

Occurrence of antibiotic resistance genes in reclaimed water and river water in the Werribee Basin, Australia

Fiona Barker-Reid, Ellen M. Fox and Robert Faggian

ABSTRACT

The purpose of this study was to investigate the occurrence of antibiotic resistance genes (ARGs) in water used for irrigation in the Werribee River Basin, Australia, including river water and reclaimed effluent water (reclaimed water). Samples of reclaimed water, collected over a one-year period, were screened for the occurrence of ARGs using PCR detection assays. The presence of ARGs in the reclaimed water samples were contrasted with that of water samples taken from the Werribee River Basin, collected over the same time period, from five points selected for varying levels of urban and agricultural impact. Of the 54 river water samples collected, 2 (4%), 2 (4%), 0 and 0 were positive for methicillin, sulfonamide, gentamicin and vancomycin-resistant genes, respectively, while 6 of 11 reclaimed water samples were positive for methicillin (9%) and sulfonamide (45%). The presence/absence of ARGs did not appear to correlate with other measured water quality parameters. The low detection of ARGs in river water indicates that, regardless of its poor quality, the river has not yet been severely contaminated with ARGs. The greater prevalence of ARGs in reclaimed water indicates that this important agricultural water source will need to be monitored into the future.

Key words | irrigation, recycled water, seasonal study, waste water

Fiona Barker-Reid (corresponding author)
Department of Natural Resource
Management and Geography,
The University of Melbourne and the
Department of Primary Industries,
Parkville, Victoria 3010,
Australia
Tel.: +613-8341-2413
Fax: +613-9347-6056
E-mail: s.barker-reid@pgrad.unimelb.edu.au

Ellen M. Fox
Department of Primary Industries,
1 Stratford Road,
Maffra, Victoria 3860,
Australia

Robert Faggian
Department of Primary Industries,
PO Box 4166,
Parkville, Victoria 3052,
Australia

TERMS AND ABBREVIATIONS

ARG	antibiotic resistance gene
BOD	biological oxygen demand
MRSA	methicillin-resistant <i>Staphylococcus aureus</i>
PCR	polymerase chain reaction
SS	suspended solids
UV	ultraviolet
WID	Werribee Irrigation District
WTP	Western Treatment Plant

INTRODUCTION

Antibiotic resistance genes (ARGs) are recognized as emerging environmental contaminants (Rysz & Alvarez 2004; Snow *et al.* 2007; Martinez 2008) that move readily

doi: 10.2166/wh.2010.102

between ecological niches (JETACAR 1999). The proliferation of these genes in the environment has been linked to the widespread use of antibiotics in humans and animals (JETACAR 1999; Pruden *et al.* 2006). Water is a vector for the transfer of ARGs from human and animal waste into the natural environment (JETACAR 1999; Witte 2000; Chee-Sanford *et al.* 2001; Koike *et al.* 2007; Baquero *et al.* 2008) and the aquatic environment has been identified as a reservoir for antibiotic resistance (Biyela *et al.* 2004; Martinez 2008). Only in recent years has there been increased interest in the prevalence of antibiotic-resistant organisms or genes in waterways and waste water (Zhang *et al.* 2009), although very few studies have investigated highly treated effluent (Bockelmann *et al.* 2009). This research was aimed at evaluating the possible input of antibiotic resistance from a relatively new source – reclaimed water – which has the potential to act as another contamination source back into agriculture.

Two sources of agricultural irrigation water were selected for investigation: river water and treated human effluent (reclaimed water). The Werribee River (historically the major source of irrigation water for food crops in the Werribee Basin) was selected because of the diversity of land uses along its length that impact on water quality. Extreme drought conditions in recent years have resulted in restricted use of both river and groundwater. Since 2005, reclaimed water (highly treated human effluent meeting 'Class A' standards, using UV light and chlorination) has been available for purchase by growers. Guidelines vary between states, but in Victoria, Australia, Class A reclaimed water is subject to Environmental Protection Agency Guidelines, which dictate the following indicative water quality objectives (median values over 12-month period): $< 10 E. coli$ org/100 mL, turbidity < 2 NTU (24 h median value pre-disinfection), $< 10/5$ mg/L BOD/SS, pH 6–9 (90th percentile), 1 mg/L Cl_2 residual (or equivalent disinfection) (EPAV 2003).

Reclaimed water is now the main source of irrigation water in the Werribee Irrigation District (WID) due to a combination of reduced rainfall and inflows, and declining quality (particularly with respect to salinity) of river water and other conventional sources of irrigation water that are still available. At present, reclaimed water is used predominantly for irrigation of vegetable crops, many of which are consumed raw. Very few studies have investigated the prevalence of ARGs in Australia (Boon 1992; Boon & Cattnach 1999; Watkinson *et al.* 2007) and to our knowledge this is the first seasonal study of ARGs in an Australian river.

The aim of this study was to look for the presence of ARGs along a gradient of water quality and where ample opportunity exists for their introduction to the water system. The best means of achieving this goal was with a method like polymerase chain reaction (PCR). Despite its shortcomings (that is, a positive test result does not necessarily indicate that the host of the ARG was viable), PCR is rapid, can be used on a variety of substrates (soil, water, sediment) and does not require that the host organism be culturable. It is therefore ideal for determining the presence of ARGs in water in order to assess their potential as environmental contaminants. This was not a comparison of methods but rather a survey of water sources using a proven method.

METHODS

Study area and sampling sites

Water samples were collected from five sites within the Werribee Basin (Figure 1). These sites were chosen to correspond with routine state-wide river sampling locations (DSE 2008), as well as to represent a gradient of human and agricultural inputs. Although the majority of the basin has been cleared for agriculture (67%) and urban development (5%), small patches of remnant vegetation still remain (Melbourne Water 2007). Agricultural land is predominantly used for horticulture (44%), improved pasture (41%) or cropping (typically consisting of broadacre cereal and legume production; 19%) (DEWHA 2007). The Werribee River, for the most part, represents a degraded river system that is highly impacted by anthropogenic activities (Da Costa *et al.* 2008). The flow regimes of the river are highly regulated, with water diversions for irrigation and domestic use strictly controlled by the state government (DSE 2005).

