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Title page

Impacts of reclaimed water irrigation on soil antibiotic resistome in urban parks of Victoria, Australia

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Running title: Impact of reclaimed water irrigation on soil resistome

Abstract

The effluents from wastewater treatment plants have been recognized as a significant environmental reservoir of antibiotics and antibiotic resistance genes (ARGs). Reclaimed water irrigation (RWI) is increasingly used as a practical solution for combating water scarcity in arid and semiarid regions, however, impacts of RWI on the patterns of ARGs and the soil bacterial community remain unclear. Here, we used high-throughput quantitative PCR and terminal restriction fragment length polymorphism techniques to compare the diversity, abundance and composition of a broad-spectrum of ARGs and total bacteria in 12 urban parks with and without RWI in Victoria, Australia. A total of 40 unique ARGs were detected across all park soils, with genes conferring resistance to β-lactam being the most prevalent ARG type. The total numbers and the fold changes of the detected ARGs were significantly increased by RWI, and marked shifts in ARG patterns were also observed in urban parks with RWI compared to those without RWI. The changes in ARG patterns were paralleled by a significant effect of RWI on the bacterial community structure and a co-occurrence pattern of the detected ARG types. There were significant and positive correlations between the fold changes of the integrase *intI1* gene and two β-lactam resistance genes (KPC and IMP-2 groups), but no significant impacts of RWI on the abundances of *intI1* and the transposase *tnpA* gene were found , indicating that RWI did not improve the potential for horizontal gene transfer of soil ARGs. Taken together, our findings suggested that irrigation of urban parks with reclaimed water could influence the abundance, diversity, and compositions of a wide variety of soil ARGs of clinical relevance.

One-sentence summary: Irrigation of urban parks with treated wastewater significantly increased the abundance and diversity of various antibiotic resistance genes, but did not significantly enhance their potential for horizontal gene transfer.

Keywords

Antibiotic resistance gene; reclaimed water; class 1 integron; β-lactamase; soil resistome

Introduction

The discovery of antibiotics and their extensive clinical use have made great contributions to treating infectious diseases, promoting livestock's growth, and protecting human and animal health (Hu et al., 2010; Nesme and Simonet, 2015). However, antibiotics are poorly absorbed by the body of humans and animals, and most of these antibiotic compounds and their metabolites are excreted and finally released into soils and municipal wastewater (Michael et al., 2013), which may exert selective pressure on resident microbial community and contribute to development of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) within the environment. The increasing emergence and propagation of ARGs are threatening the achievement of modern medicine and posing major risks to human health and ecological security in the 21st century (Udikovic-Kolic et al., 2014; Berendonk et al., 2015). In recent years, the magnitude of ARGs has been reported to reach alarming levels in many parts of the world (WHO, 2014), which attracted increasing worldwide concerns (Levy and Marshall, 2004). Therefore, ARGs have been recognized as a new type of emerging environmental contaminant (Pruden et al., 2006).

In contrast to chemical contaminants, bacterial ARGs might be more persistent in the environment, as ARGs can be not only multiplied in their hosts but also transferred to other microbial populations including human and animal commensals and pathogens through horizontal gene transfer (HGT) mechanisms via mobile genetic elements (MGEs), such as integrons, transposons, and plasmids (Gogarten and Townsend, 2005; Yu et al., 2012). It is well known that integron and transposon are responsible for the acquisition of ARGs and have frequently been found in antibiotic-resistant strains (Carattoli et al., 2001a; Scott, 2002).

Integrons possess a site specific recombination system which could capture and express mobile gene cassettes (Heuer *et al.* 2011a), and they are reported to often localize in broad-host range IncP-1ε plasmids with a wide distribution in the environment which further facilitates their mobility potential (Heuer *et al.* 2012; Wolters *et al.* 2015). Similarly, transposons include transposase genes such as *tnpA*, *tnpR* and *tnpM* and sites required for transposition (Carattoli, 2001b), and carry accessory genes conferring resistance to several classes of antibiotics, and therefore they can horizontally transfer ARGs with them (Reid et al., 2015). Resistance determinants can be horizontally transferred under broad host range through integron and transposon being carried by or incorporated into conjugative plasmids (Carattoli, 2001b; Butaye et al., 2003). Horizontal transmission of ARGs via integron, transposon, and plasmid facilitates the dissemination of ARGs in environment and may raise the risk of public health.

Soil is the original habitat for most currently-known antibiotics, and soil microbes might have developed resistance even before the production of modern antibiotics in the 1940s (Wright, 2000; Davelos et al., 2004). Therefore, soil may be a reservoir for novel ARGs that can horizontally transfer to human and animal commensals and pathogens (Dantas and Sommer, 2014). Due to the intensive anthropogenic activities such as aquaculture, land application of manure and biosolids, and large inputs of ARGs from the reuse of reclaimed water (LaPaRa et al., 2011; Cytryn, 2013; Zhu et al., 2013; Wang et al., 2014a), soil has been recognized as the largest environmental reservoir of antibiotic resistance (Nesme et al., 2014). Reclaimed water irrigation (RWI) is a practical solution for overcoming water resource shortage, and has been widely utilized in arid and semi-arid regions of the world (Fahrenfeld et al., 2013; Berendonk et al., 2015). Given the increasingly exacerbated water scarcity owing to urbanization, population growth and less available freshwater, it is anticipated that RWI will likely be more widely applied in the future (Wang et al., 2014a). However, a large

