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Removal of antibiotics and antibiotic resistance genes in rural wastewater by an integrated constructed wetland

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Abstract Integrated constructed wetlands (ICWs) are regarded as one of the most important removal technology for pollutants in rural domestic wastewaters. This study investigated the efficiency of an ICW consisting of a regulating pool, four surface and subsurface flow-constructed wetlands, and a stabilization unit for removing antibiotics and antibiotic resistance genes (ARGs) from rural domestic wastewaters. The results showed that antibiotics leucomycin, ofloxacin, lincomycin, and sulfamethazine, and ARGs *sul*1, *sul*2, *tet*M, and *tet*O were the predominant antibiotics and ARGs in the influent, respectively. The ICW system could significantly reduce most of the detected antibiotics and ARGs with their aqueous removal rates of 78 to 100 % and >99 %, respectively. Based on the measured concentrations, the total pollution loadings of antibiotics were 3,479 μg/day in the influent and 199 μg/day in the final effluent. Therefore, constructed wetlands could be a promising technology for rural wastewater in removing contaminants such as antibiotics and ARGs.

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Introduction

Antibiotics have been extensively used in the treatment of humans and animals for various bacterial infections and/or growth promotion (Sarmah et al. 2006; Kümmerer 2009). After administration, some antibiotics could end up in the environment due to direct discharge of wastewaters or incomplete removal in wastewater treatments plants (WWTPs) (Batt et al. 2006; Watkinson et al. 2007; Xu et al. 2007). It is reported that wide use in humans and animals and subsequent occurrence of antibiotics in the environment could affect aquatic and terrestrial organisms (Costanzo et al. 2005; Kotzerke et al. 2008; Liu et al. 2009), alter microbial activity and community composition (Underwood et al. 2011), and lead to environmental contamination by antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) (Pruden et al. 2006; Tao et al. 2010; Su et al. 2012). In recent years, antibiotic residues and ARGs in the environment have been recognized as an emerging environmental issue (Barnes et al. 2008; Yang et al. 2011; Zhou et al. 2011).

Many previous studies have showed incomplete removal of various antibiotics in conventional municipal WWTPs with variable rates, for example, 23 to 94 % for sulfadiazine, 34 to 84 % for sulfamethoxazole, −9 to 97 % for tetracycline, 26 to 97 % for oxytetracycline, 78 to 100 % for chlortetracycline, 43 to 70 % for doxycycline, 5 to 98 % for ciprofloxacin, −38to 93 % for norfloxacin, 3.2 to 84 % for ofloxacin, and 7 to 85 % for roxithromycin (Lindberg et al. 2005; Xu et al. 2007; Li and Zhang 2010; Gao et al. 2012; Jia et al. 2012; Zhou et al. 2013). Biodegradation and adsorption onto sludge have

been identified as the two major processes responsible for the removal of antibiotics in conventional WWTPs (Jia et al. 2012; Zhou et al. 2013). As emerging environmental contaminants, ARGs have also been detected in diverse environmental compartments (Durham et al. 2010; Tamminen et al. 2011; Pruden et al. 2012; Cheng et al. 2013; Coleman et al. 2013; Su et al. 2012). The removal of ARGs in conventional WWTPs have been widely investigated, and the results showed that the conventional activated sludge treatment processes only had limited removal efficiency for ARGs or even could increase the absolute abundance of ARGs within the system due to growth and reproduction of ARBs (Auerbach et al. 2007; Munir et al. 2011; Rizzo et al. 2013). In order to improve the removal rates of antibiotics and ARGs, more research is required to explore the mechanisms involved in various treatment processes (Langford and Lester 2003).

On the other hand, fewer studies have been done on the treatment of rural wastewaters (Huang et al. 2012; Liu et al. 2013a, b). But it is important for those countries like China with a large rural population. China is currently facing the serious challenge of treating increasing amounts of domestic sewage in rural area. Rural domestic sewage is often a composite wastewater with household wastewater from human daily activities and wastewater and manure from small-scale livestock farms. Mostly, the rural domestic sewage has been directly discharged into nearby rivers and lakes (Huang et al. 2012). Owing to dispersed rural population and the high cost in collecting sewage, centralized wastewater treatment plants based on activated sludge that are utilized in cities are not so suitable in rural area (Ye and Li 2009). Constructed wetlands (CWs), especially integrated constructed wetlands (ICWs), have been scientifically tested and constructed for on-site treatment in rural area for small towns and villages due to their low investment and operation costs (Shao et al. 2013). Therefore, it would be interesting to study the removal mechanisms of antibiotics and ARGs in small-scale ICWs for rural domestic wastewaters.

The objective of this study was to investigate the removal of antibiotics and ARGs in rural wastewaters by an ICW. The ICW was applied to treat rural domestic sewage and animal wastewater from small-scale piggeries in a small village of South China. Mass loading analysis was employed to evaluate the removal efficiencies of the antibiotics detected in the ICW and explore their potential removal mechanisms and pollution loadings to the receiving environment.

