(80) TGA. *Import volumes of antibiotics into Australia for human, veterinary and feed application 1992−2003*; Canberra: Therapeutic Goods Administration, 2003.
(81) Segura, P. A.; Francois, M.; Gagnon, C.; Sauve, S. Review of the occurrence of anti-infectives in contaminated wastewaters and natural and drinking waters. *Environ. Health Perspect.* **2009**, *117*, 675−684.
(82) Ahmed, W.; Sidhu, J. P. S.; Toze, S. Evaluation of the nifH Gene Marker of *Methanobrevibacter smithii* for the detection of sewage pollution in environmental waters in southeast Queensland, Australia. *Environ. Sci. Technol.* **2012**, *46*, 543−550.