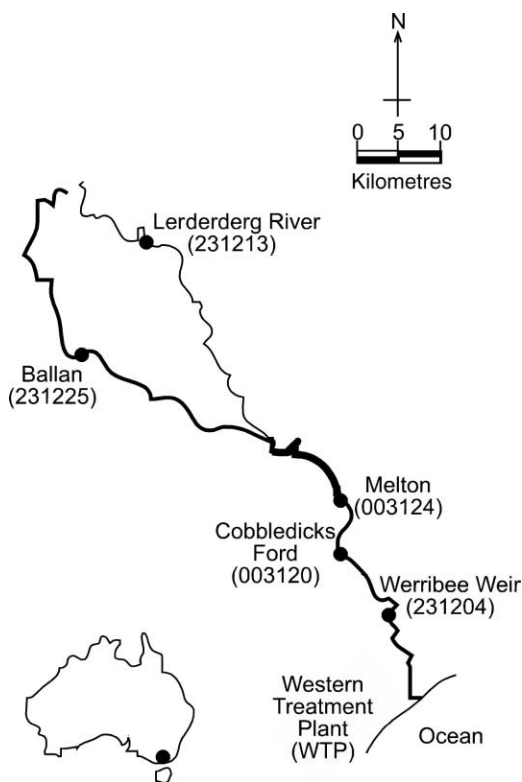


Figure 1 | Map of study area and sampling locations, with corresponding state-wide sampling numbers in parentheses (DSE 2008).

The Lerderderg River, a tributary of the Werribee River, originates in the Lerderderg State Forest and at its source is considered to be in excellent environmental condition (Melbourne Water 2007). This sampling location (1; 231213) represents a relatively pristine environment that is not impacted by agriculture or urban development. At its origin, the Werribee River is in moderate condition (DSE 2005). The river passes through the town of Ballan (population 1,700), already in poor condition, and the sampling point (2; 231225) was just south of the town. The river continues through Bacchus Marsh (population 12,000), one of two major irrigation areas (orchards and market gardens), and then flows into a major storage, Melton Reservoir. The sampling point (3; 003124) was downstream of the reservoir. From there, the river passes through an area of predominantly dryland grazing, some irrigated pasture, forestry, cropping and intensive animal production. The river crosses a small road (Cobbledicks Ford) at sampling point 4 (003120), becoming increasingly degraded as the surrounding landscape becomes more urbanized; over ~70 km, the landscape transforms from predominantly rural to predominantly urban. Further downstream, small areas of pasture, irrigated horticulture and intensive animal production remain, although horticulture is the major food-producing land use. A weir is located close to the mouth of the river and the final sampling point (5; 231204) was at the outflow of the weir. The final stretch of the river runs through the WID, a major area of vegetable production, although accessible sampling points were not identified.

Reclaimed water is predominantly used in the WID, with ~22,000 ML used in agriculture in 2005–2006 (the first year available). Smaller volumes of reclaimed water are used in Ballan, Melton and Bacchus Marsh (up to 4,400 ML) (DSE 2006).

Sampling

Water samples (2 L) were collected in sterile plastic containers on 11 different occasions between October 2007 and September 2008 (08-Oct-07, 03-Jan-08, 21-Jan-08, 18-Mar-08, 30-Mar-08, 14-Apr-08, 28-May-08, 05-Jul-08, 29-Jul-08, 15-Aug-08 and 17-Sep-08). Samples (2 L) of Class A reclaimed water from the Western Treatment Plant

(WTP) in Werribee, Victoria, were collected from a local facility which receives water directly (via pipeline) from the WTP, with a total of 11 samples collected over a similar time period. At the time of sampling, the quality of the water was assessed using a multiprobe field analyser (TPS 90FL-T) to measure pH, turbidity, temperature and salinity. Water samples were transported at room temperature and stored at 4°C within five hours of sampling. Further water quality measurements were obtained from the Victorian Water Resources Data Warehouse (DSE 2008).

Sample processing and DNA extraction

Each water sample was removed from cool storage and mixed by shaking. Sub-samples (500 mL) were filtered using a three-branch manifold system with suction, through 0.45 µm sterile cellulose nitrate membranes (Sartorius, USA). All equipment was sterilized with 70% ethanol between samples. After filtration, membranes were placed into a sterile falcon tube (50 mL) and frozen at –20°C.

To extract total DNA, membranes were brought to room temperature, cut into small pieces (~5 mm²) and suspended in 10 mL PBS (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride; Sigma, USA). The mixture was vortexed for 2 min and then centrifuged for 5 min (10,000 × *g*). DNA was prepared from the resulting supernatant using the DNeasy Plant Mini Kit (Qiagen), according to the manufacturer's protocol. One DNA extraction was performed per sample and DNA quality was verified by agarose gel electrophoresis.

PCR assays for detection of ARGs

Qualitative PCR assays were performed for broad-scale screening of the presence/absence of five ARGs conferring resistance to: methicillin (*mecA*), gentamicin (*aac(3)-I*), vancomycin (*vanA* and *vanB*) and sulfonamide (*sul(I)*). Primers for the amplification of these genes are listed in Table 1. Oligonucleotide primer pairs were checked for specificity by testing methicillin-resistant bacteria (obtained from Dr Margaret Deighton, RMIT University, Australia) and vancomycin- and sulfonamide-resistant bacteria (obtained from Dr Andrew Watkinson, SEQWater, Australia). The primer pair for amplifying the gentamicin

