Antibiotic resistance genes in freshwater biofilms along a whole river

Cynthia L. Winkworth

ABSTRACT

A key problem challenging public health officials' efforts to stem the spread of antibiotic resistance is the potential increase of resistance in the environment. Yet, despite recent and significant changes to agricultural land in New Zealand, as well as the sector's high antibiotic use, the influence on antibiotic resistance in the environment remained uncharacterised. Spatial and temporal dynamics of antibiotic resistance genes in freshwater biofilms from NZ's fourth longest river as it transitioned between low and high intensity farming were examined for 1 year. Polymerase chain reaction was employed to gauge the level of resistance present. Biofilms were screened for 10 genes conferring resistance to antibiotics used in humans only and both humans and agricultural animals. Three genes were detected, one which conferred resistance to the important human-only use antibiotic vancomycin. Detected at the two downstream sites only, and those subject to the highest combined land-use stressors, the three genes indicated an elevated presence of antibiotic resistance in relation to surrounding land use; 7.7% versus 2% across the whole river system. The detection of a gene conferring resistance to an important human-only use antibiotic was particularly concerning and highlighted human-based contamination sources along the river, in addition to those of agricultural origin.

Key words | antibiotic resistance genes, biofilm, freshwater, public health

Cynthia L. Winkworth
Department of Zoology,
University of Otago,
PO Box 56,
Dunedin 9054,
New Zealand
E-mail: *cynthia.winkworth@otago.ac.nz*

INTRODUCTION

Microorganisms are vital components of stream communities, performing a range of tasks crucial for effective ecosystem functioning like leaf decay and nutrient processing, as well as being a basal food source (Costanzo *et al.* 2005). Land use intensification is typically characterised by the input of a wide range of contaminants to streams, with the potential to affect resident organisms. Knowledge about microbial resilience or susceptibility to such contaminants, in particular antibiotics and their chemical by-products, is limited. Despite being recognised as emerging chemicals of concern in aquatic environments (Engemann *et al.* 2008), limited information exists about their effects on microbial processes, on altering the level of antibiotic-resistant microbes, or changing the spectrum of antibiotic resistance genes observed, themselves considered emerging contaminants of concern (Engemann *et al.* 2008; Ding & He 2010).

While antibiotic resistant microbes in freshwater may reflect normal, intrinsic levels of resistance, they may also reflect responses to human and agricultural pressures in the catchment (Wright 2010). Antibiotics and antibiotic-resistant microbes from anthropogenic use may reach aquatic environments via either direct human contact or indirectly via medical waste, the flushing of unused prescriptions, discharge from municipal wastewater treatment facilities and leakages from septic waste storage facilities (Halling-Sørensen *et al.* 1998; Kim & Aga 2007; Jury *et al.* 2011). The agricultural sector in New Zealand comprises similar contamination pathways, with significant land-use conversions over the past two decades

doi: 10.2166/wh.2013.223

Downloaded from https://iwaponline.com/jwh/article-pdf/11/2/186/395472/186.pdf
by guest
on 23 October 2018

of low-density beef and sheep livestock farming to intensive dairy farming (PCE 2004) of particular concern. For instance, high antibiotic use exists in the sector with 57% of antibiotics administered in New Zealand in 2000 being for agricultural use (Sarmah *et al.* 2006), with roughly 50–70% of the administered antibiotics reportedly excreted either unmetabolised or as degraded, yet still active compounds (Sarmah *et al.* 2006; Hillis *et al.* 2007; Watanabe *et al.* 2008). Designed to cause biological effects (Halling-Sørensen *et al.* 1998), the detection of antibiotics in waterways on agricultural land (Costanzo *et al.* 2005; Watkinson *et al.* 2009) is concerning given the potential to generate environmental reservoirs of antibiotic resistance genes and antibiotic resistant microbes (Storteboom *et al.* 2010). While no data exist on agricultural antibiotic presence in New Zealand waterways to date, numerous overseas studies have detected antibiotics in freshwater environments (Kemper 2008; Zhang *et al.* 2009), suggesting similar patterns may occur in New Zealand, with yet unmeasured consequences for freshwater microbes. Therefore, quantifying antibiotic resistance levels and overall gene spectrum in freshwater microbial communities is a crucial first step in evaluating potential risks from the spread of antibiotic-resistant microbes in the environment for human health (Allen *et al.* 2010).

Few studies worldwide have evaluated antibiotic resistance in streams (Schwartz *et al.* 2003; Barker-Reid *et al.* 2010; Moore *et al.* 2010; Storteboom *et al.* 2010; Tao *et al.* 2010), and whole river systems remain largely unstudied. The exception is a spatiotemporal survey of an Australian river (Barker-Reid *et al.* 2010). The reported rarity of antibiotic resistance genes in that study may have been due to the dominance of horticulture on the landscape, the focus on microbes isolated from the water column or simply reflect tightly regulated antibiotic usage in the Australian agricultural sector (JETACAR 1999). Irrespective, a more appropriate antibiotic resistance study focus would be stream biofilms, which include long-term resident microbes, given they experience the longest contact time with freshwater contaminants (Lear *et al.* 2008). Hence, a spatiotemporal survey was undertaken (a) to quantify for the first time the presence of antibiotic resistance genes in biofilms along an entire river, (b) to determine whether detection levels change in regions of higher dairy farming intensification along the river and (c) to describe any seasonal variation that occurs.

Individual biofilm samples were collected approximately every 6 weeks for 1 year at each of six sites along the Taieri River, the fourth longest in New Zealand. To overcome the difficulties of culturing microbes from the environment (Pusch *et al.* 1998; D'Costa *et al.* 2006), as well as the prospect of only obtaining those few organisms that grow under laboratory conditions (Volkmann *et al.* 2007), biofilm was screened directly and in its entirety using molecular techniques for a subset of 10 genes conferring resistance to antibiotics used in humans only (vancomycin *vanA*, *vanB*) and both humans and agricultural animals (animoglycoside *aacA-aphD*; β-lactamase *mecA*; macrolide *ermA, ermB* and tetracycline *tetA, tetB, tetK, tetM;* Norris *et al.* 2005; Sarmah *et al.* 2006).

METHODS

Field sites

Situated in the southeastern region of Otago, South Island, New Zealand, the Taieri River is 288 km long, has a catchment of approximately 5,650 km^2 and traverses a range of land uses including livestock farming, cropping, market gardening, forestry, native grasslands and sparsely populated townships (Figure 1). The Taieri River is unmodified by large dams and had a mean annual flow rate of 39.89 cubic metres per second (m^3/sec) or 3,446.5 megalitres per day at the second most downstream site (Outram Glen; S5) over the study's year-long duration. Six sites along the river were sampled to evaluate the possible influence of different land use on microbial biofilm communities along the river continuum (Figure 1; Table 1). Sample sites spanned the whole river and were chosen based on the presence of suitably-sized rocky substrates deemed sufficient for biofilm development and proximity to permanent Otago Regional Council river monitoring stations. In order of headwaters to sea, the six sites along the Taieri River were at Lammermore (S1), Waipiata (S2), Hyde (S3), Middlemarch (S4), Outram Glen (S5) and Momona (S6). While equal sample site spacing along the river was preferable, several physical features prevented ideal site selection. Closer site

