Chemical Engineering Journal 308 (2017) 692–699

Contents lists available at ScienceDirect

Chemical Engineering Journal

journal homepage: www.elsevier.com/locate/cej

Removal of antibiotics and resistance genes from swine wastewater using vertical flow constructed wetlands: Effect of hydraulic flow direction and substrate type

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HIGHLIGHTS

- Antibiotic removal from wastewater using CWs is greatly affected by substrate type.
- Surface soil and selected substrates behaved differently to OTC and DIF adsorption.
- Variation of ARG relative abundances are determined by hydraulic flow direction.
- Most $\sum$*tet* relative abundances were amplified in effluents of up-flow treatments.

ARTICLE INFO

Article history:
Received 23 July 2016
Received in revised form 21 September 2016
Accepted 22 September 2016
Available online 23 September 2016

Keywords:
Constructed wetlands
Substrate type
Hydraulic flow direction
Antibiotics
Antibiotic resistance genes

ABSTRACT

The fate of antibiotics and antibiotic resistance genes (ARGs) in constructed wetlands has received more concerns recently. This study aimed to assess the influence of hydraulic flow direction (down-flow or up-flow) and substrate type (brick rubble or oyster shell) on removal of antibiotics and ARGs from swine wastewater. For antibiotic removal, all of treatments can remove more than 84% of oxytetracycline and difloxacin during two stages of operation. Brick-based columns had stronger antibiotic removal capacity due to properties of brick, including large porosity and micropore size and 32% of Fe_2O_3. The surface soils tended to adsorb much more oxytetracycline than difloxacin, as was opposite to brick and oyster shell. For ARGs removal, the removal efficiencies of tetracycline-resistance genes (*tet*) and integrase gene of Class 1 integrons ranged from 33.2 to 99.1%, without significant difference among treatments ($p > 0.05$). However, most of $\sum$*tet* relative abundances in effluents of up-flow treatments were higher than those in influents, which indicated a risk to release relatively more antibiotic-resistant bacteria in proportion to total bacteria into environment. Significantly correlated with antibiotic concentrations, ARGs relative abundances in surface soils increased as the time progressed for all the treatments. Our results demonstrate that antibiotic removal using constructed wetlands is greatly affected by substrate type, whereas variation of ARG relative abundances in effluents and soils are determined by hydraulic flow direction.

1. Introduction

Veterinary antibiotics are a group of emerging compounds that have been extensively used in animal husbandry as feed additives, and for prophylactic and therapeutic purposes [1]. They have received great public concern because of their presence and persistence in aquatic and soil environments [2], and potential toxic effects and ARGs induction on indigenous microorganisms [3]. During animal production, the majority of antibiotics cannot be completely metabolized in animal's gut. Meanwhile, they select for resistance in intestinal microbiota. On-farm lagoon is widely

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Abbreviations: ARGs, antibiotic resistance genes; CWs, constructed wetlands; HLR, hydraulic loading rate; HRT, hydraulic retention time; ORP, oxidation–reduction potential; OTC, oxytetracycline; DIF, difloxacin; *tet*, tetracycline-resistance genes; *intI*1, integrase gene of Class 1 integrons; BD, brick and down-flow; BU, brick and up-flow; SD, oyster shell and down-flow; SU, oyster shell and up-flow.

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http://dx.doi.org/10.1016/j.cej.2016.09.110
1385-8947/

used for animal waste treatment. However, recent studies have demonstrated that lagoon treatment is not efficient enough to reduce antibiotics and ARGs in manure [4–5]. The reported concentration of antibiotics ranged from 23.8 to 685.0 μg/L in swine wastewater, with some varying from trace amounts to as high as ppm levels in manure slurry [6–7]. Relative abundances of some ARGs tended to increase after lagoon treatment [8]. Manure application would therefore cause antibiotics deposition and ARGs transfer in farmland soils [1] and possibly affect the quality of surface water and groundwater furthermore [2,9].

In order to reduce the discharge of antibiotics and ARGs from animal feeding operation, post-treatment process after waste lagoon storage is required. Constructed wetlands (CWs) are engineered systems that have been widely used as secondary wastewater treatment due to their easy operation and low maintenance costs [10–11]. During the treatment process, nutrients, heavy metals as well as pathogens are the primary constituents of concern [12–13]. Recently, CWs were successfully used to remove a variety of micro-pollutants such as pharmaceuticals from municipal and agricultural wastewater [14–17]. Several studies attempted to remove veterinary antibiotics by CWs and obtained different treatment efficiencies depending on the configurations, hydraulic loads and compounds they chose [15,18]. Meanwhile, the presence and variation of ARGs within CWs have received some attention gradually [19–20]. Antibiotic resistance investigation in CWs previously focused on comparing the number of fecal indicator bacteria tolerant to antibiotics in influents and effluents, which only covered a small fraction of microorganisms [13]. Currently, the abundance and diversity of ARGs during treatment process have been investigated in whole microbial communities by 16S rRNA gene quantification [20–21]. However, these investigations were mostly carried out in pilot or field scale with certain system configuration and operation condition. The effect of different operation conditions such as easily available substrates, water flow directions and seasons, on removal of antibiotics and ARGs by CWs require further estimation.