89 amount of antibiotics, ARB and ARGs can still persist in the reclaimed water after traditional
90 wastewater treatments which are mainly designed to remove organic matter, inorganic
91 nitrogen and phosphorous, and suspended solids, but not for the removal of antibiotics and
92 ARGs (Berendonk et al., 2015; Rodriguez-Mozaz et al., 2015; Xu et al., 2015). The presence
93 of abundant and diverse ARGs of clinical relevance was frequently reported in the effluents of
94 wastewater treatment plants (WWTPs), even after rigorous tertiary disinfection and mixed-
95 media filtration (LaPara et al., 2011; Gatica and Cytryn, 2013). For example, genes conferring
96 resistance to ampicillin, vancomycin, and methicillin were found in wastewater samples
97 collected from five municipal WWPTs in Germany (Volkmann et al., 2004). Wang et al.
98 (2015) detected the concentrations of 10 subtypes of ARGs and antibiotics in five
99 pharmaceutical WWTPs in Northern China, and found that the levels of typical ARGs ranged
100 from 2.86×10^3 to 3.68×10^6 copies ml^{-1} and antibiotic residues still remain in the final
101 WWTP effluent. Likewise, Gao et al. (2012) detected high abundances of the *tetO*, *tetW* and
102 *sulI* genes, as well as residues of tetracycline and sulfonamide in the final effluent from a
103 WWTP in Michigan, USA. The continuous release of ARB, ARGs and antibiotic residues
104 from effluents of WWTPs could cause the dissemination of ARGs in environments receiving
105 these effluents (Czekalski et al., 2014; Wang et al., 2014b; Rodriguez-Mozaz et al., 2015),
106 which has become a global concern (Berendonk et al., 2015).
107 Although reclaimed water has been recognized as an important reservoir of ARB and
108 ARGs (Fahrenfeld et al., 2013), only a limited number of studies have assessed the fate of
109 reclaimed water-derived ARGs in downstream environments(Negreanu et al., 2011;
110 Fahrenfeld et al., 2013), and people are not aware of the potential health risks they are facing.
111 To date, impacts of treated wastewater on antibiotic resistance have been reported in rivers
112 (LaPara et al., 2011), agricultural soils (McLain and Williams, 2012; Negreanu et al., 2012;
113 Fahrenfeld et al., 2013; Chen et al., 2014) and sediments (Czekalski et al., 2014), but only a

few studies focused on the occurrence and prevalence of ARGs in urban park soils irrigated by reclaimed water (Wang et al., 2014a, 2014b). Public urban parks play a vital role in the social life of human beings, and provide a potentially important pathway for the spread of ARGs from soil to human pathogens. Therefore, this study was designed to investigate the impacts of RWI on the patterns of ARGs and the soil bacterial community in 12 public urban parks in Victoria, Australia. Pristine soil samples from two remote national parks without any known exposure to antibiotics and with minimal human-induced selective pressure were collected as control. We tested the following hypotheses: (i) the occurrence and prevalence of ARGs might be strongly affected by RWI, owing to the possible selective pressure of antibiotics and ARGs in treated wastewater; (2) RWI would influence the mobility potential of ARGs in urban parks, as measured by the abundances of the *intI1* and *tnpA* genes, and (iii) RWI might also result in significant changes of the soil bacterial community, which has been suggested as a critical determinant of soil ARGs (Forsberg et al., 2014).

Materials and methods

Sampling sites and soil collection

Soil samples were collected from 12 public urban parks and two remote national parks in Victoria, Australia in January 2015. Of these parks, six urban parks were irrigated with reclaimed water including Werribee Park (WP), Werribee Rose Garden (WRG), Yarra Park (YP), HD Graham Reserve (HDGR), Altona Green Park (AGP) and Werribee Campus of the University of Melbourne (WCUM); six urban parks were irrigated with potable water including Royal Botanic Gardens (RBG), Carlton Garden (CG), Fitzroy Gardens (FZG), Princes Park (PP), Yarra Bend Park (YBP) and Flagstaff Garden (FSG); and the control soil samples were taken from pristine forests in two remote national parks far away from the Melbourne city: Lake Eildon National Park (LENP) and Yarra Ranges National Park (YRNP). The two national parks have no known history of antibiotics exposure and have minimal

human-induced selective pressure. The detailed information about the reclaimed water and potable water irrigation in the 12 urban parks is shown in Table S1. In each park, three replicate soil samples (5 cm in diameter) from the upper 10 cm were collected at a distance of 20 m from each other, and each sample was thoroughly homogenized by mixing five subsamples taken within an area of 50 m^2. All samples were transported on ice to the laboratory, and then gently crumbled to pass through a 2-mm sieve and homogenized thoroughly. Soil samples were stored at 4°C prior to measurement of soil basic properties, and stored at -20°C before DNA extraction within two weeks after collection.