Table 1 | PCR primers targeting antibiotic resistance genes

Gene (antibiotic)	Primer	Sequence (5' – 3')	Length (bp)	Product size (bp) (reference)
<i>aac(3)-I</i> (gentamicin)	Faac3-1	TTCATCGCGCTTGCTGCYTTYGA	239	Heuer <i>et al.</i> (2002)
	Raac3-1	GCCACTGCGGGATCGTCRCRCA		
<i>MecA</i> (methicillin)	mecA1-For	AAAATCGATGGTAAAGGTTGGC	533	Murakami <i>et al.</i> (1991)
	mecA2-Rev	AGTTCTGCAGTACCGGATTTGC		
<i>sul(I)</i> (sulfonamide)	<i>sulI</i> -FW	CGCACCGGAAACATCGCTGCAC	163	Pei <i>et al.</i> (2006)
	<i>sulI</i> -RV	TGAAGTCCGCCGCAAGGCTCG		
<i>VanA</i> (vancomycin)	vanA 1	TCTGCAATAGAGATAGCCGC	377	Klein <i>et al.</i> (1998)
	vanA 2	GGAGTAGCTATCCCAGCATT		
<i>VanB</i> (vancomycin)	vanB-FOR	CATGATGTGTCGGTAAATC	885	Patel <i>et al.</i> (1997)
	vanAB-REV	ACCGGGCAGRGTATTGAC		

resistance gene was tested against plasmid pDONR207 (Invitrogen, USA). Amplified fragments of these ARGs were used as positive controls for the PCR detection assays. The presence of the 16S gene (forward: 5'-CAG GCC TAA CAC ATG CAA GTC-3', and reverse: 5'-GGG CGG WGT GTA CAA GGC-3') (Marchesi *et al.* 1998) was used to confirm the presence or absence of amplifiable DNA within each sample.

The PCR reaction mix (25 μ L) consisted of 1 unit of *Taq* polymerase (Qiagen, USA), 10 \times buffer (2.5 μ L), 25 mM MgCl₂ (2 μ L), 10 mM dNTPs (1 μ L) (Promega, USA), deionized water (16.3 μ L), 10 pm/ μ L primer (1 μ L of each pair) and 30 ng of extracted DNA (1 μ L). In the negative controls, the extracted DNA was replaced with RNase- and DNase-free water. Reaction mixtures were incubated for 15 min at 95°C, followed by 5 cycles of 30 s at 95°C, 30 s at 55°C and 30 s at 72°C, followed by 35 cycles of 30 s at 95°C, 30 s at 58°C and 30 s at 72°C, followed by 30 s at 72°C and 2 min at 30°C. PCR products were separated in 2% agarose gels, and visualized under UV light following staining with ethidium bromide.

The genes were selected because of their previous detection in environmental samples from rivers (*sul(I)* and *aac(3)-I*) (Heuer *et al.* 2002; Pei *et al.* 2006; Pruden *et al.* 2006), waste water samples (*vanA*, *mecA* and *aac(3)-I*) (Heuer *et al.* 2002; Schwartz *et al.* 2003; Volkmann *et al.* 2004) and clinical isolates (*vanB*) (Patel *et al.* 1997). Furthermore, microorganisms with resistance to methicillin, sulfonamides, gentamicin and vancomycin have been

isolated from various environments in Australian studies (Bucknell *et al.* 1997; Boon & Cattanaach 1999; Jordan *et al.* 2005; Akinbowale *et al.* 2006; Christiansen *et al.* 2007).

RESULTS

Water quality

The presence/absence of ARGs was monitored within the Werribee Basin in various sampling campaigns over approximately one year (October 2007 to September 2008). Water quality measurements were quite variable, both spatially and temporally (Figure 2, Table 2) and for most of the year there was very little flow. Most samples followed a similar trend in pH, with values rising between May and August (winter), while all samples remained within the range of 7.2 to 8.8. Salinity measurements showed the strongest geographical trend, with a strong rise as the river approached the outflow (Figure 3). The highest salinity values were recorded at the Werribee Weir and at Cobblesticks Ford. The salinity values reflect a number of changes along the river including urban and agricultural land uses (DPI 2008), as well as natural salt contributions from ephemeral waterways (in excess of 8,000 μ S/cm, sampling point 231231 (DSE 2008)). Reclaimed water salinity values remained relatively consistent at \sim 2100 μ S/cm. River water temperatures remained $>$ 3°C at the surface and clearly reflected seasonal changes (maximum of 30°C). There were no apparent