Materials and methods

Wetland system and sample collection

An ICW was built to treat rural wastewater from a small village in Kaihui of Hunan province, South China. The ICW serves a population of 60 people and about 200 pigs, and is designed to have a flow of 10 m^3/day. In this small village, each family has built swine houses. The swine houses were flushed daily with well water, and the mixed flush water was collected by a sewer line and then directly discharged into the ICW system. Part of the manure was applied onto vegetable fields nearby. Wastewaters from households were collected by the sewer line and directly discharged into the ICW system. Domestic sewage accounts for 70 % of the influent, while livestock sewage accounts for 30 %. After treatment, final effluent is directly discharged into a nearby small river. The ICW system consists of a regulating pool (CW0), free-water surface flow-constructed wetland (CW1), subsurface flow-constructed wetland (CW2), surface flow-constructed wetland with floating macrophytes (CW3), surface flow-constructed wetland with emerging macrophytes (CW4), and a stabilization lagoon (CW5) (Fig. 1). The total land area of this ICW is approximately 981 m^2 (CW1, 30 m^2; CW2, 30 m^2; CW3, 192 m^2; CW4, 304 m^2; CW5, 425 m^2). Among the subsystems, CW1 is a free-water surface flow-constructed wetland (FW-SFCW) without substrate and planted with *Myriophyllum verticillatum* L., and CW2 has chaff and soil used as the wetland medium. *Pontederia cordata* and *M. verticillatum* L. were planted on the soil surface of the subsystems CW3 and CW4, respectively. The ICW system has been under stable operation since June 2012. The daily treatment capacity of this ICW system was 6.5 m^3/day with the hydraulic loading rate of 7 mm/day, and the hydraulic retention time (HRT) was round 36 h during the sampling campaign period in July 2013.

In the sampling campaign, seven wastewater samples (influent, W1; CW1 effluent, W2; CW2 effluent, W3; CW3 effluent, W4; CW4 effluent, W5; final effluent, W6; and the receiving river water, W7) from each sampling location (Fig. 1) were collected as the 24-h composite samples, while the five solid samples (CW2 medium, S1; CW3 medium, S2; CW4 medium, S3; CW5 medium, S4;sediment of the receiving river, S5) were grab collected as the five spot-composite samples (depth 0–5 cm). For analysis of antibiotics, the water samples were collected in 1-L precleaned brown glass bottles, while medium samples were collected in 1-L glass jars. For analysis of ARGs, the water samples were collected in 0.5-L sterile polypropylene bottles, while the medium samples were collected in 50-mL sterile centrifuge tubes. Three parallel samples were collected for each sample type. For antibiotic analysis, about 50 mL of methanol was added to each bottle (1 L) of the water samples and the pH was adjusted to 3 using 4 M H_2SO_4 in the field to inhibit microbial activity. One gram of sodium azide was added to each medium sample to suppress microbial activity. All the samples were transported in a cooler to a laboratory where they were stored in the dark at 4 °C prior to analysis. Medium samples were freeze-dried,

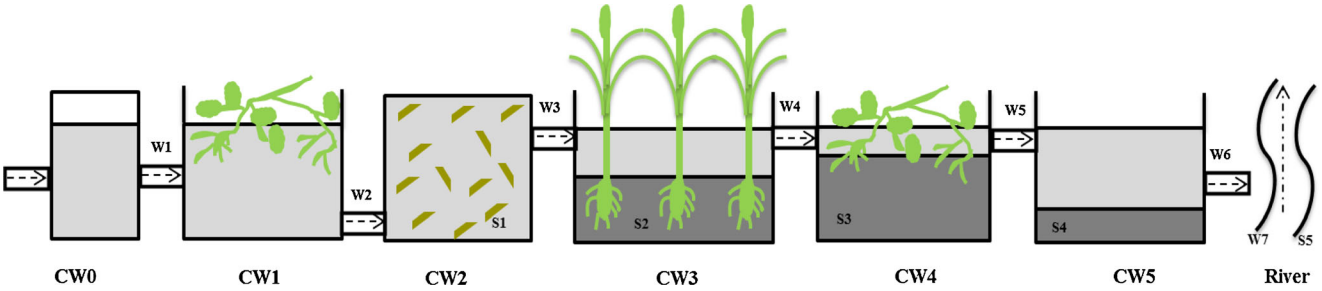

Fig. 1 The scheme of the integrated constructed wetland (ICW) showing wetland treatment units and sampling locations. *W1~W7* water samples, *S1~S5* medium samples. *CW0* is a regulating pool, *CW1* a free-water surface flow-constructed wetland (FW-SFCW), *CW2* a subsurface flow-constructed wetland (SSFCW), *CW3* a floating macrophyte surface flow-constructed wetland (FM-SFCW), *CW4* an emerging macrophyte surface flow-constructed wetland (EM-SFCW), and *CW5* a stabilization unit

homogenized, and passed through a 60-mesh standard sieve and then kept at −20 °C in the dark until extraction.

Analyses of chemical oxygen demand (COD), biochemical oxygen demand (BOD_5), total nitrogen (TN), ammonia nitrogen (NH_3-N), and total phosphorus (TP) were performed according to the standard methods (Clesceri et al. 2001). COD was measured using the potassium dichromate method. BOD_5 was measured by the 5-day BOD test using the azide modification of the iodometric method. TN and NH_3-N were determined by a UV–vis spectrophotometer (Shimadzu Instrument Co. Ltd., UV-2450, Japan) (APHA 1998; Shao et al. 2013). Total organic carbon (TOC) was measured by a TOC analyzer (LiquiTOC, Elementar Analysensyteme Co., Germany). The pH and DO were monitored online by a pH/DO meter (YSI-Pro2030; YSI Incorporated, Yellow Springs, OH, USA).