Physicochemical analysis and DNA extraction

Soil moisture content (H_2O%) was determined according to the weight loss at 105°C for 24 h. Soil pH was measured using a ratio of 1:2.5 (fresh soil to water) with an Orion Star A211 pH-meter (Thermo Scientific Inc., Melbourne, Australia). Total carbon (TC) and total nitrogen (TN) were determined using the Dumas method of combustion by an isotope-ratio mass spectrometry (Sercon Hydra, Crewe, UK). The soil types in the urban parks were generally classified into loamy sand, and the basic soil properties are shown in Table S2.

Total genomic DNA was extracted from 0.25 g of soil sub-samples from three replicate soil samples for each park with the MoBio PowerSoil DNA isolation kit (MoBio Labroratories, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration and quality of the extracted DNA were measured using a NanoDrop ND2000c spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The A260/A280 ratios of all the extracted DNAs were higher than 1.8.

High-throughput profiling of ARGs by quantitative PCR (qPCR) arrays

The occurrence and prevalence of ARGs were analyzed using the Antibiotic Resistance Genes Microbial DNA qPCR arrays (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The current version of qPCR array can simultaneously target a

broad spectrum of 84 ARGs encoding resistance to all major classes of antibiotics (Table S3), including aminoglycosides, Classes A, B, C, and D β-lactam, erythromycin, quinolones and fluoroquinolones, marcrolide lincosamide streptogramin_b (MLS_b), multidrug, tetracycline, and vancomycin.

All the qPCR analyses of ARGs were carried out on a Bio-Rad CFX96^{TM} Real-Time system (Bio-Rad Laboratories, Hercules, CA, USA). The 25 µl qPCR reaction mixture contained 5~10 ng of template DNA, 12.5 µl of HotStart DNA Polymerase Mastermix (Qiagen), l µl of 20 µM bovine serum albumin, and Microbial DNA-free water (Qiagen). The 96-well qPCR array plate contained one pair of pre-dispensed, gene-specific primers and one fluorescent hydrolysis probe in each well. Two Pan-bacteria assays were used as positive controls for the presence of bacterial species by targeting the evolutionarily conserved regions of the 16S rRNA gene, and one positive PCR control assay was also included to test the presence of inhibitors and the efficiency of the qPCR. The amplification conditions were as following: 10 min at 95°C for the initial PCR activation step, followed by 40 cycles of 15 s at 95°C as the denaturation step and 60°C for 2 min as the annealing/extension step in which the FAM fluorescence was detected. The baseline and threshold values were manually set to the same level for all qPCR runs *as per* manufacturer's recommendation, and a threshold cycle (C_T) value of 37 was used as the detection limit. The fold change values of ARGs in urban parks were calculated using the Template Excel Software (Qiagen) with the $\Delta\Delta C_T$ method of relative profiling (Zhu et al., 2013) as compared to the ARGs profiles in the two national parks.

Quantitative PCR analysis of the intI1, tnpA, and bacterial 16S rRNA genes

The *intI1* gene encoding the integrase of class 1 integrons, the *tnpA* gene of the IS6 family transposons, and the bacterial 16S rRNA gene were amplified on a Bio-Rad CFX96^{TM} Real-Time system (Bio-Rad). The 20 µl PCR reaction mixture for the *IntI1* and *tnpA* genes

consisted of 1 μl of five-fold diluted template DNA, 0.5 μl of each primer (10 μM), 10 μl of SYBR Premix Ex Taq™ (TaKaRa Biotechnology, Otsu, Shiga, Japan) and nuclease-free water. The total bacterial 16S rRNA gene was quantified using the BACT1369F/PROK1492R with the probe TM1389F and Premix Ex Taq™ (TaKaRa). The primer sets and thermo-cycling conditions used in the qPCR assays are listed in Table 1.

Standard curves for qPCR assays were constructed as follows: the PCR products of the *intI1*, *tnpA* and bacterial 16S rRNA genes were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), and ligated into the pGEM-T Easy Vector system (Promega). The resultant ligation products were transformed into JM109 competent cells (Promega) *as per* the manufacturer's instructions. Plasmids of the positive clones from each target gene were selected for sequencing, and the obtained sequences were blasted against the NCBI database to confirm their identities. Standard curves for the target genes were generated from 10-fold serial dilutions of the plasmids containing correct inserts of the target genes ranging from 10^2 to 10^9 copies μl^{-1}. Melting curve analysis was performed at the end of each qPCR run to check the specificity of amplicon products, before confirmation by standard agarose gel electrophoresis. The amplification efficiency of all qPCR runs ranged from 86 to 103%. The fold changes of the target genes were also calculated using the $\Delta\Delta C_T$ method (Zhu et al., 2013), and the relative abundance of the *intI1* and *tnpA* genes was calculated by normalizing to the bacterial 16S rRNA gene abundance.