Downloaded from https://iwaponline.com/jwh/article-pdf/11/2/186/395472/186.pdf
by guest
on 23 October 2018

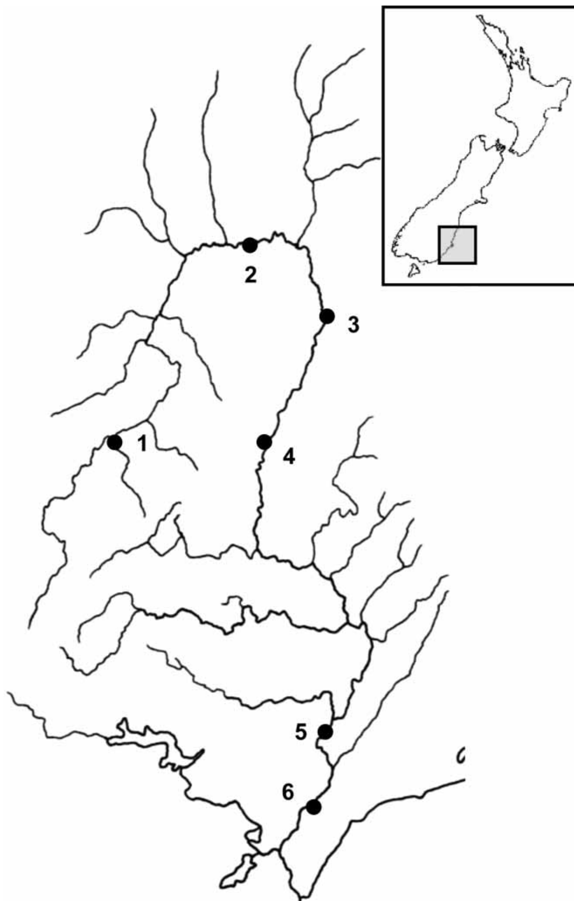

Figure 1 | Geographical distribution of the six sampling sites (S1–S6) along the Taieri River, Otago Region, New Zealand (inset).

selection between the Lammermore (S1) headwaters and Waipiata (S2) was prevented due to deep river channelisation and between Middlemarch (S4) and Outram Glen (S5) due to a steep-sided gorge. Conversely, twice-daily intertidal influences prevented greater site spacing between the two most downstream sites of Outram Glen (S5) and Momona (S6). Relatively equidistant site selection was possible along the river's middle reaches between Waipiata (S2), Hyde (S3) and Middlemarch (S4).

Land use differed markedly along the river, ranging from ungrazed tussock to intensively grazed exotic pastures. The Lammermore region of the river was largely dominated by ungrazed tall tussock grasslands (93%), while land uses in the Waipiata, Middlemarch, Outram Glen and Momona regions were characterised by low and high producing grassland grazing, ranging between 57 and 74%. Tussock grasslands and low producing grassland grazing characterised the Hyde catchment at 53%. Animal type and stocking densities differed considerably, with low producing grassland areas typically supporting beef and sheep at 3–8 stock units per hectare, while high producing grassland areas supported dairy cattle at eight times that (Otago Regional Council, personal communication). The ratio of high to low intensity farming land-use also differed considerably between the sites with no high producing grassland farming occurring at Lammermore (S1; 0:1) compared

Table 1 | Taieri River site information in ascending order of distance from headwaters, local and cumulative catchment size, percentage pastoral use (high and low intensity use), ratio of high to low farming intensity land use in each catchment, mean annual low flow and highest recorded flow

Site code	Site description (headwaters to sea)	Distance from headwaters (km)	Catchment size (cumulative, km^2)	% Pastoral use[a] (H, L)	Ratio high:low intensity	Mean annual low flow[b]	Highest recorded flow[b] (year)
S1	Taieri River at Lammermore	25	159 (159)	2 (0, 2)	0:1	N/A	N/A
S2	Taieri River at Waipiata	118	1,526 (1,685)	57 (39, 18)	2:1	5	189 (1993)
S3	Taieri River at Hyde	146	381 (2,067)	29 (2, 28)	0.1:1	7	521 (1993)
S4	Taieri River at Middlemarch	180	610 (2,677)	74 (32, 42)	0.8:1	10	560 (1993)
S5	Taieri River at Outram Glen	235	2,122 (4,800)	61 (36, 25)	1.4:1	15	2526 (1993)
S6	Taieri River at Momona	251	238 (5,038)	62 (59, 3)	22:1	N/A	N/A

[a]Calculated by dividing total pastoral land area (both high and low intensity use) by catchment size; values obtained from Otago Regional Council Land Catchment Data Base (2011).
[b]Recorded in m^3/sec; cumecs.
N/A: Not available.

Downloaded from https://iwaponline.com/jwh/article-pdf/11/2/186/395472/186.pdf
by guest
on 23 October 2018

with almost the reverse at Momona (S6; 22:1). Ratios were relatively similar between Waipiata (S2; 2:1), Middlemarch (S4; 0.8:1) and Outram Glen (S5; 1.4:1), with roughly equal amounts of high and low intensity farming land-use within each catchment.

Apart from the steep-sided gorges, year-round animal grazing occurred to the river's edge at five of the six sampling sites (Sites 1–4, 6), with no fencing present to prevent animals physically reaching the river's edge. The exception was at Outram Glen (S5); a recreational site with an expansive beach-like shoreline used heavily by local communities in the summer for swimming and in the winter for dog walking and fly-fishing. Three main intensive livestock dairy farming areas exist along the Taieri River: 1. the Upper Taieri Plain between Lammermore (S1) and Waipiata (S2); 2. the Strath Taieri between Hyde (S3) and Middlemarch (S4); and 3. the Lower Taieri Plain between Outram Glen (S5) and the river mouth beyond Momona (S6). Twice-daily milking occurs August to May at each of the three intensive dairy farming areas.

Water sampling

All six sites were visited on the same day for each of nine sampling occasions on 22 May 2010, 16 July 2010, 12 August 2010, 28 September 2010, 3 November 2010, 16 December 2010, 1 February 2011, 11 March 2011 and 19 April 2011. Physicochemical measurements of water temperature and salinity were measured at each sampling site for every sampling date using a handheld YSI meter (Professional Plus, Yellow Springs, OH, USA). Water samples were also collected at each site for every sampling date to evaluate nutrient concentrations. Unfiltered water grab samples were collected for analysis of total nitrogen (TN $\mu g\ L^{-1}$) and total phosphorus (TP $\mu g\ L^{-1}$), while water samples collected using a 100-mL plastic syringe with microfilter (GF/F; Whatman Inc., Clifton, NJ, USA; pore size 0.7 μm) were analysed for dissolved nitrate-N, dissolved nitrite-N and ammonium-N (summed and reported as dissolved inorganic nitrogen; DIN $\mu g\ L^{-1}$) and dissolved reactive phosphate (DRP $\mu g\ L^{-1}$). Samples were transported on ice and stored at −20 °C until analysis using standard colorimetric protocols (APHA 1998) with a SANPlus segmented flow autoanalyser (Skalar Analytical B.V., Breda, The Netherlands). Flow rates for each sampling date were obtained from Otago Regional Council monitoring sites located at Waipiata (S2), Tiroti (approximately 20 km downstream from Waipiata and 8 km upstream of Hyde (S3)), Sutton (approximately 8 km downstream of Middlemarch (S4)) and Outram Glen (S5). Nutrient concentrations for DRP, DIN, TN and TP were log-transformed (natural logarithm) to aid with graphical representation (Figure 2).

