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Impact of reclaimed water irrigation on antibiotic resistance in public parks, Beijing, China

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ABSTRACT

The abundance and distribution of antibiotics and antibiotic resistance genes (ARGs) in soils from six parks using reclaimed water in Beijing, China, were characterized. Three classes of commonly used antibiotics (tetracycles, quinolones, and sulfonamides) were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The highest concentrations of tetracyclines and quinolones were 145.2 μg kg^{-1} and 79.2 μg kg^{-1}, respectively. Detected *tet*G, *tet*W, *sul*I, and *sul*II genes were quantified by quantitative PCR. ARGs exhibited various abundances for different park soils. The integrase gene (*int*I1) as an indicator of horizontal gene transfer potential was also detected in high abundance, and had significant positive correlation with *tet*G, *sul*I, and *sul*II genes, suggesting that *int*I1 may be involved in ARGs dissemination. Both *sul*II and *int*I1 clones had high homology with some classes of pathogenic bacteria, such as *Klebsiella oxytoca*, *Acinetobacter baumannii*, *Shigella flexneri*, which could trigger potential public health concern.

1. Introduction

Antibiotics are probably the most successful family of drugs so far developed for treating human diseases. Antibiotics have also been widely used to prevent/treat animal diseases and promote animal growth in livestock production (Hu et al., 2010). However, most antibiotics are poorly absorbed in both animals and humans, and a large proportion of antibiotics can be excreted in an unaltered state (Kumar et al., 2005) and then dispersed into environment. Indeed, antibiotics have been detected in municipal sewage (Zhang and Zhang, 2011), hospital wastewater (Lindberg et al., 2004), surface water (Luo et al., 2011; Stoll et al., 2012), groundwater (Lindsey et al., 2001), as well as in soil, sediment and sludge samples (Kim and Carlson, 2007). Residual antibiotics may exert selection pressure on environmental microorganisms, contributing to proliferating antibiotic resistance in microorganisms (Martinez, 2009).

Many developing countries including China are facing the challenge of water shortage, and landscape irrigation has to some extent exacerbated this problem with the acceleration of urbanization, growing demand and declining supplies of freshwater. Reclaimed water from wastewater treatment plants (WWTPs) could partly relieve this issue. Antibiotics in WWTPs are mainly from hospital, pharmaceutical plant wastewater, and domestic wastewater. However, the conventional biological processes in WWTPs could not remove the antibiotics and resistant bacteria completely, which could be influenced by compound-specific properties and treatment processes in specific WWTPs, such as hydraulic retention time (HRT) and solid retention time (SRT) (Rizzo et al., 2013; Lindberg et al., 2005; Li and Zhang, 2010). Numerous studies have reported the occurrence of antibiotics in wastewater, sludge, and the removal efficiency of antibiotics by WWTPs (Leung et al., 2012; Liu et al., 2012; Zhang and Zhang, 2011; Zhang et al., 2011). For example, Gao et al. (2012a) investigated the occurrence of antibiotics in eight sewage treatment plants in Beijing, China, and found that the removal efficiency of antibiotics varied significantly among the eight WWTPs, with average removal efficiency of fluoroquinolones, sulfonamides, and macrolides ranging from 48% to 72%, 39% to 64%, and −34% to 69%, respectively. In addition, although the resistance genes was decreased by 1–3 log units after sewage treatment, tetracycline resistance genes could still be detected from 10^2 to 10^6 copies per milliliter effluent water and 10^8 to 10^9 copies per

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milliliter of biosolids (Auerbach et al., 2007). The continuously release of residual antibiotic compounds, antibiotic resistant bacteria (ARB) and ARGs from WWTPs effluent could result in the dissemination of ARGs in downstream environment (Negreanu et al., 2012; Pruden et al., 2012), which has raised great concerns (Le-Minh et al., 2010). Furthermore, the propagation of ARGs between similar bacteria, gram-positive bacteria and gram-negative bacteria, and even between pathogenic bacteria and non-pathogenic bacteria could be further facilitated through horizontal gene transfer mechanisms via self-transmissible mobile genetic elements, such as plasmids, integrons, transposons, and insertion sequences (Gogarten and Townsend, 2005; Yu et al., 2012), which could pose potential threat to human health.