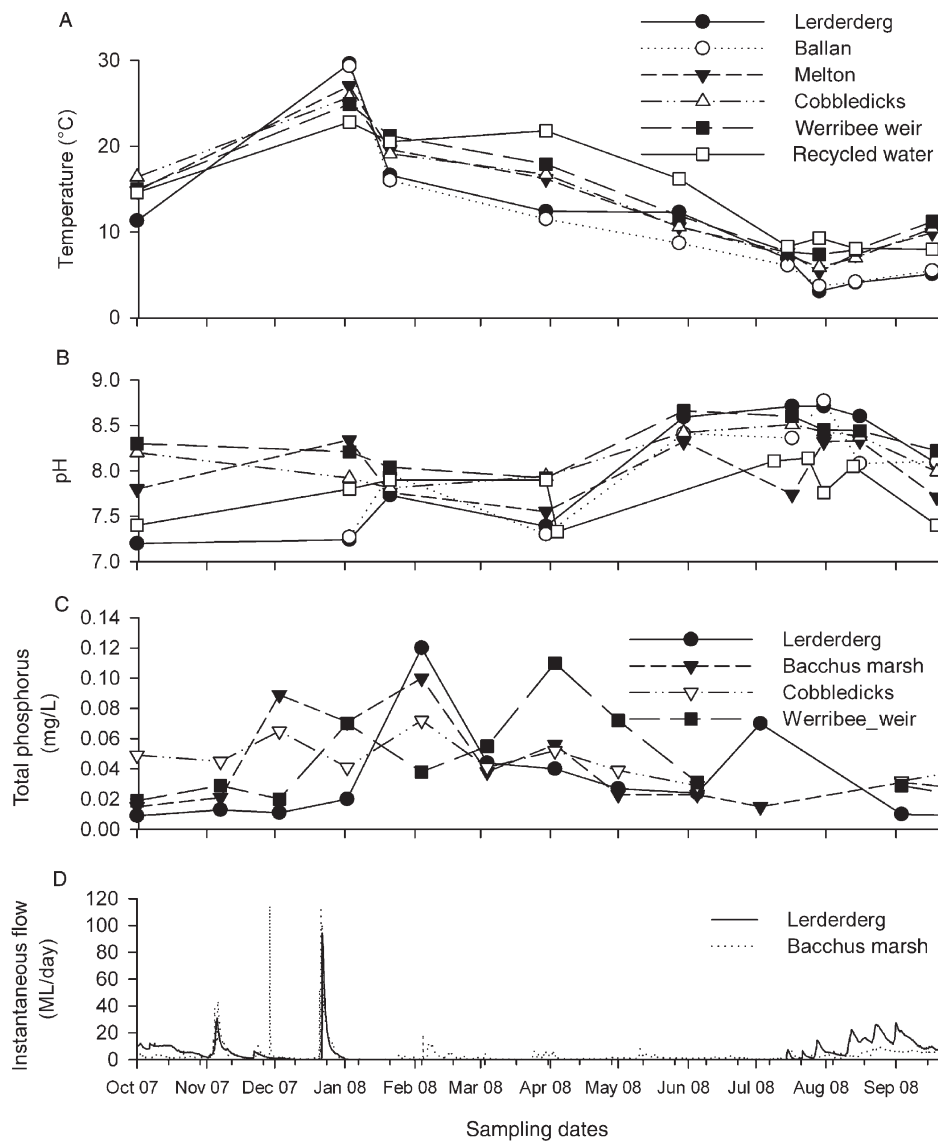


Figure 2 | Water-quality measurements at each sampling date for A. temperature, B. pH, C. total phosphorus and D. instantaneous flow. Data were compiled from manual measurements, the Victorian Water Resources Data Warehouse (DSE 2008) and Melbourne Water (pers. comm.). In some cases, data were not available for each sampling point. Data for Bacchus Marsh is presented to approximate conditions at Melton.

seasonal or geographical trends in turbidity, suspended solids or dissolved oxygen. Total phosphorus levels were sporadic, ranging from 0.01 to 0.12 mg/L. Previous research detected faecal coliforms and *Escherichia coli* in the river water but not in reclaimed water (Engleitner *et al.* 2008).

PCR assays for detection ARGs

16S rDNA was detected in 62 of the 65 water samples tested (95%) and in all samples in which ARGs were detected.

The qualitative occurrence data for all five resistance genes are presented in Table 3. Of the 54 river water samples collected, 2 (4%), 2 (4%), 0 and 0 were positive for methicillin, sulfonamide, gentamicin and vancomycin-resistant genes, while 6 of 11 reclaimed water samples were positive for either methicillin (9%) or sulfonamide (45%). ARGs were not detected in any of the Lerderderg River or Melton samples. In October 2007, *sul(I)* was detected at Cobbledicks Ford and the Werribee Weir, while *mecA* was

Table 2 | Mean values \pm 1 standard deviation of water quality data from 1 October 2007 to 17 September 2008 (n = 9) for all six sampling sites

Parameter	Lerderderg	Ballan	Melton	Cobbledicks	Werribee Weir	Recycled
pH	8.0 \pm 0.6	8.0 \pm 0.5	8.0 \pm 0.3	8.2 \pm 0.3	8.3 \pm 0.2	7.8 \pm 0.4
Turbidity (NTU)	7.8 \pm 8.2	5.9 \pm 4.7	12.6 \pm 6.8	12.1 \pm 2.9	3.5 \pm 4.6	1.6 \pm 1.2
Dissolved oxygen (mg/L)	5.6 \pm 3.5	na	7.8 \pm 1.8*	8.1 \pm 2.3	9.8 \pm 1.7	na
Suspended solids (mg/L)	5.7 \pm 4.7	na	25.8 \pm 26.1*	9.6 \pm 3.7	6.0 \pm 4.0	na

*Werribee River at Bacchus Marsh.

na = data not available.

detected in March and April 2008 at the Weir and Ballan, respectively. All ARGs were detected in the first half of the sampling period (October 2007 to April 2008).

DISCUSSION

Sewage treatment plants (STPs) may receive waste water of very different origins and qualities, including hospital waste water, domestic waste water, surface water or stormwater, industrial waste water and groundwater. The mixing of water sources may facilitate gene exchange and spread and, as some ARGs may not be removed by waste water treatment, may result in their discharge into the environment with effluent water (Zhang *et al.* 2009). While there is currently no direct evidence of ARG transfer from the environment to humans (Zhang *et al.* 2009), studies have identified the possibility that ARGs can spread and be exchanged among environmental organisms (Chee-Sanford *et al.* 2001; Agero & Sandvang 2005) and highlighted the risk of ARG contamination of the foodchain (Chee-Sanford *et al.* 2001; Rodríguez *et al.* 2006).

Detection of ARGs in river water

The very low rate of detection in river water samples was surprising given the poor quality of the river system and the number of potential pollution sources within close proximity (intensive animal production, leaking sewerage systems, disposal of treated waste water and agricultural run-off). The specific location and timing of the detections did not appear to correspond to measured water quality parameters, although most detections were in the lower (more urbanized) reaches of the river.

Antibiotic resistance genes were detected at three sites along the Werribee River: Ballan, Cobbledicks Ford and Werribee Weir. The absence of ARGs at the Lerderderg site perhaps corresponds with its classification as relatively 'pristine'. However, the absence of ARGs at Melton, and the sporadic detection at the other sites, was somewhat surprising given the agricultural and residential land use in the surrounding area and upstream. The results at the Melton site can probably be explained by the upstream reservoir providing a diluting effect on water samples.