Additional unfiltered water grab samples were collected at each of the six sampling sites to assess antibiotic presence in the water column for vancomycin, aminoglycoside, *β*-lactamase, macrolide and tetracycline antibiotics, corresponding to the 10 antibiotic resistance genes screened. Difficulties with analytical processing and reliable detection limits have prevented the analysis of water samples for antibiotic presence; thus, these samples will not be discussed further.

Biofilm sampling

Three rocks of roughly 10 cm diameter and likely to have been continuously submerged were collected randomly from the riverbed surface at each site. A toothbrush previously sterilised in a 10% bleach solution for 1 hour and rinsed thrice using Milli-Q water was used to scrub the entire surface area of each rock. The loosened biofilm was collected in a 50 mL conical tube (BD Falcon™, Franklin Lakes, NJ, USA) using water from the same site. The conical tubes were immediately stored on ice and frozen at the end of each collection day for long-term storage at −20 °C.

Molecular procedures

Nucleic acid was isolated from all biofilm samples using the QIAamp® DNA Stool Mini kit (Qiagen®, Valencia, CA, USA) following the manufacturer's recommended protocol and stored at −20°C until use. Polymerase chain reaction (PCR) amplifications were performed on all extracted nucleic acid using primers detailed in Table 2. The PCR mix for all primer sets comprised the Promega GoTaq® Green Master Mix (Promega Corp, Madison, WI, USA) of 1 × GoTaq® Master Mix, 200–300 ηM each of the forward and reverse primers (Invitrogen™ New Zealand Ltd, Auckland, NZ), purified DNA and Milli-Q water to a total

Downloaded from https://iwaponline.com/jwh/article-pdf/11/2/186/395472/186.pdf
by guest
on 23 October 2018

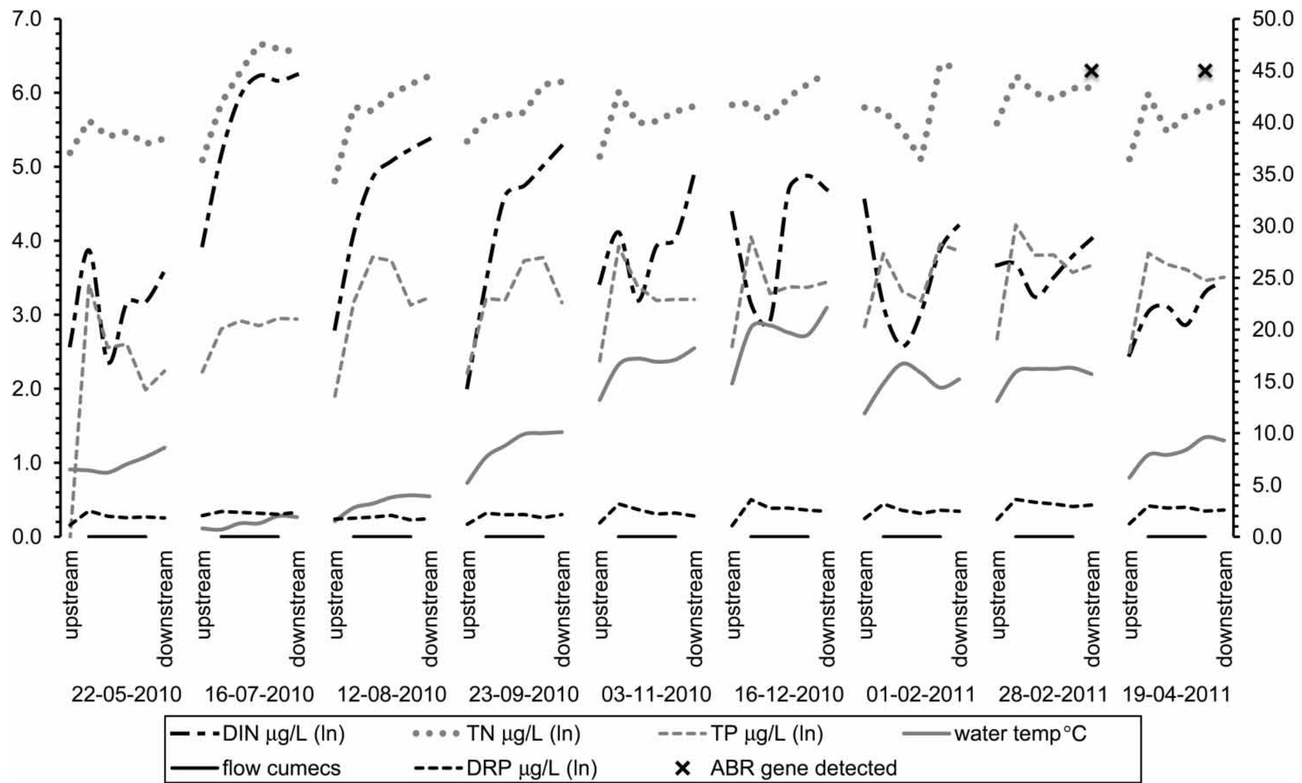

Figure 2 | Physiochemical values and antibiotic resistance gene detection data for the nine sampling occasions at six sites along the Taieri River. Total nitrogen, total phosphorus, dissolved inorganic nitrogen and dissolved reactive phosphate (all natural log transformed) on left axis, flow and temperature on right axis.

volume of 25 µL. For all primer pairs except *tetM* and *mecA*, PCR consisted of an initial denaturing step of 95 °C for 5 minutes, a set of 35 cycles comprising 95 °C for 45 sec, annealing at the recommended temperature for 45 seconds and 72 °C for 60 sec, followed by a final extension at 72 °C for 7 minutes. For the *tetM* and *mecA* primer pairs, PCR consisted of an initial denaturing step of 95 °C for 5 minutes, a set of five cycles comprising 95 °C for 30 sec, 64 °C for 30 sec and 72 °C for 60 sec, a second set of 30 cycles comprising 95 °C for 30 sec, 50 °C for 45 sec and 72 °C for 90 sec, followed by a final extension at 72 °C for 7 minutes. All PCR products were visualised on Tris-buffered 1% agarose gels (VWR International Ltd, Poole, UK; Pure Science Ltd, Wellington, NZ) containing 3% SYBR Safe DNA gel stain (Invitrogen).

Amplified products were purified for sequencing using a PureLink™ PCR Purification Kit (Invitrogen) following the manufacturer's instructions and eluted in 30 µL. Purified products were labeled with the ABI Prism BigDye® Terminator Version 3.1 Ready Reaction Cycle Sequencing kit (Applied BioSystems™, Foster City, CA, USA) and comprised 3.2 ρmole primer, 3.5 µL sequencing buffer, 1 µL Big Dye Terminator version 3.1, 10–20 ηg template and Milli-Q water to a total volume of 20 µL. PCR products were sequenced in two overlapping bi-directional reactions following the manufacturer's recommended protocol and separated using capillary analysis on a ABI™ 3730×1 DNA Analyser at the University of Otago Genetic Analysis Service (Dunedin, New Zealand). Bi-directional sequences were assembled using Sequencher® Version 4.10 (Gene Codes Corp., Ann Arbor, MI, USA) and compared to previously published sequences of the corresponding antibiotic resistance profiles to confirm resistance identification.