Chemical analysis

Chemicals and materials

We have selected 11 classes of 50 antibiotics for this investigation based on our previous study (Zhou et al. 2012): sulfonamides, diaminopyrimidines, tetracyclines, fluoroquinolones, macrolides, polyether ionophores, polypeptides, lincosamides, chloramphenicol derivatives, and β-lactams. High purity standards of antibiotics and materials used in the analysis and the physicochemical properties of the target compounds are given in our previous study (Zhou et al. 2012). HPLC-grade methanol and acetonitrile were purchased from Merck (Darmstadt, Germany). Stock solutions of chemicals (100 mg/L) were prepared in methanol and stored at −20 °C for later use. Working standard solutions were prepared weekly. All glassware was hand washed with tap water, rinsed with HPLC-grade water and methanol, and baked at 450 °C for at least 4 h before use.

Sample extraction and instrumental analysis

Water and medium samples were extracted and analyzed by following our previous method (Zhou et al. 2012), with the procedures being described briefly as follows. The water samples were extracted by Oasis HLB cartridges (6 mL, 500 mg), while the medium samples were extracted by ultrasonic-assisted extraction with solvents (acetonitrile and citric acid buffer), followed by an enrichment and cleanup step with solid-phase extraction using SAX-HLB cartridges in tandem. The target antibiotic compounds in the extracts were analyzed by rapid-resolution liquid chromatography-tandem mass spectrometry (RRLC-MS/MS). The RRLC-MS/MS used in the analysis was Agilent liquid chromatography 1200 series RRLC system coupled to an Agilent 6460 triple quadrupole MS equipped with an electrospray ionization (ESI) source (Agilent, Palo Alto, CA, USA). The quantitative analysis of the target compounds was carried out in multiple reaction monitoring (MRM) mode (Zhou et al. 2012). Quantification of the target compounds was obtained using the internal standard method. Laboratory blanks were also analyzed along with the samples to assess potential sample contamination. Sample concentrations were not corrected for the method recoveries.

DNA extraction and purification

Each water sample (0.5 L) was filtered through a sterile membrane filter (0.45-μm pore diameter) with a vacuum filtration apparatus. Then, the membrane filter was aseptically removed by using a sterile forceps, rolled and put into the tube provided by the PowerSoil DNA Isolation Kit (Mobio, USA). The DNA extraction procedures used here followed the protocol provided by the manufacturer.

The medium samples were freeze-dried, ground with a mortar, and sieved through a 100-mesh screen. DNA was extracted from each medium sample using the PowerSoil DNA Isolation Kit (Mobio, USA). Exactly 0.5 g of each medium sample was used for DNA extraction. The DNA extraction steps followed the protocol provided by the manufacturer. Then, DNA was further purified using the DNA Spin Kit (Tiangen Biotech, China) to minimize PCR inhibition.

ARG quantification

External reference method was used to quantify the selected 11 ARGs in the wastewater and medium samples (Table S1). The two integrase genes and nine ARGs included *intI*1, *intI*2, *sul*1, *sul*2, *sul*3, *tet*B/P, *tet*M, *tet*O, *tet*X, *erm*B, and *erm*C, which are commonly reported in wastewaters (Pruden et al. 2012; Cheng et al. 2013; Su et al. 2014). SYBR Green Real-Time qPCR Kit (TAKARA, Japan) was applied to quantitatively determine the abundance of resistance genes. The specific primers of the selected ARGs are listed in Table S1. Positive controls consisted of cloned and sequenced PCR amplicons obtained from the sludge of WWTPs and manure of livestock farms. Both positive and negative controls (Milli-Q water) were included in every run. A total of 40 cycles was applied to improve the chances of product formation from low initial template concentrations. A 20-μL PCR reaction solution was employed: 2× THUNDERBIRD SYBR® qPCR Mix 10 μL, 0.05 mM each primer 0.08 μL, 50× ROX reference dye 0.04 μL, template DNA 2 μL (DNA <80 ng), and distilled water 7.8 μL (DNase I treated). The qPCR assays were run on an Applied Biosystems 7500 Fast Real-Time PCR System (ABI, USA). The temperature program for quantification of ARGs consisted of initial denaturing at 95 °C for 1 min, followed by 40 cycles for 15 s at 95 °C, 55 °C for 30 s (some primers of ARGs have different annealing temperatures, see Table S1), 72 °C for 30 s, and a final step for melting curve. The external reference method was used to calculate the copy number of ARGs, with the square of related coefficient (r^2) of the standard curve >0.99 and the amplification efficiency ranging between 95 and 110 %.

Mass loading analysis

Mass loading analysis was used to analyze the mass flow of an antibiotic entering and leaving each unit of the ICW in water phase by multiplying concentrations of each antibiotic in aqueous phase by average daily flow.

$$M_i = C_{i,\ \mathrm{water}} \times Q \quad (1)$$

where M_i is the mass loading of the compound i in the water phase, $C_{i,\ \mathrm{water}}$ represents the concentration of compound i in water, and Q is the average daily water flow in the ICW.