Community profiling of the bacterial 16S rRNA gene by terminal restriction fragment length polymorphism (T-RFLP)

The community structure of total bacteria was analyzed by the T-RFLP analysis of the bacterial 16S rRNA genes. Briefly, the bacterial 16S rRNA genes were amplified with the primer pairs 27F/1492R labeled with 6-carboxyfluorescein (FAM) (Weisburg et al., 1991), and the resultant PCR products were purified with the Wizard SV Gel and PCR Clean-Up

System (Promega) and digested with restriction enzyme HhaI (BioLabs, Sydney, NSW, Australia) *as per* manufactures' instructions. Terminal restriction fragments (TRFs) were resolved using an ABI PRISM 3500 Genetic analyzer (Applied Biosystems, CA, USA). T-RFLP profiles were analyzed using a local southern size calling method (peaks > 50 bp in size) and a peak amplitude threshold setting of 50, using Genemapper version 4.0 (Applied Biosystems). TRFs with peak height comprising less than 2% of the total peak height were removed from downstream analysis, and peaks that differed by less than 1 bp were binned into the same TRF (Singh and Thomas, 2006). The relative fluorescence abundances of all TRFs were exported for further analysis.

Network analysis and visualization

The co-occurrence patterns between the detected ARGs in the high-throughput qPCR array were explored using network analysis with the CoNet Cytoscape plug-in (Soffer et al., 2014). Briefly, Pearson correlation, Spearman correlation, mutual information, Bray-Curtis dissimilarity, and Kullback-Leibler dissimilarity were calculated for all the pairwise interactions, and the ARGs with a minimum occurrence of less than three were discarded to exclude the potential spurious correlations. The ReBoot procedure was conducted with 100 permutations to control the potential false-positive correlations, and the resulting distribution was refined with 1000 bootstraps. The co-occurrence networks were constructed based on the resultant pairwise correlations between the ARGs. Network visualization was performed on the open-source interactive platform of Gephi (Bastian et al., 2009). Only associations with a correlation coefficient (ρ) above 0.8 and a significance level (P) below 0.05 were displayed (Junker and Schreiber, 2008).

Statistical analysis

One-way analysis of variance (ANOVA) based on the Fisher LSD test was conducted to analyze the differences in the numbers and fold changes of ARGs across the park soils by

using SPSS 13.0 (IBM, USA). ARGs were considered to be statistically enriched if the range created by two standard deviations of the mean fold change values was entirely >1 (Zhu et al., 2013). Spearman's correlation analysis was performed to test the relationships among the fold changes of total ARGs, the relative abundance of the *intI1* and *tnpA* genes, and soil parameters using SPSS 13.0 and $P < 0.05$ was considered to be statistically significant. Non-metric multidimensional scaling (NMDS) plots were used to visualize the Bray–Curtis dissimilarity matrices based on T-RFLP data of the bacterial 16S rRNA gene. A permutational multivariate analysis of variance (PERMANOVA) test with 999 permutations was performed to examine whether the bacterial community structures were statistically different between park soils with and without RWI, by using the *adonis* function of the vegan package in R platform. The heat maps of qPCR array results of ARGs were generated from log-transformed fold changes of ARGs using the *gplots* package in R.

Results

Diversity and enrichment of ARGs in urban park soils with and without RWI

Among all the investigated park soils, a total of 40 unique ARGs were detected, and the number of the detected ARGs was no more than 15 for individual parks (Fig. 1a). The numbers of the detected ARGs were significantly higher in urban parks with RWI than those without RWI including two remote national parks ($P < 0.01$), whereas there was no significant difference in the numbers of the detected ARGs between the two national parks and the urban parks without RWI (Fig. 1a). The sum of the enrichment of all unique ARGs in one sample was used to approximate the total enrichment (Zhu et al., 2013). It was found that ARGs tended to be enriched in urban parks irrespective of RWI, compared with the control remote national parks (Fig. 1b). The parks with RWI had a significantly higher level of ARG enrichment than those without RWI ($P < 0.05$). The enrichment levels of ARGs ranged from

815- to 4300-fold in parks with RWI and from 150- to 1240-fold in parks without RWI, compared with the remote national parks (Fig. 1b).

The genes conferring resistance to all major classes of antibiotics except for erythromycin and vancomycin were detected in the urban park soils with and without RWI (Fig. 2). The two national parks also harbored a diverse set of ARGs conferring resistance to Aminoglycosides, β-lactam, quinolones and fluoroquinolones, MLS_b, and multidrug (Figure S1). The composition profiles of different types of the detected ARGs were similar between the parks with and without RWI (Figs. 2a and 2c). The genes conferring resistance to Classes A, B, C, and D β-lactam were the most frequently detected ARGs, comprising a large proportion of more than 50% of the total number of the detected ARGs in urban parks with and without RWI. In particular, the most prevalent β-lactam resistance genes contained SHV(238S240K), SHV(156G) and SHV for Class A; IMP-2 group and IMP-5 group for Class B; ACT-1 group, MIR, and ACT 5/7 group for Class C; and OXA-51 Group, OXA-60 and OXA-50 Group for Class D. Other frequently detected ARGs encompassed resistance genes for MLS_b (19.07% and 16.34% in parks with and without RWI, respectively) and quinolones and fluoroquinolones (10.70% and 11.76% in parks with and without RWI, respectively). In terms of the enrichment levels of different types of ARGs, there were some differences in the composition profiles in urban parks with and without RWI (Figs. 2b and 2d). The genes conferring resistance to Class C β-lactam (25.92%), MLS_b (23.85%) and Class B β-lactam (18.70%) were the most enriched ARGs in parks with RWI (Fig. 2b), whereas the genes resistant to Class C β-lactam (35.66%), tetracycline (29.05%) and MLS_b (12.43%) constituted the main types of enriched ARGs in parks without RWI (Fig. 2d).