Data analysis

All environmental samples collected were checked with generic bacterial 16S *r*RNA primers (Table 3) to confirm successful nucleic acid extraction prior to

Downloaded from https://iwaponline.com/jwh/article-pdf/11/2/186/395472/186.pdf
by guest
on 23 October 2018

Table 2 | Descriptions for the antibiotic resistance gene targets, gene size and primer information

Target profile	Target gene (bp)	Primer name	Sequence 5′-3′	Anneal temp (°C)	Reference
16s rRNA	bacterial (various)	8F 1492R	AGAGTTTGATCCTGGCTCAG GGTTACCTTGTTACGACTT	47	Lane (1991)
Vancomycin resistance	vanA (231)	vanABF vanAR	GTAGGCTGCGATATTCAAAGC CGATTCAATTGCGTAGTCCAA	53	Bell *et al.* (1998)
	vanB (330)	vanABF vanBR	GTAGGCTGCGATATTCAAAGC GCCGACAATCAAATCATCCTC	53	Bell *et al.* (1998)
Methicillin resistance	mecA (310)	mecA1 mecA2	GTAGAAATGACTGAACGTCCGATAA CCAATTCCACATTGTTTCGGTCTAA	64/50	Ramos-Trujillo *et al.* (2003)
Erythromycin resistance	ermA (190)	ermA1 ermA2	AAGCGGTAAACCCCTCTGA TTCGCAAATCCCTTCTCAAC	53	Strommenger *et al.* (2003)
	ermB (364)	ermBF ermBR	GATACCGTTTACGAAATTGG GAATCGAGACTTGAGTGTGC	57	Chen *et al.* (2007)
Tetracycline resistance	tetA (957)	tetAF tetAR	GTAATTCTGAGCACTGTCGC CTGCCTGGACAACATTGCTT	55	Nawaz *et al.* (2009)
	tetB (415)	tetBF tetBR	CTCAGTATTCCAAGCCTTTG ACTCCCCTGAGCTTGAGGGG	52	Agerso & Petersen (2007)
	tetK (360)	tetKF tetKB	GTAGCGACAATAGGTAATAGT GTAGTGACAATAAACCTCCTA	55	Strommenger *et al.* (2003)
	tetM (171)	tetMF tetMR	ACAGAAAGCTTATTATATAAC TGGCGTGTCTATGATGTTCAC	64/50	Strommenger *et al.* (2003)
Aminoglycoside resistance	aacA-aphD (227)	aacAaphDF aacAaphDR	TAATCCAAGAGCAATAAGGGC GCCACACTATCATAACCACTA	55	Strommenger *et al.* (2003)

Table 3 | Antibiotic resistant bacterial strain descriptions (New Zealand Reference Culture Collection Medical Section, Institute of Environmental Science and Research Ltd, Wellington, NZ, ESR; Southern Community Laboratories, Dunedin, NZ, SCL)

Antibiotic resistance profile	Isolate source	Bacterial host	Accession number	Type source	Genbank accession	Detection limit
vanA	ESR	*Enterococcus faecium*	3602	BM 4147	N/A	5 ρg/μL
vanB	ESR	*Enterococcus faecalis*	3488	ATCC 51299	JN801173	5 ρg/μL
mecA	ESR	*Staphylococcus aureus*	3529	ATCC43300	JN801172	1 ηg/μL
tetA	Wastewater	Unknown	Tahuna	N/A	JN801167	5 ρg/μL
tetB	Wastewater	Unknown	Tahuna	N/A	JN801171	1 ηg/μL
tetK	SCL	*Staphylococcus*	SCL-T2	N/A	JN801166	0.1 ηg/μL
tetM	ESR	*Neisseria gonorrhoeae*	3330	NZ AS91/376	N/A	1 ηg/μL
ermA	ESR	*Staphylococcus aureus*	4315	ATCC BAA-977	N/A	<0.5 ρg/μL
ermB	Wastewater	Unknown	Tahuna	N/A	JN801168	0.1 ηg/μL
aacAaphD	SCL	*Staphylococcus*	SCL-T2	N/A	N/A	0.1 ηg/μL

N/A: Not applicable.

antibiotic resistance gene screening, using a laboratory-grown *Escherichia coli* strain as a positive nucleic acid control. Nucleic acid from antibiotic-resistant bacterial strains were used as positive controls in all molecular analyses (details in Table 3). Strains were obtained from the New Zealand Reference Culture Collection Medical Section (Institute of Environmental Science and Research Ltd, Wellington, NZ), resistant infections presented to a local medical

Downloaded from https://iwaponline.com/jwh/article-pdf/11/2/186/395472/186.pdf
by guest
on 23 October 2018

screening laboratory (Southern Community Laboratories, Dunedin, NZ) and water samples collected from the intake at the Tahuna Municipal Wastewater Treatment plant (Dunedin, NZ). All positive control genes were confirmed with sequencing and included in every set of PCR amplifications prepared to verify correct reaction conditions had been achieved. Blank controls comprising sterile water instead of template nucleic acid were also included with every prepared PCR set to discount both non-specific amplification and nucleic acid contamination.

To reduce screening costs, three sets of multiplex-PCR were possible and comprised seven of the 10 genes investigated (*vanA*, *vanB* and *ermA*; *tetM* and *mecA*; *tetK* and *aacA-aphD*), while single primer-pair amplification reactions were used for the remaining three genes (*ermB, tetB, tetA*). To confirm minimal or no interference existed between primer pairs used in the three different multiplex reactions, 1 ηg aliquots for each positive control in the three different multiplex reaction sets were mixed and subjected to PCR amplification both in the presence and absence of a representative environmental sample (22 ηg). Nucleic acid extracted from a Momona (S6) environmental sample was chosen (collected 19 April 2010), as it was reasonable to expect it to contain the highest degree of inhibitory influence being sourced from the most downstream site. No cross-reaction or interference was detected for any of the seven antibiotic resistance genes used in three multiple reaction sets (Figure 3). Mild PCR inhibition was detected in the individual multiplex PCR reactions when all positives were mixed and amplified together, as would be expected when several primer pairs compete to amplify target nucleic acid (Figure 3). The impact of primer competition affecting amplification in the *tetK/aacA-aphD* multiplex reaction could not be directly evaluated for each gene individually, like that for *vanA, vanB* and *ermA* or *tetM* and *mecA*, as the positive control contained both antibiotic resistance genes. Rather, each gene was amplified in a single primer pair reaction with and without the environmental sample and then compared to equivalent amplifications from the multiplex reaction.