In order to assess the contributions of sorption and degradation of the antibiotics during the treatment processes in the ICW, raw wastewater was taken as initial total mass loading, while the system output consisted of CW6 effluent. The difference between the mass inflow and outflow (in aqueous phase) for each antibiotic was expressed as the loss due to the total effect of sorption and degradation in each treatment unit within the ICW and calculated by following equation:

$$M_{\mathrm{Loss}} = M_{\mathrm{Influent}} - M_{\mathrm{Effluent}} \quad (2)$$

where M_{Influent} and M_{Effluent} are the mass loadings (aqueous phase) of an antibiotic in the influent and each treatment unit effluent or final effluent (μg/d), respectively, and M_{Loss} stands for the mass loss of the antibiotic during the whole ICW treatment.

Statistical analysis

One-way ANOVA was used to evaluate the statistical significance of difference with p value <0.05 using SPSS version 13.0 (IBM, NY). Averages and standard deviations were calculated with Microsoft Excel, 2010.

Results

Operational performance of the ICW

The general performance of the ICW system for rural domestic wastewater is summarized in Table 1. The removal rates observed for the general water quality parameters (BOD_5, COD, NH_3-N, TN, and TP) ranged between 80.9 to 99.6 %, except for COD (64.9 %) in the ICW system. In the influent, NH_3-N accounted for 94 % of TN and was the dominant type of N. Wastewater pH was relatively stable within the range of 7.5 to 8.0 in the whole ICW system. In the final effluent, NH_3-N, TN, and TP were significantly decreased to very low concentrations of 0.27 to 0.89 mg/L and BOD_5 and COD were detected at the concentrations of 8.12 and 41.8 mg/L, which had already met the Chinese standards (Class I-A Criteria of GB18918-2002, BOD_5<10 mg/L and COD<50 mg/L) of wastewater discharge.

Occurrence and removal of antibiotics in the ICW

The occurrence of all selected 50 antibiotics was investigated in the ICW system. The concentrations of antibiotics in wastewater samples from the ICW system are summarized in Table S2. Out of the 50 target antibiotics, 10 antibiotics including 5 sulfonamides and trimethoprim, 2 macrolides, lincomycin, ofloxacin, and salinomycin were detected in both influent and final effluent samples (Table S2 and Fig. 2). Concentration levels of the antibiotics ranged from 1.93±0.12 to 120±7.59 ng/L in the influent and from <LOQ to 14.3±0.99 ng/L in the final effluent. The predominant compounds in the influent of the ICW were leucomycin and ofloxacin with their concentrations of 120±7.59 and 193±75.8 ng/L, respectively.

Table 1 Water (mg/L) and media (g/kg) quality parameters in the integrated constructed wetland system

Parameters	Water samples (mg/L)							Total removal rate (%)
	W1	W2	W3	W4	W5	W6	W7	
BOD_5	42.6	33.4	98.9	59.6	10.8	8.12	3.68	81
COD	119	127	269	209	82.1	41.8	10.1	65
TP	11.7	12.9	11.8	11.4	0.70	0.39	0.09	97
TN	71.8	73.9	65.9	45.8	4.58	0.89	1.57	99
NH_3-N	67.4	68.2	62.1	42.8	2.12	0.27	0.21	100
	Medium samples (g/kg)							
			S1	S2	S3	S4	S5	–
TP			0.48	0.49	0.45	0.41	0.22	–
TN			188	58.9	76.1	25.9	26.6	–
NH_3-N			2.80	1.86	1.51	1.39	0.54	–
TOC			108	39.6	29.7	27.6	12.2	–

BOD_5 biochemical oxygen demand, *COD* chemical oxygen demand, *TP* total phosphorus, *TN* total nitrogen, *NH_3-N* ammonia nitrogen, *TOC* total organic carbon

In terms of aqueous-phase removal, four detected antibiotics (ofloxacin, leucomycin, sulfamonomethoxine, and trimethoprim) in wastewater were totally eliminated by the ICW treatment processes. Compared to the other three treatment units, CW2 and CW3 were more efficient in the removal of these four antibiotics. CW2 treatment unit contributed 73 and 62 % in the total removal of ofloxacin and trimethoprim, respectively (Table S3). For the removal of sulfamonomethoxine and leucomycin, CW3 treatment unit contributed 80 and 41 %, respectively. The total removal rates in aqueous phase observed for the other detected antibiotics ranged between 78 to 95 %, except for sulfadiazine, sulfacetamide, and salinomycin with their removal rates between only 10 to 24 % (Table S3).