Detection of ARGs in reclaimed water

The ARGs were more frequently detected in the samples of reclaimed water. This indicates that the river, even in its very poor condition, hasn't yet been severely impacted by ARG-contaminated wastes such as agricultural run-off and human sewage (treated or otherwise). Previous studies have detected ARGs in treated waste water and the results from this current study confirm that even very high levels of

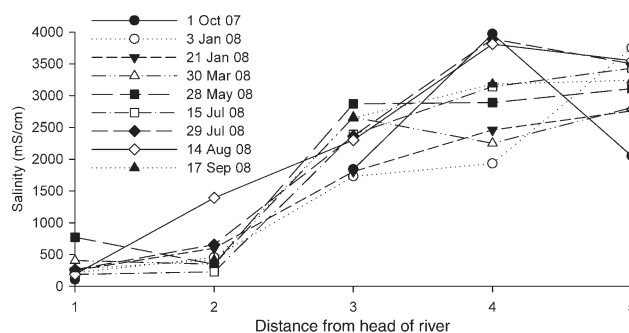


Figure 3 | Salinity measurements for the Werribee River over a one-year period (2007–2008), presented as a function of their relative distance from the headwaters (1 – Lerderderg, 2 – Ballan, 3 – Melton, 4 – Cobbledicks Ford and 5 – Werribee Weir). The July 2008 salinity measurement for Melton failed; in its place, a reading from Bacchus Marsh (~20 km upstream) was used.

Table 3 | Molecular detection of *aac(3)-I* (A), *mecA* (B), *sul(1)* (C), *vanA* (D), *vanB* (E) and *16S* (F). The symbol + denotes detected and – not detected. Sampling sites: (1) Lerderderg, (2) Ballan, (3) Melton, (4) Cobblebicks Ford, (5) Werribee Weir and (6) Reclaimed

Sampling site/ Sampling time	1						2						3						4						5						6											
	A	B	C	D	E	F	A	B	C	D	E	F	A	B	C	D	E	F	A	B	C	D	E	F	A	B	C	D	E	F	A	B	C	D	E	F						
8-Oct-07	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3-Jan-08	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21-Jan-08	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18-Mar-08*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
30-Mar-08	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14-Apr-08	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
28-May-08†	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15-Jul-08	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
29-Jul-08	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15-Aug-08	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17-Sep-08	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

*No water available for sample collection at Lerderderg.

†Lerderderg sample collected from further downstream (north of Bacchus Marsh) as road closure prevented access to sampling location.

treatment are not sufficient to eliminate ARGs. Whether they come from viable microorganisms is another matter, but their presence alone certainly provides justification to further investigate the potential contribution by reclaimed water.

ARGs were detected only in the first half of the sampling period (October to April) when average daily maximum ambient temperature was ~9°C warmer than the second half (April–September) (Commonwealth Bureau of Meteorology – Laverton station #087031). It is possible that elevated water temperature may have resulted in increased microbial multiplication (Huq *et al.* 1984; Rhodes & Kator 1988) to levels that were detectable. At this time, it is also likely that water inflows to the sewage treatment plant were lower (due to a combination of higher evapotranspiration rates, reduced rainfall and community water restrictions), resulting in more concentrated effluent. Total phosphorus levels, which were elevated between January and May 2008 (0.04 to 0.11 mg/L), may also have contributed to increased microbial growth (Karl 2000).

ARGs are persistent in the environment, and many can be found in the water column for up to 14 days after release (Engemann *et al.* 2008). There have been mixed results as to the impact of waste water treatment on the persistence and proliferation of ARGs (Pei *et al.* 2007), with some studies showing that treatment processes increased the proportion of antibiotic-resistant bacteria (Murray *et al.* 1984), and others that found no significant difference between inflow and treated waste water (Da Costa *et al.* 2008), or in some cases even a decline (Baquero *et al.* 2008). One study found that UV light had no impact on detectable levels of ARGs (Auerbach *et al.* 2007). The WTP at Werribee treats ~55% of Melbourne's sewage and >70% of its industrial waste (Lawson 2003). The potential presence of ARGs in the industrial waste is not known, although it is known to contribute significantly to high salinity loads in the sewage inflows (Melbourne Water & City West Water 2004; Ibrahim *et al.* 2007).

Importance of ARGs detected

The antibiotic resistance genes were selected due to their previous detection in environmental or waste water samples in other studies. Of the five ARGs investigated,

two conferring resistance to methicillin and sulfonamide were detected in samples collected over a one-year period.

The emergence and global spread of methicillin resistance, specifically methicillin-resistant *Staphylococcus aureus* (MRSA), is a significant world-wide problem (Nimmo *et al.* 2006), first emerging in Australia in the 1960s (Monaghan 2008). Estimated MRSA-related hospitalizations in the USA more than doubled between 1999 and 2005 (Klein *et al.* 2007) while in Australia, community-associated MRSA isolates rose from 4.7% in 2000 to 7.3% in 2004 (Nimmo *et al.* 2006). The rise of MRSA infections is of particular concern as many strains of MRSA are multidrug-resistant (Decker 2008; Wu & Li 2008). Sulfonamides are generally used as therapeutic agents for humans and animals, rather than routine growth-promoters (Huovinen *et al.* 1995; Pei *et al.* 2006), and resistance is now widespread (Huovinen *et al.* 1995). In the UK, even after significant reductions in the prescription of sulfonamide for human treatment, the frequency of resistance remained quite high (Enne *et al.* 2007), although this may be influenced by the continued high use in agriculture. This study has made no attempt to attribute contamination to any specific source, but suggests that reclaimed water and heavily impacted waterways may represent a reservoir for antibiotic resistance to methicillin and sulfonamide.

Quantitative information would have been useful for further analysis of these results. Quantitative real-time PCR was attempted to determine the abundance of ARGs (data not shown); unfortunately, the sensitivity of the technique was hindered by impurities in the DNA templates derived from the environmental samples. Pei and colleagues (2006) experienced similar difficulties.

Implications for irrigation

The findings of this study are of particular relevance as ARGs were found in relatively clean Class A water. This is not unprecedented, as ARGs (Pruden *et al.* 2006; Bockelmann *et al.* 2009) and antibiotic-resistant *E. coli* (Watkinson *et al.* 2007) have previously been detected in treated waste water samples. However, the majority of water tested in the previous studies was not as comprehensively treated as the Class A water tested in this current investigation. Besides the five ARGs that were the focus of

this study, there are many others that are of importance and should be included in further monitoring and study of reclaimed water.