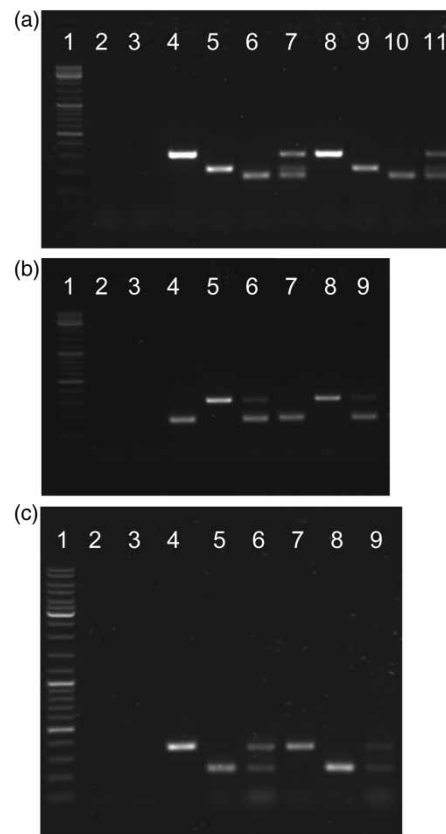

Figure 3 | Electrophoresis gel images of the multiplex interference specificity tests for antibiotic resistance gene mixes: a. *vanA, vanB, ermA*; b. *tetM, mecA*; c. *tetK/aacAaphD*. (a.) Lane 1 (L1): 100 bp ladder, L2: negative control, L3: environmental sample (22 ηg), L4: *vanB* positive control (1 ηg), L5: *vanA* positive control (1 ηg), L6: *ermA* positive control (1 ηg), L7: *vanB, vanA* & *ermA* mixed (1 ηg each), L8: environmental sample (22 ηg) & *vanB* (1 ηg), L9: environmental sample (22 ηg) & *vanA* (1 ηg), L10: environmental sample (22 ηg) & *ermA* (1 ηg) L11: environmental sample (22 ηg) & *vanB, vanA* & *ermA* mixed (1 ηg each); (b.) L1: 100bp ladder, L2: negative control, L3: environmental sample (22 ηg), L4: *tetM* positive control (1 ηg), L5: *mecA* positive control (1 ηg), L6: *tetM* & *mecA* mixed (1 ηg each), L7: environmental sample (22 ηg) & *tetM* (1 ηg), L8: environmental sample (22 ηg) & *mecA* (1 ηg), L9: environmental sample (22 ηg) & *tetM* & *mecA* mixed (1 ηg each); (c.) L1: 100 bp ladder, L2: negative control, L3: environmental sample (22 ηg), L4: *tetK* positive control (1 ηg), L5: *aacA-aphD* positive control (1 ηg), L6: *tetK & aacA-aphD* mixed (1 ηg), L7: environmental sample (22 ηg) & *tetK* (1 ηg), L8: environmental sample (22 ηg) & *aacA-aphD* (1 ηg), L9: environmental sample (22 ηg) & *tetK & aacA-aphD* mixed (1 ηg).

PCR detection limits were determined for all 10 antibiotic resistance genes using serial dilution, both in the presence and absence of the representative environmental sample from Momona. Aliquots for each antibiotic resistance gene ranging from 1 ηg/μL to 0.5 ρ/μL were screened. No meaningful differences were observed between detection limits in the presence or absence of the representative environmental sample for each of the 10 antibiotic resistance genes, although detection limits ranged from >0.5 ρg/μL to 1 ηg/μL for the 10 genes (Table 3).

Downloaded from https://iwaponline.com/jwh/article-pdf/11/2/186/395472/186.pdf
by guest
on 23 October 2018

RESULTS

Water quality

Physicochemical parameters were measured in tandem with the antibiotic resistance gene monitoring. As expected for freshwater, salinity was minimal at all six sites across the nine sampling occasions, ranging between 0.0 and 0.1 parts per thousand and confirmed sampling at Momona occurred above the zone of intertidal influence in the river. While temperature and flow generally followed an expected pattern of both increasing from the upstream to more downstream sites on all sampling occasions, nutrient measurements were quite variable both spatially and temporally (Figure 2). River temperatures followed seasonal patterns, with the lowest temperatures of 0.7–2.0 °C (upstream to downstream, respectively) measured on 16 July 2010 (austral winter) and the highest temperatures of 14.8–22.1 °C on 16 December 2010 (austral summer). The two lowest flow rates upstream to downstream respectively of 1.8–4.5 m^3/sec and 1.1–6.0 m^3/sec were observed seven months apart, with the first observed late autumn (22 May 2010) and the second mid-summer (16 December 2010). However, a Second Level Flood warning (approximately 10 times mean Average Flow) was activated on 29 May 2010, only a few days after the first sampling occasion, highlighting flow variability in the Taieri River.

Acceptable DRP levels of ≤6.0 μg/L, as referenced against the New Zealand Periphyton Guidelines (Biggs 2000), were exceeded on all nine sampling occasions at most sampling sites indicating nuisance algal growths were possible in much of the river year round. The key exceptions were at Lammermore (S1; headwaters), where DRP concentrations were routinely below acceptable levels on all but one occasion (16 July 2010; 7.7 μg/L), and at both Outram Glen and Momona on 12 August 2010 (5.1 and 5.8 μg/L, respectively). Such levels indicated that the excess of biologically available components of TP were the result of direct runoff and tributaries entering the Taieri River between Lammermore (S1) and Waipiata (S2) given the pattern of decreasing DRP levels at each site thereafter. TP in the river exceeded ANZECC guidelines of ≤33 μg/L (ANZECC 2000) on seven of nine sampling occasions (compared to all nine for DRP) and did so at fewer sites on those occasions (18 out of 54). Guidelines for TP were never exceeded at Lammermore (S1), while Waipiata (S2) exceeded acceptable levels on five occasions; again highlighting the influence of either direct runoff and/or tributaries entering the river between these two sites. New Zealand Periphyton guidelines for DIN of ≤75 μg/L (Biggs 2000), the biologically available component of TN, were exceeded on seven of nine sampling occasions, while TN guidelines of 614 μg/L (ANZECC 2000) were only exceeded on a single sampling occasion (16 July 2010) at the three most downstream sites of Middlemarch (S4), Outram Glen (S5) and Momona (S6).

Molecular analyses

Positive controls included in every set of PCR successfully amplified confirming correct reaction conditions were achieved for both 16s *r*RNA and antibiotic resistance gene screening. None of the blank controls showed amplification, indicating neither non-specific amplification nor nucleic acid contamination had occurred during sample screening. Successful 16s *r*RNA PCR amplifications were obtained for all samples collected during the study confirming good quality nucleic acid had been extracted for the subsequent antibiotic resistance gene screening. Fifteen biofilm samples could not be collected on five sampling occasions at Momona (S6; 22 May 2010, 23 September 2010, 3 November 2010, 16 December 2010 and 1 February 2011) due to the unavailability of suitable rock substrates above riverbed sediment; only four sampling dates for this site were included in the analyses. The remaining 147 samples were screened for the presence of the 10 antibiotic resistance genes using single primer reactions and multiplex PCR. Only three antibiotic resistance genes were detected and confirmed using Sanger sequencing during the year-long survey.

The antibiotic resistance gene *ermB* was detected at Momona (S6) on 28 February 2011 (S6; GenBank accession number JN801170), two weeks following a notable runoff event (7 February 2011; 13 times mean annual low flow). Both other antibiotic resistance genes of *vanB* and *tetB* were detected at the popular swimming spot of Outram

Downloaded from https://iwaponline.com/jwh/article-pdf/11/2/186/395472/186.pdf
by guest
on 23 October 2018

Glen (S5) on the same day in late summer after water temperatures had been warmer for several months (19 April 2011; *vanB* sequence available on request from the author; *tetB* GenBank accession number JN801169). No antibiotic resistance genes were detected from biofilms collected at Lammermore (S1), Waipiata (S2), Hyde (S3) and Middlemarch (S4) during the year-long study. Although three different genes were detected, they were only observed from one of the three individual rock biofilm replicate samples collected at each site, indicating antibiotic resistance gene presence was not widespread at the three locations.