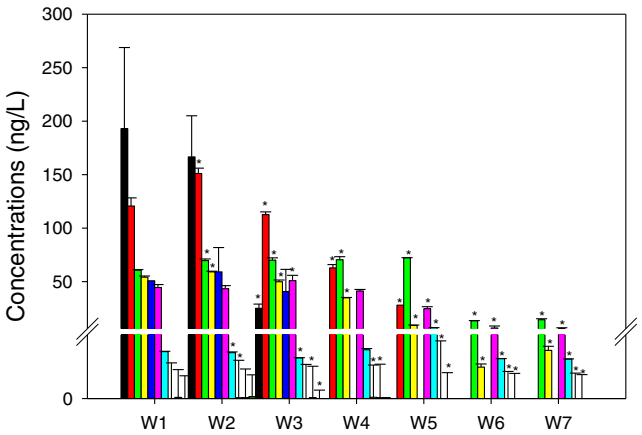

Fig. 2 Concentrations (ng/L) of antibiotics in wastewater samples from the integrated constructed wetland system. *W1* influent, *W2* CW1 effluent, *W3* CW2 effluent, *W4* CW3 effluent, *W5* CW4 effluent, *W6* final effluent, *W7* the receiving river water. *$p<0.05$, significantly different values when compared to the influent (*W1*)

Three target antibiotics including ofloxacin, anhydroerythromycin, and sulfamethazine were quantified in all the medium samples of ICW units. It should be noted that the concentrations of these three detected antibiotics in the medium of ICW were relatively low and in the similar range from 1.12±0.46 to 4.88±1.59 ng/g (Table S4 and Fig. 3).

Occurrence and removal of ARGs in the ICW

ARGs were found in all ICW units. Eleven ARGs from four classes were all detected positively in both water and medium samples of the ICW. The concentrations of the ARGs varied from 8.57 copies/mL (*intI*2, CW5) to 5.10×10^6 copies/mL (*sul*1, CW2) for the water samples (Table S5 and Fig. 4) and ranged from 3.47×10^3 copies/g (*intI*2, CW3) to 7.22×10^8 copies/g (*tet*M, CW2) for the medium samples (Table S6 and Fig. 5). For the water samples, *sul*1 was the most abundant ARG in the ICW units, with its average concentration of 1.97×10^6 copies/mL, followed by *sul*2, *tet*M, *tet*O, *erm*B, *intI*1, *tet*B/P, *tet*X, *erm*C, and *sul*3. The lowest ARG was *intI*2, with an average concentration of 2.18×10^3 copies/mL (Table S5 and Fig. 4). Additionally, *sul*1 was also the most abundant in the medium samples, with an average concentration of 1.61×10^8 copies/g, followed by *tet*M, *erm*B, *sul*2, *intI*1, *tet*O, *tet*B/P, *tet*X, *erm*C, and *sul*3. And *intI*2 was also the least abundant, with an average concentration of 8.79×10^4 copies/g (Table S6 and Fig. 5).

The aqueous removal rates of ARGs were rather high in the ICW. All the selected ARGs were significantly reduced by 1–3 orders of magnitude from the influent to the effluent ($p<0.05$). The removal rates for *intI*2, *tet*B/P, *tet*M, and *tet*X were the highest, by 100 %, followed by *tet*O and *erm*B, by 99 %, and *erm*C had the lowest removal rate, by 43 %

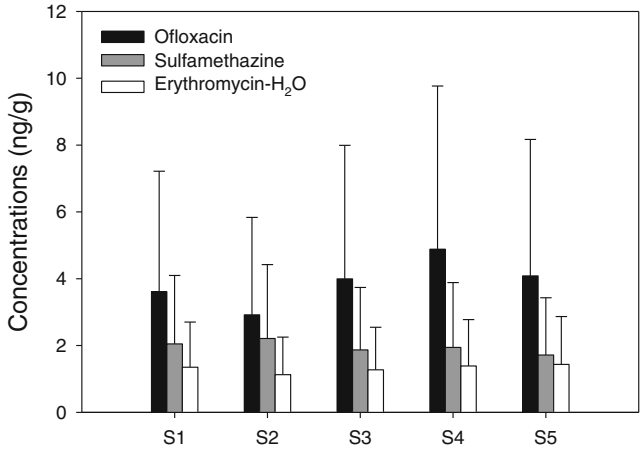

Fig. 3 Concentrations (ng/g) of antibiotics in medium samples from the integrated constructed wetland system. *S1* CW2 medium, *S2* CW3 medium, *S3* CW4 medium, *S4* CW5 medium, *S5* sediment of the receiving river

(Table 3). The removal rates for the other ARGs, *sul*3, *intI*1, *sul*2, and *sul*1, ranged between 83 and 97 %. In the five wetland treatment units, CW3 showed the highest contribution to the total removal rate of ARGs in the ICW, with the contributing rate of 43.6 %, followed by CW2 (27.5 %), CW1 (11.9 %), and CW4 (11.9 %). The least-contributing treatment unit was CW5, with a contributing rate of 2.6 % (Table S7).