The distribution patterns of ARGs in urban park soils with and without RWI

The log-transformed fold changes of the 84 target ARGs across parks with and without RWI are shown in a heat map (Fig. 3). Overall, the ARG profiles in parks without RWI

tended to cluster together, while the ARGs profiles of parks with RWI formed different clusters with the exception of HDGR. The specific types of ARGs were selectively enriched in parks with RWI: for example, ARGs conferring resistance to MLS_b (e.g. *ermC* and *mefA* with an enrichment of 1108.5- and 143.4-fold, respectively) were found to be abundant in WRG. The *IMP-12* group encoding resistance to Class B β-lactam was enriched up to 575.8-fold in WP. The MLS_b resistance gene *ermC* and Class B β-lactam resistance gene *MIR* were the two most abundant genes in AGP enriched by 140.1- and 150.5-fold, respectively. The genes *ermC* resistant to MLS_b and the *ACT-1* group resistant to class C β-lactam in WCUM were found to be enriched by 115.6- and 237.0-fold, respectively. The two genes conferring resistance to Class B β-lactam, including the *ACT-1* group and *MOX* in YP, were enriched by 238.3- and 111.0-fold, respectively. However, no obvious enrichment of ARGs was found in HDGR compared with other parks with RWI. For the parks without RWI, no striking enrichment (fold changes > 100) was found in any types of ARGs.

Changes in the intI1 and tnpA genes in urban park soils with and without RWI

To estimate the impact of RWI on the mobility potential of ARGs, the class 1 integrase *intI1* gene and the IS6 family transposase *tnpA* gene were quantified from all park soils. The bacterial 16S rRNA gene was also quantified to calculate the fold changes of the relative abundance of the *intI1* and *tnpA* genes in urban parks compared to the remote national parks. There was no significant enrichment of the *intI1* and *tnpA* genes in urban parks with or without RWI, which was illustrated by the fold changes from -2.72 to 1.94 and from 0.86 to 7.28 for the *intI1* and *tnpA* genes, respectively (Fig. 4). In addition, no significant difference in the fold changes of these two genes was observed between urban parks with and without RWI.

Spearman's correlations between the fold changes of ARGs and the relative abundance of the *intI1* and *tnpA* genes were further conducted to assess the mobility potential of ARGs in

urban park soils (Fig. 5). Among the 40 detected ARGs, only the β-lactam resistance genes *KPC* (R = -0.453, P < 0.01) and the *IMP-2* group (R = -0.381, P < 0.05) exhibited significantly positive correlations with the *intI1* gene (Fig. 5a). The *tnpA* gene was also found to be significantly and positively correlated with the *intI1* gene (R = -0.488, P < 0.01), but no significant relationship was found between the *tnpA* gene and any of the detected ARGs. To understand the impact of soil properties on the enrichment patterns of ARGs and MGEs, spearman's correlations were performed between the fold changes of total ARGs, the *intI1* and *tnpA* genes and soil properties. Soil pH had a significantly positive correlation with the enrichment of total ARGs (R = 0.427, P < 0.01), which was significantly and negatively related to TN (R = -0.475, P < 0.01) (Fig. 5b). No obvious relationship was found between the examined soil physicochemical parameters and the relative abundance of the *intI1* and *tnpA* genes.

Co-occurrence patterns among the detected ARGs

The co-occurrence patterns among the detected ARGs were tested using the network analysis based on strong ($\rho > 0.8$) and significant ($P < 0.05$) correlations. The resulting network was composed of 20 nodes (unique ARGs) and 19 edges (Fig. 6). The high modularity index of 0.694 demonstrated the presence of a modular structure in the network (Newman, 2006), which could be separated into three modules (Fig. 6). The ARGs clustered into one module had more frequent interactions among themselves compared with those in other modules (Li et al., 2015). The most densely connected nodes (i.e. hubs), conferred resistance to the major types of antibiotics including Classes C and D β-lactam, tetracycline, and quinolones and fluoroquinolones. Each module contained various types of ARGs, and no single module harbored only one type of ARGs. For instance, the module with the hub QnrB-4 group resistant to quinolones and fluoroquinolones consisted of the co-occurring genes OXA-18 and *mecA* with Class D β-lactam resistance, the IMP-12 group with Class B β-lactam

resistance, the *aphA6* gene with aminoglycosides resistance, and the *oprm* gene with multidrug resistance, indicating that these genes might be carried on some specific microbial populations or some specific MGEs (even in various microbial groups).