Considering only the two sites where resistance was detected, of the 39 samples collected at Outram Glen (S5) and Momona (S6) (three samples on nine and four sampling occasions respectively for each site), antibiotic resistance genes were detected in 7.7% of the samples. Considering all 147 samples from the river, gene detection dropped to 2%.

DISCUSSION

Antibiotic resistance in the environment has been recognised by the World Health Organization as a global threat and critical public health challenge (WHO 2000; Storteboom *et al.* 2010) due to the growing evidence of vast environmental reservoirs (Wright 2010). Examples of terrestrial reservoirs of antibiotic resistance have been identified; such as significant resistance profile correlations between antibiotic-resistant microbes in agricultural manure and wild bird populations residing at the same location (Blanco *et al.* 2009). However, while knowledge of antibiotic resistance reservoirs from aquatic reservoirs is improving (Zhang *et al.* 2009), studies have been somewhat limited both in scale and duration (Schwartz *et al.* 2003; Moore *et al.* 2010; Storteboom *et al.* 2010). Given aquatic environments are significant food and drinking water sources, as well as for pursuing recreational activities, there is an obvious importance in characterising baseline levels of antibiotic resistance in such reservoirs in order to appropriately assess potential public health concerns. The current study addressed missing baseline information for antibiotic resistance in aquatic biofilms in a New Zealand river, but also addressed key ecological questions of presence along an entire river and what influences, if any, changing livestock farming intensities and seasonal variation may have. As New Zealand's fourth longest river and one fairly representative for the country by displaying increasing livestock farming intensification as it flows downstream, the Taieri River was an obvious choice to explore such questions. Antibiotic resistance genes were only detected in 2% of all samples collected from the river, indicating either minimal antibiotic resistance existed in the river or alternatively highlighted limitations of the methods employed.

Previous antibiotic resistance gene screening of freshwater biofilm communities has been unsatisfactory to date, with work largely reliant on cultivation (McArthur & Tuckfield 2000; Fincher *et al.* 2009; Moore *et al.* 2010). As microbes sampled from the environment and recovered by cultivation can be less than 1% viable when compared to total cell counts (D'Costa *et al.* 2006), more recent studies have screened biofilm directly and in its entirety using PCR (Engemann *et al.* 2008; Barker-Reid *et al.* 2010; Storteboom *et al.* 2010). Although molecular based approaches also have multiple shortcomings, PCR has been shown previously to be reliable and an appropriate method for identifying antibiotic resistance genes from environmental samples (Barker-Reid *et al.* 2010). Nevertheless, the identification and subsequent sequencing confirmation of only three antibiotic resistance genes from three of 147 samples suggested possible limitations with the detection method employed in the current study. Some potential limitations included the presence of reaction inhibitors preventing successful amplification, the mismatching of primers to sample DNA, non-specific amplification, false positives and low DNA concentrations or false negatives (Zhang *et al.* 2009). Successful amplifications for all 147 samples with a generic bacterial 16s *r*RNA primer pair prior to antibiotic resistance gene screening ruled out the presence of strong reaction inhibitors preventing amplification. While perhaps more suitable, similar amplifications with a low-copy number housekeeping gene were not performed. The mismatching of primers to sample DNA was considered unlikely given the use of control samples from environmental and clinical sources known to carry the target antibiotic resistance genes. Lastly, non-specific amplification resulting in false positives was discounted due to the use of Sanger

Downloaded from https://iwaponline.com/jwh/article-pdf/11/2/186/395472/186.pdf
by guest
on 23 October 2018

sequencing to confirm all possible antibiotic resistance gene amplifications.

The incorrect classification of samples as false negatives in the current study, observed as the failure to detect antibiotic resistance genes present in DNA concentrations below particular thresholds, could not be discounted. However, several factors suggested the low level of presence detected in the current study was not unreasonable given previous findings reported in the literature. Similarly, low detection rates of 0, 0 and 4% were reported for three of the same antibiotic resistance genes (*vanA*, *vanB* and *mecA*, respectively) from a recent Australian river study (Barker-Reid *et al.* 2010). However, it should be noted that Barker-Reid *et al.* (2010) did not state their antibiotic resistance detection limits. In fact, minimum detection limit ranges for antibiotic resistance genes, determined both in the presence and absence of representative environmental samples, have not typically been reported in the literature. Of all the studies referenced herein, only Schwartz *et al.* (2003) stated their actual antibiotic resistance gene detection limits. Detection limits determined in the current study ranged between <0.5 *p*g and 1 ng for the 10 different antibiotic resistance genes, with no discernable difference detected in the presence and absence of a representative environmental sample. Understandably, with only one published study stating detection limits to compare to Schwartz *et al.* (2003), it was not possible to evaluate whether the current study's levels of 10–2,000 times that were prohibitively too high or not. However, detection limits of the three genes detected in the current study were 5 *p*g, 0.1 ng, 1 ng, suggesting that the higher detection limits in comparison to Schwartz *et al.* (2003) were not prohibitively too high for detecting antibiotic resistance gene presence in the environmental samples. Therefore, it is concluded that the failure to detect antibiotic resistance genes in 98% of all collected samples most likely indicated minimal antibiotic resistance was present along the Taieri River.

Focussing now on where the three antibiotic resistance genes were identified from, a stretch of river subject to the greatest combination of human use, as well as cumulative and locally intensive dairy farming, the detection level of genes in biofilms from the Taieri River at those two sites increased to 7.7% from 2% overall. Interestingly, the presence of substantial locally intensive dairy farming, evident as high-producing exotic grasslands land-use cover, was not unique to the two downstream sites of Outram (S5; 36%) and Momona (S6; 59%). Comparably high-producing exotic grassland land usage existed at Waipiata (S2; 39%) and Middlemarch (S4; 32%). Yet, despite similar land use cover, no antibiotic resistance genes were detected at Waipiata or Middlemarch throughout the year-long study, suggesting dairy farming intensity alone may not necessarily result in detectable biofilm resistance. In fact, no genes were detected throughout the year at the four upstream sites. Rather, human usage, either independently or in combination with intensive dairy farming, appeared to be an important driver of antibiotic resistance in the Taieri River.

Lending further weight to the likelihood of a human source being responsible for the three antibiotic genes detected was the type of resistance detected and seasonal timing. While both erythromycin and tetracycline are used in the agricultural sector as well as for the treatment of human disease, vancomycin is only available for human use in New Zealand and is a crucial antibiotic for treating serious, multi-drug resistant infections (Gold & Moellering 1996). Avoparcin usage in animals has been shown to also promote *vanA* gene resistance (Haenni *et al.* 2009), yet neither *vanA* nor *vanB* have been reported from New Zealand cattle to date and avoparcin use in the New Zealand agricultural sector was phased out over 12 years ago (Manson *et al.* 2004). The detection of the three genes during the austral summer/early autumn months correlated with the greatest use of the river by humans, both directly at Outram Glen and indirectly via increased tourism (and hence increased wastewater) to the Middlemarch Township. If agricultural influences (namely, faecal deposits in farm fields) were more meaningful, antibiotic resistance genes would have been expected in the biofilm following surface runoff events, in particular those typical in the austral winter and spring months when the ground was already saturated. However, no such patterns were observed following the substantial flood event in late May 2010 or after the higher river flows observed in September 2010.