The use of reclaimed water is increasing rapidly, particularly in countries experiencing significant water stress such as Australia, where reclaimed water is now used in many areas for irrigation of food crops. This increasing reliance on reclaimed water carries some level of risk associated with contaminants of emerging concern such as ARGs. Given that ARGs can survive in an aquatic environment, there is a risk that ARGs may enter the food chain directly from irrigation water. There has been very limited investigation of food crops as a potential reservoir of ARGs. Sørum & L'Abée-Lund (2002) reported that ARGs spread between ecological niches, with identical genes being found in plant pathogens, as well as bacteria from human and animal sources. Rodríguez and colleagues found tetracycline ARGs in lettuce samples collected from farms in Costa Rica (Rodríguez *et al.* 2006), Kapperud and colleagues found antibiotic-resistant *Escherichia coli* on iceberg lettuce (Kapperud *et al.* 1995), while Bezanson and colleagues found antibiotic-resistant isolates on Romaine lettuce, Savoy spinach and alfalfa sprouts (Bezanson *et al.* 2008). Further research is required to evaluate the transfer of ARGs from irrigation water to plant surfaces and tissues and their persistence and prevalence in the environment. As the use of reclaimed water for agricultural irrigation increases, monitoring is needed to evaluate the risk of ARG dissemination into the food chain and the environment.

CONCLUSIONS

This study found a very low proportion of ARG detections in river water, while detections in reclaimed water were proportionally quite high. This indicates that the river, even in its very poor condition, hasn't yet been severely impacted by ARG-contaminated wastes such as agricultural run-off and human sewage (treated or otherwise). Reclaimed water will need to be monitored into the future, as its use in agricultural irrigation increases, to evaluate the risk of ARG dissemination into the food chain and the environment.

ACKNOWLEDGEMENTS

This research was supported by the State Government of Victoria's Our Rural Landscape (ORL) program. We are grateful to Andrew Watkinson (National Research Centre for Environmental Toxicology, University of Queensland) and Margaret Deighton (Biotechnology and Environmental Biology, School of Applied Sciences, RMIT University) for the provision of isolates, to Siegfried Engleitner (Department of Primary Industries Victoria – Werribee, Australia) for the collection of reclaimed water samples, and to Arati Agarwal (Department of Primary Industries Victoria – Knoxfield, Australia) for her assistance with laboratory analyses.

REFERENCES

- Agerso, Y. & Sandvang, D. 2005 Class 1 integrons and tetracycline resistance genes in *Alcaligenes*, *Arthrobacter*, and *Pseudomonas* spp. isolated from pigsties and manured soil. *Appl. Environ. Microbiol.* **71**(12), 7941–7947.
- Akinbowale, O. L., Peng, H. & Barton, M. D. 2006 Antimicrobial resistance in bacteria isolated from aquaculture sources in Australia. *J. Appl. Microbiol.* **100**(5), 1103–1115.
- Auerbach, E. A., Seyfried, E. E. & McMahon, K. D. 2007 Tetracycline resistance genes in activated sludge wastewater treatment plants. *Water Res.* **41**(5), 1143–1151.
- Baquero, F., Martinez, J. L. & Canton, R. 2008 Antibiotics and antibiotic resistance in water environments. *Curr. Opin. Biotechnol.* **19**(3), 260–265.
- Bezanson, G. S., MacInnis, R., Potter, G. & Hughes, T. 2008 Presence and potential for horizontal transfer of antibiotic resistance in oxidase-positive bacteria populating raw salad vegetables. *Int. J. Food Microbiol.* **127**(1–2), 37–42.
- Biyela, P. T., Lin, J. & Bezuidenhout, C. C. 2004 The role of aquatic ecosystems as reservoirs of antibiotic resistant bacteria and antibiotic resistance genes. *Water Sci. Technol.* **50**, 45–50.
- Bockelmann, U., Dorries, H. H., Ayuso-Gabella, M. N., De Marcay, M. S., Tandoi, V., Levantesi, C., Masciopinto, C., Van Houtte, E., Szewzyk, U., Wintgens, T. & Grohmann, E. 2009 Quantitative PCR monitoring of antibiotic resistance genes and bacterial pathogens in three European artificial groundwater recharge systems. *Appl. Environ. Microbiol.* **75**(1), 154–163.
- Boon, P. I. 1992 Antibiotic resistance of aquatic bacteria and its implications for limnological research. *Mar. Freshwater Res.* **43**(4), 847–861.
- Boon, P. I. & Cattanach, M. 1999 Antibiotic resistance of native and faecal bacteria isolated from rivers, reservoirs and sewage treatment facilities in Victoria, south-eastern Australia. *Lett. Appl. Microbiol.* **28**(3), 164–168.
- Bucknell, D. G., Gasser, R. B., Irving, A. & Whithear, K. 1997 Antimicrobial resistance in *Salmonella* and *Escherichia coli* isolated from horses. *Aust. Vet. J.* **75**(5), 355–356.
- Chee-Sanford, J. C., Aminov, R. I., Krapac, I. J., Garrigues-Jeanjean, N. & Mackie, R. I. 2001 Occurrence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. *Appl. Environ. Microbiol.* **67**(4), 1494–1502.
- Christiansen, K. J., Turnidge, J. D., Bell, J. M., George, N. M. & Pearson, J. C. 2007 Prevalence of antimicrobial resistance in *Enterococcus* isolates in Australia, 2005: report from the Australian Group on Antimicrobial Resistance. *Commun. Dis. Intell.* **31**(4), 392–397.
- Da Costa, P. M., Vaz-Pires, P. & Bernardo, F. 2008 Antimicrobial resistance in *Escherichia coli* isolated in wastewater and sludge from poultry slaughterhouse wastewater plants. *J. Environ. Health* **70**(7), 40–45.
- Decker, C. F. 2008 Pathogenesis of MRSA infections. *Disease-a-Month* **54**(12), 774–779.
- DEWHA 2007 *Landscape – carbon, nutrients, water and productivity – Werribee River*. Australian Natural Resources Atlas – Natural Resource Topics, Commonwealth of Australia, Department of the Environment, Water, Heritage and the Arts (DEWHA), Canberra, ACT. Retrieved 21.10.08, from <http://www.anra.gov.au/topics/land/carbon/vic/basin-werribee-river.html>
- DPI 2008 *A Guide to the Inland Angling Waters of Victoria – Werribee River Basin 31*. Department of Primary Industries (DPI), Victoria. Retrieved 20.10.08, from <http://www.dpi.vic.gov.au/angling/31-Werribee/Basin%20TEMPLATE%20ED21Jan-worked.htm>
- DSE 2005 *Index of Stream Condition: The Second Benchmark of Victorian River Condition*. The State of Victoria Department of Sustainability and Environment (DSE), Melbourne. Retrieved 08.10.08, from http://www.vicwaterdata.net/vicwa terdata/data_warehouse_content.aspx?option=5
- DSE 2006 *State Water Report 2005–2006*. The State of Victoria Department of Sustainability and Environment (DSE), Melbourne. From http://www.ourwater.vic.gov.au/_data/assets/pdf_file/0004/14746/State-Water-Report-2005-06-26-Werribee-Basin.pdf
- DSE 2008 *Victorian Water Resources Data Warehouse*. The State of Victoria Department of Sustainability and Environment (DSE), Melbourne. Retrieved 08.10.08, from <http://www.vicwa terdata.net/vicwaterdata/home.aspx>
- Engemann, C. A., Keen, P. L., Knapp, C. W., Hall, K. J. & Graham, D. W. 2008 Fate of tetracycline resistance genes in aquatic systems: migration from the water column to peripheral biofilms. *Environ. Sci. Technol.* **42**(14), 5131–5136.
- Engleitner, S., Barker-Reid, F., Wos, M., Faggian, R. & Boland, A. -M. 2008 Recycled water for irrigation of vegetables on a Sodosol in the Werribee Irrigation District, Victoria, *Proceedings of the Vth International Symposium on Irrigation of Horticultural Crops. Acta Horticulturae* **792**(June 2008), 273–281.