Discussion

Removal mechanisms of antibiotics and ARGs in the ICW

Ten out of the 50 target antibiotics were quantitatively detected in wastewaters and mediums of the ICW. High aqueous-phase removals (78 to 100 %) were achieved for ofloxacin, leucomycin, sulfamonomethoxine and trimethoprim, lincomycin, sulfamethazine, and anhydro-erythromycin, while only low to moderate removals for other three antibiotics sulfadiazine, sulfacetamide, and salinomycin (10 to 24 %) (Table 2). Three antibiotics, ofloxacin, anhydro-erythromycin, and sulfamethazine, have been found accumulated in the mediums of all CW units at several nanograms per gram levels (1.12 to 4.88 ng/g) (Table S4 and Fig. 3). It is obvious that adsorption onto medium is an important aqueous-phase removal mechanism for the antibiotics like ofloxacin, anhydro-erythromycin, and sulfamethazine. The results are in good consistence with the previous reports that the predominant removal mechanism for ofloxacin was adsorption onto sludge rather than biodegradation (Golet et al. 2002; Lindberg et al. 2005; Li and Zhang 2010; Jia et al. 2012). It should be noted that we can only show the accumulated concentrations of the target antibiotics in the medium and the aqueous-phase removal in the CWs, due to lack of data from plant uptake, photolysis, and volatilization. Numerous studies have documented that photodegradation contributes to the abiotic transformation of trace organic contaminants such as pharmaceutical and personal care products (PPCPs) and pesticides in wetlands, and the transformation rates are strongly affected by pH and dissolved organic carbon (Zeng and Arnold 2013; Jasper and Sedlak 2013). Several chemical species, such as reduced sulfur species (e.g., bisulfide and polysulfides), dissolved organic matter, and Fe(II)-organic matter complexes, could also contribute to the dissipation of organic contaminants (e.g., pesticides and *p*-cyanonitrobenzene) in wetland systems (Zeng et al. 2012; Zhang and Weber 2013).

CW2 is a subsurface flow-constructed wetland (SSFCW) with chaff used as its substrate, which exhibited nearly

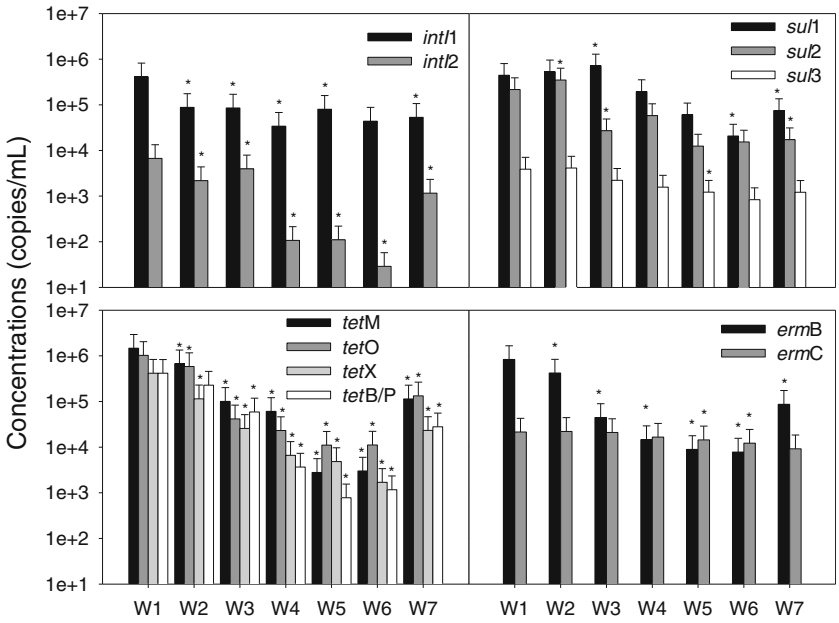

Fig. 4 Concentrations (copies/mL) of antibiotic resistance genes in wastewater samples from the integrated constructed wetland system. *W1* influent, *W2* CW1 effluent, *W3* CW2 effluent, *W4* CW3 effluent, *W5* CW4 effluent, *W6* final effluent, *W7* the receiving river water. *$p<0.05$, significantly different values when compared to the influent (*W1*)

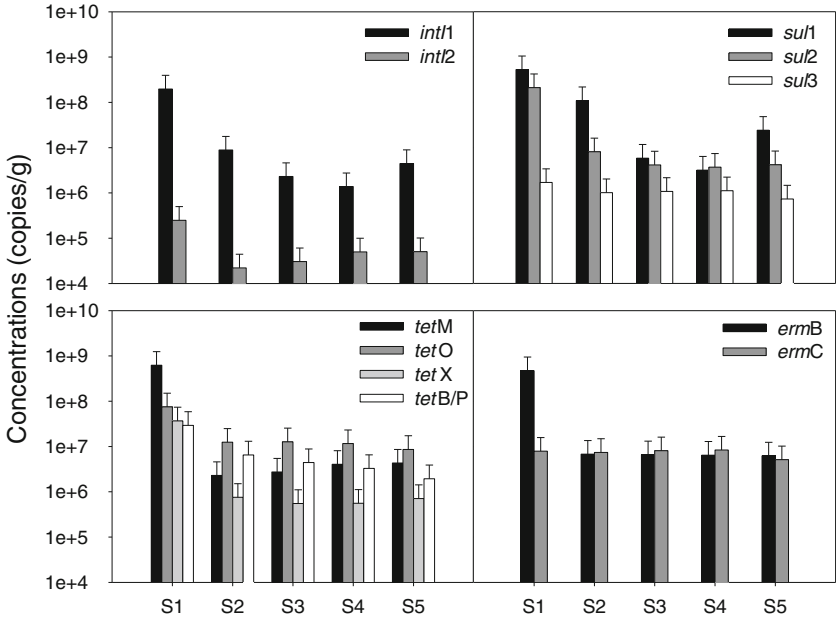

Fig. 5 Concentrations (copies/g) of antibiotic resistance genes in medium samples from the integrated constructed wetland system. *S1* CW2 medium, *S2* CW3 medium, *S3* CW4 medium, *S4* CW5 medium, *S5* sediment of the receiving river