Two key situations exist along the river that could explain a human-origin contamination pathway resulting in the biofilm resistance patterns observed. Firstly, a municipal wastewater treatment facility outlet (up to 360 m^3 treated domestic effluent per day discharged at 4.1 L per

Downloaded from https://iwaponline.com/jwh/article-pdf/11/2/186/395472/186.pdf
by guest
on 23 October 2018

second into the river) approximately 50 km upstream of Outram Glen (S5) at the Middlemarch Township (165 permanent inhabitants recorded in the 2006 New Zealand Census) and secondly, heavy recreational use of the river at Outram Glen (S5). Certainly, contamination could have resulted via either direct human faecal deposition at the recreational site itself or alternatively from the treatment plant given known contamination pathways for antibiotics and antibiotic resistant microbes to waterways (Halling-Sørensen *et al.* 1998; Kim & Aga 2007; Jury *et al.* 2011). Isolating which of the human pathways discussed, or indeed confirming any or all, as the probable contamination source could not be resolved with the scale of sampling employed in the current study. Delays in the identification of antibiotic resistance from the Outram Glen samples prevented additional site-targeted sampling upstream at the municipal treatment plant outflow for an appropriately timed comparison. Such sampling may have helped clarify the source of resistance observed at the popular recreational site. The location and possible influence of municipal wastewater treatment outlets along the Australian river system (if any) on the rate of antibiotic resistance genes detected was not explicitly stated by Barker-Reid *et al.* (2010), yet similar conclusions were reached regarding correlations between antibiotic resistance detection and river characteristics. Owing to the river's physiochemical variability and the limited detection of antibiotic resistance genes, it was not appropriate to make predictions or state probable correlations between specific physiochemical variables and the likely presence of antibiotic resistance genes.

It was not possible to establish whether the limited detection of antibiotic resistance genes in the current study equated to a limited presence of antibiotics in the river. Unfortunately, water samples collected from the six sites throughout the year-long study could not be reliably analysed to low enough concentrations based on limits reported in previous freshwater studies (Kemper 2008). Given antibiotics are designed to affect microbes (Halling-Sørensen *et al.* 1998), even at relatively low concentrations, the presence of sub-inhibitory level concentrations can have consequences for river ecosystem functioning and, in turn, important ecological processes (Ding & He 2010). Accordingly, two separate studies are underway to: (1) quantify the levels of antibiotics present in the Taieri River, if any, and (2) characterise the specific bacterial communities present at each of the six sampling sites throughout the year, along with their associated ecological processes, in relation to the antibiotic resistance patterns observed.

CONCLUSIONS

Considering all the evidence for human and agricultural contamination stressors influencing the level of antibiotic resistance along the river, it appeared that while resistance levels were low across the whole river, sites subjected to the combined influences of greater human usage and intensive dairy farming showed an increased level of resistance; albeit nominally of 7.7% compared with 2% overall. The detection of a gene conferring resistance to a key human-only use antibiotic was particularly concerning and highlighted the importance of considering human sources of antibiotic resistance contamination along the river, in addition to dairying intensification, when assessing and managing potential public health concerns. Therefore, this study supports the partitioning of rivers into higher and lower human-use areas to help predict potential environmental reservoirs of antibiotic resistance, as opposed to solely considering agricultural land usage. These findings suggest additional studies are necessary to gauge a more complete understanding of human-based environmental reservoirs of antibiotic-resistant microbes. In particular, sites targeted above and below municipal wastewater treatment outlets would be useful for assessing and quantifying the influence of human contamination of freshwaters.

ACKNOWLEDGEMENTS

I thank Colin Townsend for editorial suggestions, Emily Nichol for assistance with field collections and laboratory work, Nicky McHugh for nutrient analysis, the Otago Regional Council for Taieri River catchment data and the anonymous reviewers whose comments improved this manuscript. This work was supported by a Project Grant-in-Aid from the Maurice & Phyllis Paykel Trust New Zealand and a New Zealand Foundation for Science Research and Technology Postdoctoral Fellowship.

Downloaded from https://iwaponline.com/jwh/article-pdf/11/2/186/395472/186.pdf
by guest
on 23 October 2018

REFERENCES

Agerso, Y. & Petersen, A. 2007 The tetracycline resistance determinant Tet 39 and the sulphonamide resistance gene sulII are common among resistant Acinetobacter spp. isolated from integrated fish farms in Thailand. *J. Antimicrob. Chemother.* **59**, 23–27.

Allen, H., Donato, J., Wang, H., Cloud-Hansen, K., Davies, J. & Handelsman, J. 2010 Call of the wild: antibiotic resistance genes in natural environments. *Nat. Rev.* **8**, 251–259.

ANZECC 2000 *Australian and New Zealand Guidelines for Fresh and Marine Water Quality, Volume 1, The Guidelines*. Australian and New Zealand Environment and Conservation Council, Canberra.

APHA 1998 *Standard Methods for the Examination of Water and Wastewater*. American Public Health Association, Washington, DC, USA.

Barker-Reid, F., Fox, E. & Faggian, R. 2010 Occurrence of antibiotic resistance genes in reclaimed water and river water in the Werribee Basin, Australia. *J. Water Health* **8**, 521–531.

Bell, J. M., Paton, J. C. & Turnidge, J. 1998 Emergence of vancomycin-resistant enterococci in Australia: phenotypic and genotypic characteristics of isolates. *J. Clin. Microbiol.* **36**, 2187–2190.

Biggs, B. 2000 *New Zealand Periphyton Guideline: Detecting, Monitoring and Managing Enrichment of Streams*. M. f. t. Environment, Wellington.

Blanco, G., Lemus, J. A. & Grande, J. 2009 Microbial pollution in wildlife: linking agricultural manuring and bacterial antibiotic resistance in red-billed choughs. *Environ. Res.* **109**, 405–412.

Chen, J., Yu, Z., Michel, F., Wittum, T. & Morrison, M. 2007 Development and application of real-time PCR assays for quantification of *erm* genes conferring resistance to macrolides-lincosamides-streptogramin B in livestock manure and manure management systems. *Appl. Environ. Microbiol.* **73**, 4407–4416.

Costanzo, S., Murby, J. & Bates, J. 2005 Ecosystem response to antibiotics entering the aquatic environment. *Mar. Pollut. Bull.* **51**, 218–223.

D'Costa, V., McGrann, K., Hughes, D. & Wright, G. 2006 Sampling the antibiotic resistome. *Science* **311**, 374–377.

Ding, C. & He, J. 2010 Effect of antibiotics in the environment on microbial populations. *Appl. Microbiol. Biotechnol.* **87**, 925–941.

Engemann, C., Keen, P., Knapp, C., Hall, K. & Graham, D. 2008 Fate of tetracycline resistance genes in aquatic systems: migration from the water column to peripheral biofilms. *Environ. Sci. Technol.* **42**, 5131–5136.