- Enne, V. I., Livermore, D. M., Stephens, P. & Hall, L. M. C. 2007 Persistence of sulfonamide resistance in *Escherichia coli* in the UK despite national prescribing restriction. *Lancet* **357**, 1325–1328.
- EPAV 2003 *Guidelines for Environmental Management: Use of Reclaimed Water*. Environmental Protection Agency Victoria, Southbank, Victoria. Retrieved 30.06.08 from <http://epa.note2.epa.vic.gov.au/EPA/Publications.nsf/PubDocsLU/464.2?OpenDocument>
- Heuer, H., Krogerrecklenfort, E., Wellington, E. M. H., Egan, S., Elsas, J. D., Overbeek, L., Collard, J. M., Guillaume, G., Karagouni, A. D., Nikolakopoulou, T. L. & Smalla, K. 2002 Gentamicin resistance genes in environmental bacteria: prevalence and transfer. *FEMS Microbiol. Ecol.* **42**(2), 289–302.
- Huovinen, P., Sundstrom, L., Swedberg, G. & Skold, O. 1995 Trimethoprim and sulfonamide resistance. *Antimicrob. Agents. Chemother.* **39**(2), 279–289.
- Huq, A., West, P. A., Small, E. B., Huq, M. I. & Colwell, R. R. 1984 Influence of water temperature, salinity, and pH on survival and growth of toxigenic *Vibrio cholerae* serovar O1 associated with live copepods in laboratory microcosms. *Appl. Environ. Microbiol.* **48**, 420–424.
- Ibrahim, T. R., Meehan, B., Carpenter, P. & Corby, N. 2007 TDS in sewage: sources and trends in Melbourne's west. *Water - J. Aust. Water Assoc.* **34**(5), 44–47.
- JETACAR 1999 *The Use of Antibiotics in Food-producing Animals: Antibiotic-resistant Bacteria in Animals and Humans*. Commonwealth Department of Health and Aged Care, Canberra. from <http://www.health.gov.au/internet/main/publishing.nsf/Content/health-pubhlth-publicat-document-jetacar-cnt.htm>
- Jordan, D., Morris, S. G., Gill, P., Andersen, L. M., Chowdhury, A., Stevenson, A. E. & Spence, S. A. 2005 Mass screening for antimicrobial resistant *Escherichia coli* in dairy cows in northern New South Wales. *Aust. Vet. J.* **83**(11), 688–694.
- Kapperud, G., Rorvik, L. M., Hasseltvedt, V., Hoiby, E. A., Iversen, B. G., Staveland, K., Johnsen, G., Leitao, J., Herikstad, H. & Andersson, Y. 1995 Outbreak of *Shigella sonnei* infection traced to imported iceberg lettuce. *J. Clin. Microbiol.* **33**(3), 609–614.
- Karl, D. M. 2000 Phosphorus, the staff of life. *Nature* **406**, 31–33.
- Klein, E., Smith, D. L. & Laxminarayan, R. 2007 Hospitalizations and deaths caused by methicillin-resistant *Staphylococcus aureus*, United States, 1999–2005. *Emerg. Infect. Dis.* **13**(12), 1840–1846.
- Klein, G., Pack, A. & Reuter, G. 1998 Antibiotic resistance patterns of *Enterococci* and occurrence of vancomycin-resistant *Enterococci* in raw minced beef and pork in Germany. *Appl. Environ. Microbiol.* **64**(5), 1825–1830.
- Koike, S., Krapac, I. G., Oliver, H. D., Yannarell, A. C., Chee-Sanford, J. C., Aminov, R. I. & Mackie, R. I. 2007 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.* **73**(15), 4813–4823.
- Lawson, J. 2003 *Managing Melbourne's Water Resources: Water Recycling to Protect the Environment and Support Melbourne's Growth*. A submission outlining strategies to implement Melbourne 2030 – Planning for Sustainable Growth. Lawson Consultancy Pty Ltd, South Yarra, Victoria. from <http://cleanocean.org/fifthestate/archives/110.030/316/2030submission.pdf>
- Marchesi, J. R., Sato, T., Weightman, A. J., Martin, T. A., Fry, J. C., Hiom, S. J. & Wade, W. G. 1998 Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Appl. Environ. Microbiol.* **64**(2), 795–799.
- Martinez, J. L. 2008 Antibiotics and antibiotic resistance genes in natural environments. *Science* **321**(5887), 365–367.
- Melbourne Water 2007 *Werribee Catchment*. Port Phillip and Westernport Regional River Health Strategy, State of Victoria, Melbourne. from http://www.melbournewater.com.au/content/publications/reports/rivers_and_creeks_reports.asp?bhcp=1
- Melbourne Water & City West Water 2004 *Western Treatment Plant Salinity Management Plan: A Plan to Manage Salinity in Recycled Water from Western Treatment Plant to Facilitate Sustainable Water Recycling*. From www.citywestwater.com.au/business/docs/Western_Treatment_Plant_Salinity_Management_Plan_-_FINAL_Dec_2004.doc
- Monaghan, W. M. 2008 The evolution of antibiotic resistance in *Staphylococcus aureus* in Australia. *Aust. J. Med. Sci.* **29**(4), 131–146.
- Murakami, K., Minamide, W., Wada, K., Nakamura, E., Teraoka, H. & Watanabe, S. 1991 Identification of methicillin-resistant strains of staphylococci by polymerase chain reaction. *J. Clin. Microbiol.* **29**(10), 2240–2244.
- Murray, G. E., Robin, R. S., Junkins, B. & Kushner, D. J. 1984 Effect of chlorination on antibiotic resistance profiles of sewage-related bacteria. *Appl. Environ. Microbiol.* **48**(1), 75–77.
- Nimmo, G. R., Coombs, G. W., Pearson, J. C., O'Brien, F. G., Christiansen, K. J., Turmidge, J. D., Gosbell, I. B., Collignon, P., McLaws, M. L., Faoagali, J., George, N., Harper, J., Schooneveldt, J., Bradbury, S., Tiley, S., Gottlieb, T., Funnell, G., Fernandes, C., Benn, R., Yan, B., Ziochos, H., Vickery, A., Mitchell, D., Ryder, S., Branley, J., Spelman, D., Franklin, C., Garland, S., Gonis, G., Waters, M. J., Joyce, L., Ward, P., Andrew, J., McGregor, A., Peterson, R., Bell, J., Lim, I., Pratt, R., Pruell, H., Mulgrave, L., McGeachie, D., Francis, G., Lurn, G., Paul, M., Robson, J., Lee, P. C. & Benson, S. 2006 Methicillin-resistant *Staphylococcus aureus* in the Australian community: An evolving epidemic. *Med. J. Aust.* **184**(8), 384–388.
- Patel, R., Uhl, J. R., Kohner, P., Hopkins, M. K. & Cockerill, F. R., III 1997 Multiplex PCR detection of *vanA*, *vanB*, *vanC-1*, and *vanC-2/3* genes in Enterococci. *J. Clin. Microbiol.* **35**(5), 703–707.
- Pei, R., Cha, J., Carlson, K. H. & Pruden, A. 2007 Response of antibiotic resistance genes (ARG) to biological treatment in dairy lagoon water. *Environ. Sci. Technol.* **41**(14), 5108–5113.