anaerobic condition in this unit. The aqueous-phase removal results showed that 36 % of sulfamonomethoxine by concentration was eliminated in the CW2 treatment unit. It means that biodegradation, especially anaerobic degradation, could play a significant role in elimination of sulfamonomethoxine in this unit. Sulfonamides such as sulfamethazine and sulfamonomethoxine have been reported to be easily biodegradable (Mohring et al. 2009; Li and Zhang 2010; Garcia-Galan et al. 2011; Xu et al. 2007) and transformed in anaerobic sludge digestion (Golet et al. 2002). For leucomycin, the overall removal percentage was 100 % and no accumulation was found in any medium of the ICW units. The present study and previous reports all showed that the removal of macrolides mainly occurred in biological treatment (Zhou et al. 2013). It should also be noted that negative aqueous removal percentages for some detected antibiotics in the present study were obtained when the concentration for an antibiotic in effluent was higher than in influent in some ICW units. This could be explained by sampling variations and presence of antibiotic conjugates and/or metabolites that are reverted back during treatment into its original form (Garcia-Galan et al. 2008).

As emerging environmental contaminants, ARGs were detected worldwide in various environmental compartments (Tamminen et al. 2011; Pruden et al. 2012; Su et al. 2014; Coleman et al. 2013). And the elimination of ARGs is still a challenge even in the municipal wastewater treatment system. Chen and Zhang (2013) found that the *tet* genes (*tet*M, *tet*O, *tet*Q, and *tet*W) were significantly reduced by two to three orders of magnitude in four municipal WWTPs, but a smaller reduction was observed in the rural domestic sewage treatment systems (Chen and Zhang 2013). Small-scale constructed wetland is widely applied particularly in rural areas due to its low construction and operation costs. In the present study, the ICW system showed high removal rates for the ARGs. Except for *erm*C (43 %), the removal rates of the remaining

Table 2 Aqueous concentrations (ng/L) and removal rates (%) of antibiotics in the integrated constructed wetland system

Class	Compound	Influent (ng/L)	Effluent (ng/L)	Total removal rate (%)
Fluoroquinolones	Ofloxacin	193±75.8[a]	n.d.	100±0.00
Ionophores	Salinomycin	4.04±0.03	3.43±0.01	15.2±0.45
Lincosamides	Lincomycin	60.7±0.44	13.4±0.13	78.0±0.39
Macrolides	Erythromycin-H_2O	44.5±2.78	6.64±1.80	85.1±3.78
	Leucomycin	120±7.59	n.d. (<LOQ)	100±0.00
Sulfonamides	Sulfacetamide	3.04±0.10	2.32±0.01	23.8±3.27
	Sulfadiazine	2.40±0.03	2.15±0.01	10.3±1.81
	Sulfamethazine	54.1±1.09	2.70±0.27	95.1±0.71
	Sulfamonomethoxine	50.8±0.00	n.d.	100±0.00
	Trimethoprim	1.93±0.12[a]	n.d.	100±0.00

n.d. not detected, *LOQ* limit of quantification

[a] Mean±standard deviation

Table 3 Aqueous concentrations (copies/mL) and removal rates (%) of antibiotic resistance genes in the integrated constructed wetland system

Gene	Influent (copies/mL)	Effluent (copies/mL)	Total removal rate (%)
*intI*1	$(4.10\pm0.33)\times10^{5}$[a]	$(4.39\pm0.27)\times10^{4}$	89.3±0.27
*intI*2	$(6.68\pm0.04)\times10^{3}$	$(2.88\pm0.86)\times10^{1}$	99.6±0.16
*sul*1	$(2.64\pm0.35)\times10^{6}$	$(7.38\pm0.98)\times10^{4}$	97.2±0.57
*sul*2	$(1.14\pm0.01)\times10^{6}$	$(5.22\pm0.44)\times10^{4}$	95.4±0.53
*sul*3	$(1.05\pm0.07)\times10^{4}$	$(1.75\pm0.25)\times10^{3}$	83.4±1.58
*tet*M	$(1.47\pm0.11)\times10^{6}$	$(3.00\pm0.66)\times10^{3}$	99.8±0.04
*tet*O	$(1.02\pm0.13)\times10^{6}$	$(1.11\pm0.15)\times10^{4}$	98.9±0.28
*tet*X	$(4.17\pm0.72)\times10^{5}$	$(1.69\pm0.12)\times10^{3}$	99.6±0.07
*tet*B/P	$(4.16\pm0.20)\times10^{5}$	$(1.17\pm0.29)\times10^{3}$	99.7±0.23
*erm*B	$(8.33\pm0.93)\times10^{5}$	$(7.80\pm1.43)\times10^{3}$	99.1±0.23
*erm*C	$(2.14\pm0.08)\times10^{4}$	$(1.22\pm0.21)\times10^{4}$	43.1±14.1

[a] Mean±standard deviation

ten ARGs ranged from 83 % (*sul*3) to 100 % (*intI*2, *tet*B/P, *tet*M, and *tet*X) (Table 3). In the present ICW system, CW3 was founded to be the highest contribution (43.6 %) to the total removal rate of ARGs, followed by CW2 (27.5 %) (Table S7). In a lab-scale experiment with two vertical flow-constructed wetland systems, Liu et al. (2013a, b) found that the two systems significantly decreased the concentrations of target antibiotics and tetracycline resistance genes in swine wastewater, but their removal efficiencies were related to wetland medium and structure. The present and previous studies (Liu et al. 2013a, b) demonstrated that sorption onto soil or medium and biodegradation are two main mechanisms for ARG elimination in the ICW system.