Effects of RWI on the total soil bacterial communities

The changes in the patterns of ARGs suggested that the soil bacterial communities carrying these ARGs might be also concomitantly changed, which prompted us to explore the impacts of RWI on the soil bacterial community structure. The T-RFLP analysis of the bacterial 16S rRNA gene yielded 21 TRFs from restriction digestion of *HhaI*. The TRFs of 56-, 77-, 88-, 91- and 342-bp were the most dominant phylotypes across all the park soil samples. The NMDS analysis illustrated that the bacterial communities in the parks with RWI were obviously separated from the parks without RWI (PerMANOVA, $P < 0.001$), and there was no significant difference in the bacterial community structure between the remote national parks and the urban parks without RWI (Fig. 7).

Discussion

Impacts of RWI on the patterns of ARGs in urban park soils

Soil is assumed to be the largest environmental reservoir comprising as much as 30% of the currently-known ARGs in public repositories (Nesme et al., 2014), and is likely being enriched by anthropogenic activities (Dantas and Sommer, 2014). In this study, about a half of the target 84 ARGs conferring resistance to almost all the major classes of antibiotics were detected among the examined parks, supporting the argument that soil is a major component of antibiotic resistome (D'Costa et al., 2006). Our findings are in line with a previous high-throughput qPCR survey of ARGs in urban parks with RWI in China (Wang et al., 2014a), which detected 147 ARGs from the 295 target ARGs among all of the park soils. However, the average numbers and enrichment of ARGs in a single park detected by Wang et al., (2014a) were remarkably higher than that in this study. Up to 8655.3-fold of enrichment was

363 detected in several RWI parks by Wang et al. (2014a), by contrast, no higher than 4300-fold
364 enrichment was found in parks with RWI in this study. It might be attributed to the different
365 origins of treated wastewater effluent, soil conditions and anthropogenic disturbance between
366 the two studies. In terms of the numbers and enrichment levels of unique ARGs, the genes
367 conferring resistance to various classes of β-lactam were the most prevalent ARG types,
368 which was not surprising because diverse β-lactamases have been widely detected in soils
369 influenced by anthropogenic activity and in pristine undisturbed soils (Allen et al., 2009;
370 Wang et al., 2014a).
371 Anthropogenic activities such as RWI and other agricultural practices may contribute to
372 expansion of soil antibiotic resistance (Munir and Xagoraraki, 2011; Cytryn, 2013), and
373 potentially threaten human health through transfer of ARGs and ARB into human-associated
374 pathogens (Forsberg et al., 2012) and food chain (Marti et al., 2013; Wang et al., 2015). In
375 this study, the numbers and fold changes of the detected ARGs were found to be significantly
376 greater in urban parks with RWI than those without RWI, suggesting that RWI could enhance
377 the diversity and enrichment of soil ARGs. In consistence with our findings, a recent study
378 investigating the impact of RWI on soil resistome by high throughput qPCR technique
379 demonstrated that RWI was an important source of ARGs in urban park soils (Wang et al.,
380 2014a). However, conflicted results are also available: for example, Negreanu et al. (2012)
381 analyzed the abundance of ARB and ARGs using culture-dependent and qPCR methods and
382 found that the levels of resistant isolates and ARGs were similar or even lower in soils
383 irrigated by treated wastewater relative to soils irrigated by freshwater. Chen et al. (2014)
384 used similar methods to quantify the abundance of ARB and ARGs in soils of North China
385 and found that despite the significantly higher relative abundance of ARGs in wastewater-
386 irrigated soils, the relative abundance of ARB except anti-sulfadiazine bacteria was not
387 significantly different from the non-irrigated ones. One possible explanation for the conflicted

388 research findings might be attributed to the different qPCR techniques used by the above
389 studies. Wang et al. (2014a) together with our study detected a large quantity of ARGs with
390 high throughput qPCR technique, therefore the effect of RWI on the whole antibiotic
391 resistome could be more comprehensive compared to the results of Negreanu et al. (2012) and
392 Chen et al. (2014) which examined only a few specific types of ARGs. It indicated that the
393 broad-scale survey of ARGs by using high-throughput qPCR techniques is important for
394 understanding the overall characteristics of soil resistome.

395 In this study, the patterns of ARGs compositions in urban parks were analyzed. The
396 ARGs profiles in urban parks without RWI clustered together, whereas the parks with RWI
397 were generally separated into distinct clusters for individual parks (Fig. 3). It indicated that
398 apart from the numbers and abundance of ARGs, the ARG compositions could be also
399 influenced by RWI, but the effects of RWI on the ARGs profile seemed to be soil dependent.
400 Soil type has been recognized as a critical factor in determining the fate of ARGs introduced
401 by RWI (Fahrenfeld et al., 2013). It is well known that most of wastewater treatment
402 processes could not efficiently remove antibiotics, ARGs and ARB (Berendonk et al., 2015;
403 Rodriguez-Mozaz et al., 2015), which was corroborated by the diverse and abundant ARGs
404 detected ARGs in the reclaimed water samples high-throughput qPCR analysis (Figure S2).
405 Therefore, RWI could impact the soil resistome through many ways such as increasing
406 number and abundance of ARGs and changing antibiotic resistance profiles (Wang et al.,
407 2014a; Xu et al., 2015). The different shifts in ARGs distribution profiles in the RWI parks
408 might be caused by a complex set of extrinsic and intrinsic factors, such as various reclaimed
409 water origins, irrigation histories and volumes, abundance of the native ARGs, adaptation
410 capability of introduced RWI-derived ARB to new surroundings and their competition with
411 native soil microbial communities (Gatica and Cytryn, 2013; Wang et al., 2014a). Apart from
412 the effects of RWI, we also observed an enrichment of ARGs in urban parks without RWI