Until now, only a few studies have concerned about the occurrence and abundance of antibiotics and ARGs in public park soils (Malik et al., 2008; Yang et al., 2010). The hypothesis was that environmental compartments most directly impacted by urban/agricultural activities would have significantly higher concentrations of ARGs than less impacted and pristine environments. Additionally, the integrase gene *int*I1 of class I integron was tracked as an indicator of horizontal gene transfer potential. Therefore, soil samples from six public parks using reclaimed water irrigation were collected. Since antibiotic resistance is ancient (Allen et al., 2009; D'Costa et al., 2011), four pristine soil samples from Natural Scenic Resort of Ling Mountain not using reclaimed water were collected as control. The objective of this study was to evaluate whether the soil in public parks receiving reclaimed water acts as a reservoir of ARGs and has the potential for transferring ARGs to human pathogens. Therefore, this study tended to (i) evaluate the occurrence and concentration of 24 antibiotics and ARGs in 29 soil samples; (ii) determine if the pattern of ARGs prevalence was different between each public park; (iii) investigate if there was a link between the ARGs and integrase gene (*int*I1).

2. Material and methods

2.1. Soil sampling and processing

Soil samples were collected from Taoranting Park (TP; $n = 8$), Daguanyuan Park (DP; $n = 5$), Longtanhu Park (LP; $n = 2$), Beijing World Park (SP; $n = 4$), Olympic Forest Park (OP; $n = 4$), and Research Center for Eco-Environmental Sciences Park (EP; $n = 2$) using reclaimed water irrigation between August and September 2011. Samples were taken from rhizosphere soil, non-rhizosphere soil, and wetland soil according to the condition of each park. At the same time, four samples collected from Natural Scenic Resort of Ling Mountain (LM) not receiving reclaimed water, were used as control. Details about the samples were shown in Table S1. All of the samples were kept on dry ice during transportation and stored at −80 °C before DNA extraction and chemical analysis. The soil characteristics were measured using standard methods (Lu, 1999). Soil organic matter was determined by the $K_2Cr_2O_7$ oxidation method. Soil pH was determined with a soil to water ratio of 1:5. Total carbon and total nitrogen were determined by Element Analyzer (Vario EL III, Elementar, USA). All samples were analyzed in triplicate. The average results are summarized in Table 1.

2.2. Antibiotics analysis

The procedures for extraction of tetracyclines, quinolones, sulfonamides, and their degradation products in soils followed methods described by Wu et al. (2011) with some modifications. LC–MS/MS was used to separate and detect the tetracyclines, sulfonamides, and quinolones following methods described by Zhang et al. (2011). Briefly, Na_2EDTA (0.4 g) and 15 mL of a mixed solution containing phosphate buffer (pH = 2.0) and acetonitrile (2:1, vol/vol) were used as extraction solution. After sonication for 30 min, samples were then centrifuged at 7000 rpm for 5 min. The extraction process was performed three times in each sample. The obtained supernatants were combined and then diluted to 400 mL with deionized water, and acidified to pH 2.5 before SPE extraction. The samples were extracted using Oasis HLB (500 mg, 6 mL) extraction cartridges. Final extracts were transferred to 2 mL amber vials for LC–MS/MS analysis.

Five target tetracyclines, including tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC), methacycline (MTC), and doxycycline (DXC), and nine degradation products, including anhydrotetracycline (ATC), 4-epitetracycline (ETC), 4-epianhydrotetracycline (EATC), 4-epioxytetracycline (EOTC), α-apo-oxytetracycline (α-apo-OTC), β-apo-oxytetracycline (β-apo-OTC), isochlortetracycline (ICTC), 4-epianhydrochlortetracycline (EACTC), demethylchlortetracycline (DMCTC), four sulfonamides, sulfadimethoxine (SDM), sulfamerazine (SMI), sulfamethizole (SMT), sulfamethoxazole (SMZ), and six fluoroquinolones, including ofloxacin (OFL), enrofloxacin (ENRO), sarafloxacin (SARA), danofloxacin (DANO), ciprofloxacin (CIP), norfloxacin (NOR), were analyzed in this study.

2.3. DNA extraction

Total DNA was extracted from 0.5 g of soil using a FastDNA SPIN Kit for soil (Bio 101 Inc., Vista, CA), following the manufacturer's instructions. The concentration and quality of the extracted DNA was determined by spectrophotometer analysis (NanoDrop ND-1000, NanoDrop Technologies, Willmington, DE) and agarose gel electrophoresis.