The results from the present study clearly demonstrated that the detected antibiotics and ARGs could be efficiently eliminated by ICW. The aqueous removal rates for most of the detected antibiotics and ARGs were consistent with or even better than those in the reported conventional WWTPs (Lindberg et al. 2005; Xu et al. 2007; Li and Zhang 2010; Gao et al. 2012; Jia et al. 2012; Zhou et al. 2013). This suggests that ICW can be applied as an effective treatment facility in rural areas to remove antibiotics and ARGs in domestic sewage.

Pollution loading of antibiotics

Pollution loading of antibiotics in the raw wastewater (influent) could be calculated to indicate the input into an ICW and reflect to a certain degree the use pattern of antibiotics in the service area, while the pollution loading in the final effluent of an ICW could be used to estimate the pollution contribution to the receiving environment. The calculated total pollution loading of all detected antibiotics in the influent was 3,479 μg/day, while the total pollution loading to the receiving environment (a small river in the present study) based on the concentration data in the final effluent was 199 μg/day (Table 4). The results from the present study also showed that lincomycin (87 μg/day) was the main antibiotic that reached the receiving environment via discharge of effluents (Table 4). Pollution loading of antibiotics in the effluent is affected by their concentrations in the influent, fate, and removal rates during the treatment. The pollution loadings of all detected antibiotics in the final effluent ranged from n.d. to 87.1 μg/day (Table 4). This pollution loading level in the present study is

Table 4 Mass fluxes (μg/d) of antibiotics in wastewaters of the integrated constructed wetland system

Class	Compound	Wastewater						
		W1	W2	W3	W4	W5	W6	W7
Fluoroquinolones	Ofloxacin	1,255±603[a]	1,083±306	163±31.3	n.d.	n.d.	n.d.	n.d.
Ionophores	Salinomycin	26.3±0.27	25.7±0.56	22.8±0.14	27.3±0.85	45.2±1.37	22.3±0.12	22.1±0.11
Lincosamides	Lincomycin	395±3.50	452±12.2	455±16.8	458±22.2	470±42.3	87.1±1.01	93.2±7.87
Macrolides	Erythromycin-H_2O	289±22.1	282±22.8	331±39.1	268±11.4	161±5.54	43.2±14.3	40.3±5.54
	Leucomycin	784±60.4	982±39.3	732±20.6	408±24.3	182±5.01	n.d.	n.d.
Sulfonamides	Sulfacetamide	19.8±0.76	21.0±0.50	19.0±0.65	18.0±0.38	32.2±1.24	15.1±0.11	14.2±0.06
	Sulfadiazine	15.6±0.25	16.1±1.28	17.5±0.16	19.0±0.63	14.4±0.81	14.0±0.09	13.3±0.15
	Sulfamethazine	352±8.68	384±5.51	324±12.9	227±2.53	59.3±8.94	17.6±2.16	27.0±2.80
	Sulfamonomethoxine	330±0.00	384±181	265±0.00	n.d.	n.d.	n.d.	n.d.
	Trimethoprim	12.5±0.94	12.3±0.37	4.60±0.20	n.d.	n.d.	n.d.	n.d.
The mass fluxes of all detected antibiotics in each unit		3,479.2±830	3,642.1±345	2,333.8±59.9	1,425.3±53.3	964.1±44.6	199.3±16.1	210.1±12.8

n.d. not detected

[a] Mean±standard deviation

much lower than that of some previous studies calculated by using the concentrations of antibiotics in effluent from urban WWTPs (Garcia-Galan et al. 2011; Gao et al. 2012; Jia et al. 2012; Leung et al. 2012). Based on the present study, antibiotics are still a concern due to their frequent detection in rural domestic sewage. In order to reduce the potential risks, it would be necessary to apply a wastewater treatment technology such as ICW in rural areas.

Conclusions

The present study showed that leucomycin, ofloxacin, lincomycin, and sulfamethazine, and *sul*1, *sul*2, *tet*M, and *tet*O were the predominant antibiotics and ARGs, respectively, in the rural domestic sewage. The results have demonstrated that the concentration levels of most detected antibiotics and ARGs in rural domestic sewage could be effectively reduced (78 to 100 % for antibiotics and >99 % for ARGs) through the wetland treatment systems. However, additional research is needed to better illustrate the fate and removal mechanism of antibiotics and ARGs in ICWs. The total pollution loading of antibiotics to the receiving environment via ICW effluent discharge was found at the relatively low levels. Therefore, ICW could be applied as an important treatment technology for the removal of antibiotics and ARGs, and other emerging contaminants and their metabolites resulting from agricultural activities or aquaculture in rural areas.

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