Fincher, L., Parker, C. & Chauret, C. 2009 Occurrence and antibiotic resistance of *Escherichia coli* 0157:H7 in a watershed in North-Central Indiana. *J. Environ. Qual.* **38**, 997–1004.

Gold, H. & Moellering, R. 1996 Drug therapy – antimicrobial-drug resistance. *N. Engl. J. Med.* **335**, 1445–1453.

Haenni, M., Saras, E., Châtre, P., Meunier, D., Martin, S., Lepage, G., Ménard, M., Lebreton, P., Rambaud, T. & Madec, J. 2009 *vanA* in *Enterococcus faecium, Enterococcus faecalis*, and *Enterococcus casseliflavus* detected in French cattle. *Foodborne Pathog. Dis.* **6**, 1107–1111.

Halling-Sørensen, B., Nors Nielsen, S., Lanzky, P., Ingerslev, F., Holten Lützhøft, H. & Jørgensen, S. 1998 Occurrence, fate and effects of pharmaceutical substances in the environment – a review. *Chemosphere* **36**, 357–393.

Hillis, D., Lissemore, L., Sibley, P. & Solomon, K. 2007 Effects of monensin on zooplankton communities in aquatic microcosms. *Environ. Sci. Technol.* **41**, 6620–6626.

JETACAR 1999 *The Use of Antibiotics in Food-Producing Animals: Antibiotic-Resistant Bacteria in Animalsand Humans*. Commonwealth Department of Health and Aged Care, Canberra, Australia. Available from: www.health.gov.au/internet/main/publishing.nsf/content/2A8435C711929352CA256F180057901E/$File/jetacar.pdf.

Jury, K., Khan, S., Vancov, T., Stuetz, R. & Ashbolt, N. 2011 Are sewage treatment plants promoting antibiotic resistance? *Clin. Rev. Environ. Sci. Technol.* **41**, 243–270.

Kemper, N. 2008 Veterinary antibiotics in the aquatic and terrestrial environment. *Ecol. Indic.* **8**, 1–13.

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

Lane, D. (ed.) 1991 *16S/23S rRNA Sequencing. Nucleic Acid Techniques in Bacterial Systematics*. John Wiley & Sons, Chichester, UK.

Lear, G., Anderson, M., Smith, J., Boxen, K. & Lewis, G. 2008 Spatial and temporal heterogeneity of the bacterial communities in stream epilithic biofilms. *FEMS Microbiol. Ecol.* **65**, 463–473.

Manson, J., Smith, J. & Cook, G. 2004 Persistance of Vancomycin-resistant enterococci in New Zealand broilers after discontinuation of Avoparcin use. *Appl. Environ. Microbiol.* **70**, 5764–5768.

McArthur, J. & Tuckfield, R. 2000 Spatial patterns in antibiotic resistance among stream bacteria: effects of industrial pollution. *Appl. Environ. Microbiol.* **66**, 3722–3726.

Moore, J., Rao, J., Moore, P., Millar, B., Goldsmith, C., Loughrey, A. & Rooney, P. 2010 Determination of total antibiotic resistance in waterborne bacteria in rivers and streams in Northern Ireland: can antibiotic-resistant bacteria be an indicator of ecological change? *Aquat. Ecol.* **44**, 349–358.

Nawaz, M., Khan, A., Khan, S., Sung, K., Kerdahi, K. & Steele, R. 2009 Molecular characterization of tetracycline-resistant genes and integrons from avirulent strains of *Escherichia coli* isolated from catfish. *Foodborne Pathog. Dis.* **6**, 553–559.

Norris, P., Becket, G. & Ecke, D. 2005 Demographic variation in the use of antibiotics in a New Zealand town. *NZ Med. J.* **118**, 1–7.

ORC 2011 *Otago Regional Council Land Catchment Database*. Dunedin, New Zealand.

PCE 2004 *Growing for Good: Intensive Farming, Sustainability and New Zealand's Environment*. Parliamentary

Downloaded from https://iwaponline.com/jwh/article-pdf/11/2/186/395472/186.pdf
by guest
on 23 October 2018

Commissioner for the Environment, Wellington, New Zealand, pp. 236.

Pusch, M., Fiebig, D., Brettar, I., Eisenmann, H., Ellis, B., Kaplan, L., Lock, M., Naegeli, M. & Traunspurger, W. 1998 The role of micro-organisms in the ecological connectivity of running waters. *Freshwater Biol.* **40**, 453–495.

Ramos-Trujillo, E., Perez-Roth, E., Mendez-Alvarez, S. & Claverie-Martin, F. 2003 Multiplex PCR for simultaneous detection of enterococcal genes vanA and vanB and staphylococcal genes mecA, ileS-2 and femB. *Int. Microbiol.* **6**, 113–115.

Sarmah, A., Meyer, M. & Boxall, A. 2006 A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. *Chemosphere* **65**, 725–759.

Schwartz, T., Kohnen, W., Jansen, B. & Obst, U. 2003 Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. *FEMS Microbiol. Ecol.* **43**, 325–335.

Storteboom, H., Arabi, M., Davis, J., Crimi, B. & Pruden, A. 2010 Identification of antibiotic resistance gene molecular signatures suitable as tracers of pristine river, urban, and agricultural sources. *Environ. Sci. Technol.* **44**, 1947–1953.

Strommenger, B., Kettlitz, C., Werner, G. & Witte, W. 2003 Multiplex PCR assay for simultaneous detection of nine clinically relevant antibiotic resistance genes in Staphylococcus aureus. *J. Clin. Microbiol.* **41**, 4089–4094.

Tao, R., Ying, G., Su, H., Zhou, H. & Sidhu, J. 2010 Detection of antibiotic resistance and tetracycline resistance genes in Enterobacteriaceae isolated from the Pearl rivers in South China. *Environ. Pollut.* **158**, 2101–2109.

Volkmann, H., Schwartz, T., Kirchen, S., Stofer, C. & Obst, U. 2007 Evaluation of inhibition and cross-reaction effects on real-time PCR applied to the total DNA of wastewater samples for the quantification of bacterial antibiotic resistance genes and taxon-specific targets. *Mol. Cell. Probes* **21**, 125–133.

Watanabe, N., Harter, T. & Bergamaschi, B. 2008 Environmental occurrence and shallow ground water detection of the antibiotic monensin from dairy farms. *J. Environ. Qual.* **37**, S-78–S-85.

Watkinson, A., Murby, E., Kolpin, D. & Costanzo, S. 2009 The occurrence of antibiotics in an urban watershed: from wastewater to drinking water. *Sci. Total Environ.* **407**, 2711–2723.

WHO 2000 *Antimicrobial Resistance: A Global Threat. Essential Drugs Monitor*. World Health Organization, Geneva.

Wright, G. 2010 Antibiotic resistance in the environment: a link to the clinic? *Curr. Opin. Microbiol.* **13**, 589–594.

Zhang, X., Zhang, T. & Fang, H. 2009 Antibiotic resistance genes in water environment. *Appl. Microbiol. Biotechnol.* **82**, 397–414.

First received 17 October 2012; accepted in revised form 12 February 2013. Available online 8 March 2013

Downloaded from https://iwaponline.com/jwh/article-pdf/11/2/186/395472/186.pdf
by guest
on 23 October 2018