Based on water flow patterns, CWs can be generally classified into surface free water constructed wetlands (SF-CWs), horizontal subsurface flow constructed wetlands (HSSF-CWs), and vertical subsurface flow constructed wetlands (VSSF-CWs). In this study, we constructed mesocosm vertical flow wetlands to remove antibiotics and ARGs from swine wastewater. The selected main substrates, brick rubble and oyster shell, are two widely-distributed solid wastes along southeast coast of China. Microcosm experiment was carried out to further confirm the different removal capacities of substrates. Two hydraulic flow directions, up-flow and down-flow, were compared because they potentially influences the contact pattern between pollutants and substrates, hydraulic retention time (HRT) and oxidation–reduction potential (ORP). The target antibiotics, oxytetracycline (OTC) and difloxacin (DIF), belong to tetracyclines and fluroquinolones that are extensively used in livestock industry. These compounds were frequently detected in farmlands soils with high concentrations (189.8–2668.9 μg/kg) [1] which exceeded the ecological risk trigger value (100 μg/kg) established by the Steering Committee of the Veterinary Internationa1 Committee on Harmonization (VICH). Three *tet* genes (*tet*W, *tet*A, *tet*X) were chosen as representatives of three tetracycline-resistance mechanisms (ribosomal protection protein, efflux pump and enzymatic modification) due to their frequent occurrence in wastewater treatment lagoon [8]. The integrase gene of Class 1 integrons (*intI*1) was also determined since it contributes to evolution and proliferation of multiple antibiotic resistant bacteria.

The main objectives of this study were: (1) to investigate OTC and DIF removals in mesocosm and microcosm CWs, and (2) to assess *tet* genes and *intI*1 reduction in mesocosm CWs, with different substrates and water flow directions.

2. Materials and methods

This study was composed of two stages of mesocosm experiment and one microcosm experiment. The first stage of mesocosm lasted three months from October 2013 to January 2014 in winter. To reliably estimate CWs performances, the second stage of experiment was conducted from April 2015 to July 2015 in early summer. During mesocosm experiment, to keep CWs running stably, we did not collect the substrates from bottom layer and determine their antibiotic concentrations. Alternatively, microcosm experiment was conducted to compare antibiotic adsorption between two main substrates.

2.1. Mesocosm experiment

Four PVC columns, 25 cm in diameter and 60 cm in height, were situated in a controlled greenhouse. At the bottom, two columns were filled with 450 mm high layer of 10–20 mm brick particles, while the others were filled with oyster shell of similar size. At the top, all the columns were filled with 100 mm high of a mixture of red soil and humus soil (2:1). *Phragmites australis* were planted at the top layer. The CWs had been fed with tap water for several months to promote plant growth. Wastewater was taken from anaerobic lagoon of swine feedlots (in Xiamen, China) and diluted with tap water. The diluted wastewater, which already contained some antibiotics (OTC: 30.28 ± 9.38 μg/L; DIF: 0.30 ± 0.12 μg/L), was doped with a mixture of OTC and DIF (250 μg/L individually). Each system was injected with doped wastewater twice per day (1.25 L × 2), corresponding to hydraulic loading rate (HLR) of 5.1 cm/d. The doped wastewater was refreshed every 2 days. The down-flow and up-flow direction were employed for two of CWs respectively. Thus, the experimental CWs were designated as Brick-Down (BD), Brick-Up (BU), Shell-Down (SD) and Shell-Up (SU). The HRTs were 1.9, 5.8, 1.6 and 4.9 days for BD, BU, SD and SU respectively. Fig. 1 displays the configuration and water flow of CWs. Table S1 shows the physicochemical properties of OTC and DIF. Several physical and chemical properties of brick and oyster shell had been presented in Wang et al. [22]. According to X-ray diffraction patterns (XRD), bricks are composed of two crystalline species, SiO_2 (68%) and Fe_2O_3 (32%), while oyster shells contain three crystalline species, $CaCO_3$ (93%), NaCl (4%) and SiO_2 (3%).

During two stages of operation, influents, effluents, surface soils and plant blades were collected when the experiment started and every 30 days. For each treatment, waters were collected daily for five days and combined as composite samples; surface soils were taken at a depth of ~5 cm from different positions and mixed to form composite samples; five blades were taken from different plants. When the second stage was finished, the systems continued to run until October 2015 injected with tap water. The soil samples were then collected on days 120 and 180 to observe the decay of antibiotics. All the samples were stored at −20 °C before analysis.

2.2. Microcosm experiment

Microcosm experiment was carried out in laboratory under shading condition, using four small columns, 3.25 cm in diameter and 40 cm height. Columns were filled with 30 cm high of 3–5 mm brick granule or oyster shells without soil and plant. The columns were fed with deionized water doped with a mixture of OTC and DIF (250 μg/L individually) using down-flow or up-flow directions. The HLR was adjusted to 2 cm/d. The microcosm CWs