compared to the remote national parks, suggesting that not only RWI but also other human disturbance might have selected soil antibiotic resistome. The regular anthropogenic disturbance occurred in the parks without RWI such as discarded food scraps, heavy metals or pesticides, which can deliver nutrients to soil, and lead to the growth and proliferation of antibiotic resistant microbial groups (Gillings et al., 2015). Taken together, the findings suggested that RWI was probably one of the primary factors contributing to dissemination of the ARGs, and other anthropogenic disturbance might also have a minor effect.

Impacts of RWI on the mobility potential of ARGs in urban park soils

Soil is a complex ecosystem where diverse and heterogeneous habitats are located at small spatial scale (Nesme and Simonet, 2015). A large variety of microbes with high genetic diversity live close to each other, which facilitates exchange of genetic materials through the HGT mechanism and a subsequent acquisition of ARGs by human pathogens (Nesme and Simonet, 2015). Some reports argued that a large number of ARB and ARGs entering soils through RWI could not effectively compete with resident microbial community and survive in soil environment (Gatica and Cytryn, 2013), but HGT may contribute to the dissemination and proliferation of ARGs in a new environment (Gillings and Strokes, 2012; Nesme and Simonet, 2015). Integrons, plasmids and transposons are considered as the important broad-host-range MGEs involved in the development of resistance (Heuer et al., 2011a). Many ARG cassettes are located on integrons which are capable of capturing and expressing mobile gene cassettes through a site specific recombination system (Heuer et al., 2011a). In this study, the β-lactam resistance genes *KPC* and *IMP-2* group were significantly and positively correlated with the *intI1* gene, suggesting the potential important role of integrons in ARGs transfer and dissemination. In fact, it has been verified that integron-associated genes can confer resistance to a broad spectrum of β-lactam, carbapenems and fluoroquinolones (Gaze et al., 2011). The *tnpA* gene had a significantly positive relationship with the *intI1* gene, which might be

ascribed to the location of integrons on transposons (Heuer et al., 2011a). However, the horizontal transfer rate of ARGs in soil was thought to be very low relative to vertical transmission of ARGs caused by growth of microorganisms that carry these genes (Heuer et al., 2011b). In this study, we did not find any significant impacts of RWI on the enrichment of the *intI1* and *tnpA* genes, suggesting that RWI did not obviously increase the mobility potential of ARGs in the investigated urban parks. Recent functional metagenomics studies also revealed that soil ARGs had no positive correlations with mobility elements including transposases and integrases, suggesting that the potential for horizontal gene transfer of ARGs in soil bacteria is very low (Forsberg et al., 2014). Although the dissemination of ARGs among soil bacteria and the subsequent acquisition by pathogens through HGT was considered as the most possible means of ARGs spread in clinical settings (Nesme and Simonet, 2015), more investigations on its transfer mechanism are needed in complex soil environments.

Impacts of RWI on the bacterial communities in urban park soils

Previous studies have reported the significant impacts of short-term or long-term RWI on the soil microbial abundance, diversity and community structure (Elifanz et al., 2011; Adrover et al., 2012), which is also supported by our study. The bacterial communities in the parks with RWI were clearly separated from those without RWI based on the T-RFLP profile, whereas no obvious separation of the bacterial communities was found between the remote national parks and the urban parks without RWI (Fig. 7). These findings implied that RWI was probably an important factor influencing the bacterial community structures in the examined park soils. Abundant soil resident bacteria can act as hosts of various ARGs, and different types of ARGs may be carried by the same microbial groups, which can be promoted by HGT of ARGs via MGEs (Li et al., 2015). Therefore, the changes in the bacterial community composition might have contributed to the current patterns of ARGs in soils (Su

et al., 2015). In other words, the similar variation trends of ARGs and the bacterial community under continuous RWI pressure could be explained by some specific ARGs located on certain microbial groups or MGEs (Forsberg et al., 2014; Li et al., 2015). In fact, the obvious co-occurrence patterns among various types of ARGs were observed by the network analysis, indicating that genes conferring resistance to different types of antibiotics could be located on some specific taxa of microbes or MGEs (Forsberg et al., 2014; Li et al., 2015).

Besides the impact of RWI on soil resistome and the bacterial community, we also examined whether soil ARGs was shaped by soil properties, which were generally considered as important factors affecting soil microbes (Cytryn, 2013; Xu et al., 2014). In this study, soil pH (ranging from 3.76 to 7.57, Table S2) exhibited a significant and positive correlation with the enrichment of total ARGs, which was in consistence with the results of Tang et al. (2015), which found the significantly positive correlations between soil pH (ranging from 5.63 to 7.55) and the relative abundances of several genes conferring resistance to tetracyclines and sulfonamides. One possible explanation is that the majority of soil bacteria are more adaptive to neutral pH and the soil acidity has an important role in the sorption and desorption behavior of antibiotics in soil (Peng et al., 2014; Sylvia et al., 2005). By contrast, total nitrogen content (Table S2) was negatively related to the enrichment of total ARGs, which may be due to changes of structure and diversity of soil bacterial communities harboring ARGs under different nitrogen levels (Forsberg et al., 2014).