2.4. Detection and quantification of ARGs

Polymerase chain reaction (PCR) detection assays were used for broad-scale screening of the presence/absence of fifteen tetracycline genes (seven efflux pump genes (*tet*A, *tet*C, *tet*E, *tet*K, *tet*L, *tet*A/P, *tet*G), seven ribosomal protection proteins (RPPs) genes (*tet*M, *tet*O, *tet*Q, *tet*S, *tet*T, *tet*W, *tet*B/P), and one enzymatic modification gene (*tet*X)), four beta-lactamases genes ($bla_{CTX\text{-}M}$, bla_{TEM}, bla_{SHV}, bla_{ampC}), three sulfonamide genes (*sul*I, *sul*II, *sul*III), three quinolone genes (*qnr*A, *qnr*B, *qnr*S), three erythromycin genes (*ere*A, *ere*B, *mph*A), and the integrase gene of class I integron (*int*I1). Primers and annealing temperatures are described in Table S2. To ensure reproducibility, triplicate PCR reactions were performed for each sample. Sterile water was used as the negative control in every run. After PCR amplification, gel slices of an agarose gel containing the PCR products were excised and purified using Agarose Gel DNA Purification Kit (Promega). The purified PCR products were ligated into p-GEM T easy vector (Promega) and then cloned into *Escherichia coli* JM109 (Takara). Clones containing ARGs inserts were picked and sequenced. If the gene inserts were verified as the object resistance genes using the BLAST alignment tool (http://www.ncbi.nlm.nih.gov/blast/), clones that had the right ARGs inserts were chosen as the positive control and standards for quantitative polymerase chain reaction (Q-PCR). Plasmids carrying target genes were extracted with Plasmid Kit (Takara).

Four ARGs (*tet*G, *tet*W, *sul*I, *sul*II) that were frequently detected and *int*I1 were quantified by Q-PCR using a SYBR Green approach. Eubacterial 16S rRNA genes were quantified according to the TaqMan Q-PCR method described by Suzuki et al. (2000). 10-fold serial dilutions of a known copy number of the plasmid DNA were generated to produce the standard curve. Each sample DNA was diluted for minimizing threshold cycle suppression by inhibitors for that sample type (typically 1:10). All Q-PCR reactions were done in triplicate for both the standards and the microbial community DNA samples. The Q-PCR efficiencies (89.9%–107.5%) were examined to test for inhibition. R^2 values were more than 0.997 for all calibration curves. All Q-PCR analyses were performed using an iCycler IQ5 Thermocycler (Bio-Rad, Hercules, CA).

2.5. Cloning and phylogenetic analysis of sulII and intI1 genes

The clone libraries of *sul*II and *int*I1 genes amplicons obtained from six soil samples were constructed to analyze the sequence diversity. *Sul*II (191 bp) and *int*I1 (473 bp) genes fragments were cloned as described above. Between 20 and 30

Table 1
Soil physicochemical properties of each park (mean values are in brackets).

Samples	pH	Organic matter OM (g kg^{-1})	N (g kg^{-1})	C (g kg^{-1})	S (g kg^{-1})	C/N
TP	8.11–8.32 (8.23)	24.34–72.38 (46.00)	0.98–2.64 (1.92)	19.49–47.07 (33.14)	1.23–3.11 (2.02)	13.68–22.37 (17.84)
DP	7.96–8.63 (8.35)	17.54–44.65 (29.67)	0.95–2.26 (1.48)	25.03–34.20 (28.85)	1.30–1.80 (1.54)	15.12–27.53 (20.69)
LP	8.56–8.61 (8.58)	26.61–27.46 (27.04)	1.06–1.33 (1.20)	24.10–25.81 (24.96)	0.61–0.98 (0.80)	18.07–24.43 (21.25)
OP	8.13–8.57 (8.37)	13.65–73.53 (40.74)	0.42–5.56 (2.56)	20.40–66.49 (38.58)	0.52–1.56 (1.03)	12.08–48.52 (24.55)
SP	8.42–8.82 (8.64)	16.67–35.85 (26.18)	0.91–2.41 (1.79)	17.93–31.44 (26.78)	0.54–0.82 (0.62)	13.07–19.86 (15.94)
EP	8.41–8.58 (8.50)	27.09–32.32 (29.71)	1.37–1.58 (1.48)	24.46–29.19 (26.82)	1.01–1.14 (1.08)	17.91–18.47 (18.19)
LM	7.24–7.57 (7.41)	36.62–61.55 (47.27)	2.15–3.03 (2.58)	24.93–36.77 (31.08)	0.30–0.43 (0.37)	11.57–12.38 (12.02)