- Pei, R., Kim, S. -C., Carlson, K. H. & Pruden, A. 2006 Effect of river landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). *Water Res.* **40**(12), 2427–2435.
- Pruden, A., Pei, R., Storteboom, H. & Carlson, K. H. 2006 Antibiotic resistance genes as emerging contaminants: studies in Northern Colorado. *Environ. Sci. Technol.* **40**, 7445–7450.
- Rhodes, M. W. & Kator, H. 1988 Survival of *Escherichia coli* and *Salmonella* spp. in estuarine environments. *Appl. Environ. Microbiol.* **54**(12), 2902–2907.
- Rodríguez, C., Lang, L., Wang, A., Altendorf, K., García, F. & Lipski, A. 2006 Lettuce for human consumption collected in Costa Rica contains complex communities of culturable oxytetracycline- and gentamicin-resistant bacteria. *Appl. Environ. Microbiol.* **72**(9), 5870–5876.
- Rysz, M. & Alvarez, P. J. J. 2004 Amplification and attenuation of tetracycline resistance in soil bacteria: aquifer column experiments. *Water Res.* **38**(17), 3705–3712.
- 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.
- Snow, D. D., Bartelt-Hunt, S. L., Saunders, S. E. & Cassada, D. A. 2007 Detection, occurrence, and fate of emerging contaminants in agricultural environments. *Water Environ. Res.* **79**(10), 1061–1084.
- Sorum, H. & L’Abee-Lund, T. M. 2002 Antibiotic resistance in food-related bacteria - a result of interfering with the global web of bacterial genetics. *Int. J. Food Microbiol.* **78**(1–2), 43–56.
- Volkman, H., Schwartz, T., Bischoff, P., Kirchen, S. & Obst, U. 2004 Detection of clinically relevant antibiotic-resistance genes in municipal wastewater using real-time PCR (TaqMan). *J. Microbiol. Methods* **56**, 277–286.
- Watkinson, A. J., Micalizzi, G. B., Graham, G. M., Bates, J. B. & Costanzo, S. D. 2007 Antibiotic-resistant *Escherichia coli* in wastewaters, surface waters, and oysters from an urban riverine system. *Appl. Environ. Microbiol.* **73**(17), 5667–5670.
- Witte, W. 2000 Ecological impact of antibiotic use in animals on different complex microflora: environment. *Int. J. Antimicrob. Agents* **14**, 321–325.
- Wu, A. H. & Li, C. H. 2008 Antimicrobial resistance of methicillin-resistant *Staphylococcus aureus* in hospital environment and inpatients. *Acta Academiae Med. Sin.* **30**(5), 525–530.
- Zhang, X. X., Zhang, T. & Fang, H. H. P. 2009 Antibiotic resistance genes in water environment. *Appl. Microbiol. Biotechnol.* **82**(3), 397–414.

First received 15 July 2009; accepted in revised form 19 November 2009. Available online 9 March 2010