Conclusions

In conclusion, by using high-throughput qPCR array techniques, we provide evidence that RWI impose some impacts on the abundance, diversity, and compositions of a broad spectrum of ARGs in urban parks in Australia. Our results also suggested that, irrespective of the selective pressure exerted by RWI, other anthropogenic activities could also influence soil

resistome. The significant impact of RWI on soil ARGs was accompanied by an obvious divergence of the bacterial community structure between the parks with and without RWI. Although a significant correlation was found between the abundance of the *intI1* gene and two β-lactam resistance genes, no significant effects of RWI on the mobility potential of ARGs (as revealed by no obvious enrichment of the *intI1* and *tnpA* genes) was observed. In addition, soil properties (particularly soil pH) might also have affected the patterns of antibiotic resistome. However, further research is required to assess the dispersal risks of ARGs caused by RWI and to formulate reasonable measures of treated wastewater reuse to minimize dissemination of ARGs into human pathogens.

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684

685 **Table 1** Primer sets, probe and amplification conditions used in the qPCR assays

Target genes	Amplicon size (bp)	Primer pairs	Sequence (5'-3')	Amplification conditions	References
intI1	473	HS463a	CTGGATTTCGATCACGGCACG	1 cycle of 95°C for 3 min; 40 cycles of 30 s at 95°C, 45 s at 60°C, and 45 s at 72°C (plate read)	Hardwick et al. 2008
		HS464	ACATGCGTGTAAATCATCGTCG		
tnpA	101	tnpA-04F	CCGATCACGGAAAGCTCAAG	1 cycle of 95°C for 3 min; 40 cycles of 20 s at 95°C, 30 s at 60°C, and 30 s at 72°C (plate read)	Zhu et al. 2013
		tnpA-04R	GGCTCGCATGACTTCGAATC		
Bacterial 16S rRNA	140	BACT1369F	CGGTGAATACGTTCYCGG	1 cycle of 10 s at 95°C; 35 cycles of 15 s at 95°C and 60 s at 56°C (plate read)	Suzuki et al. 2000
		PROK1492R	GGWTACCTTGTTACGACTT		
		Probe TM1389F	CTTGTACACACCGCCCGTC		

686 **Figure legends**

687 **Fig. 1** The numbers of the detected ARGs (a) and the fold changes of the enriched ARGs (b)
688 in urban parks with and without RWI compared to the two remote national parks. Error bars
689 indicate standard errors (n = 3). Abbreviations: WP, Werribee Park; WRG, Werribee Rose
690 Garden; YP, Yarra Park; HDGR, HD Graham Reserve; AGP, Altona Green Park; WCUM,
691 Werribee Campus of the University of Melbourne; RBG, Royal Botanic Gardens; CG,
692 Carlton Garden; FZG, Fitzroy Gardens; PP, Princes Park; YBP, Yarra Bend Park; FSG,
693 Flagstaff Garden; YRNP, Yarra Ranges National Park; LENP, Lake Eildon National Park.

694 **Fig. 2** The comparison of the numbers (a and c) and the enrichment levels (b and d) of the
695 detected ARGs between the urban parks with and without RWI. The ARGs were classified
696 based on the antibiotics to which they confer resistance. MLS_b, Macrolide Lincosamide
697 Streptogramin_b resistance.

698 **Fig. 3** The heat map showing the fold changes of the 84 target ARGs in the 12 urban park
699 soils. The names of parks with RWI were denoted in red color, whereas the names of parks
700 without RWI in green color. The detailed information about the 84 ARGs is shown in Table
701 S3, with exactly the same orders as those appeared in the heat map.

702 **Fig. 4** The fold changes of the relative abundance of the *intI1* and *tnpA* genes in urban parks
703 with and without RWI. The names of parks with RWI were denoted in red color, whereas the names
704 of parks without RWI in green color. Error bars indicate standard errors (n = 3).

705 **Fig. 5** Correlations between the fold changes of β-lactam resistance genes and the *tnpA* gene
706 with the *intI1* gene (a) and correlations between the fold changes of total ARGs and soil pH
707 and total nitrogen (TN) content (b).

708 **Fig. 6** The network analysis illustrating the co-occurrence patterns among the detected ARGs

709 across all the urban parks. The nodes of various colors represent different types of ARGs, and
710 the edges indicate a strong ($\rho > 0.8$) and significant ($P < 0.05$) correlation between nodes. The
711 size of each node is proportional to the number of significant connections.

712 **Fig. 7** NMDS plots derived from the T-RFLP data of the bacterial 16S rRNA gene based on
713 Bray–Curtis dissimilarity matrices. The solid red spots represent the urban parks with RWI,
714 the solid green spots represent the urban parks without RWI, and the hollow green spots
715 represent the remote national parks.