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Efficient removal of antibiotics in surface-flow constructed wetlands, with no observed impact on antibiotic resistance genes

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HIGHLIGHTS

- Antibiotics were added to constructed wetlands.
- Removal of antibiotics and impact on resistance genes were assessed.
- Antibiotics were efficiently removed in the constructed wetlands.
- No impact on resistance gene abundance could be observed.
- Constructed wetlands may be useful for removing antibiotics from wastewater.

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ABSTRACT

Recently, there have been growing concerns about pharmaceuticals including antibiotics as environmental contaminants. Antibiotics of concentrations commonly encountered in wastewater have been suggested to affect bacterial population dynamics and to promote dissemination of antibiotic resistance. Conventional wastewater treatment processes do not always adequately remove pharmaceuticals causing environmental dissemination of low levels of these compounds. Using constructed wetlands as an additional treatment step after sewage treatment plants have been proposed as a cheap alternative to increase reduction of wastewater contaminants, however this means that the natural microbial community of the wetlands becomes exposed to elevated levels of antibiotics. In this study, experimental surface-flow wetlands in Sweden were continuously exposed to antibiotics of concentrations commonly encountered in wastewater. The aim was to assess the antibiotic removal efficiency of constructed wetlands and to evaluate the impact of low levels of antibiotics on bacterial diversity, resistance development and expression in the wetland bacterial community. Antibiotic concentrations were measured using liquid chromatography–mass spectrometry and the effect on the bacterial diversity was assessed with 16S rRNA-based denaturing gradient gel electrophoresis. Real-time PCR was used to detect and quantify antibiotic resistance genes and integrons in the wetlands, during and after the exposure period. The results indicated that the antibiotic removal efficiency of constructed wetlands was comparable to conventional wastewater treatment schemes. Furthermore, short-term treatment of the constructed wetlands with environmentally relevant concentrations (i.e. 100–2000 ng $\times$ l^{-1}) of antibiotics did not significantly affect resistance gene concentrations, suggesting that surface-flow constructed wetlands are well-suited for wastewater treatment purposes.

1. Introduction

Over the last decade, there have been growing concerns about the effects of pharmaceuticals and antibiotics on bacterial populations in the environment. It has been shown that these micro-pollutants enter the aquatic environment after being discharged in municipal and hospital wastes (Heberer, 2002; Segura et al., 2009; Verlicchi et al., 2012). Numerous studies have demonstrated that municipal and hospital wastewater treatment plants are not always efficient enough and do not always fully remove pharmaceuticals from the water. Consequently, antibiotics and related compounds have been detected in various bodies of water at levels ranging from ng $\times$ l^{-1} to low μg $\times$ l^{-1} (Lindberg et al., 2004; Gros et al., 2006; Loos et al., 2009; Hughes et al., 2013). A number of sophisticated treatment technologies have been developed to increase the efficiency with which pharmaceuticals are removed from

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wastewater streams before they are discharged. These include advanced oxidation and UV exposure (Zwiener and Frimmel, 2000; Ternes et al., 2003). However, while these methods may be useful, they are costly to implement. Consequently, there is a growing need to explore alternative low-cost technologies that complement conventional methods.

Recently, it has been shown that treating wastewater using constructed wetlands (CWs) can serve as a cost-effective and promising alternative to conventional wastewater treatment methods for removing or reducing levels of nitrogen, phosphorous, pharmaceuticals and the BOD (biological oxygen demand) in treated water (Kiviasi, 2001; Matamoros et al., 2008; Llorens et al., 2009; Matamoros et al., 2009; Park et al., 2009; Dordio et al., 2010; Hijosa-Valsero et al., 2010; Hijosa-Valsero et al., 2011; Breitholtz et al., 2012). CWs can facilitate the removal of pharmaceuticals via natural processes involving plants, microorganisms, solid matrix components and sunlight (Dordio et al., 2010).

The removal efficiency of pharmaceuticals in CWs has been demonstrated to be comparable to conventional wastewater treatment processes (Matamoros et al., 2008; Matamoros et al., 2009; Breitholtz et al., 2012). Studies have also indicated that passage through CWs removes antibiotics (Park et al., 2009; Hijosa-Valsero et al., 2011; Breitholtz et al., 2012) making them potentially attractive options for this purpose.

Some concerns regarding using CWs for wastewater treatment should be addressed. The ability of CWs to improve water quality in wastewater effluents depends heavily on the bacterial communities present within them. Processes such as ammonia oxidation, nitrogen fixation and denitrification are mediated by different bacterial taxa. Changes in the makeup of the bacterial community such as those that might be induced by a high concentration of antibiotics in the influx could thus, affect the functionality and water purifying properties of a constructed wetland (Scholz and Lee, 2005).

Furthermore, it is believed that environmental bacteria carrying antibiotic resistance genes (ARGs) may function as reservoirs of resistance, which may be transferred to pathogenic bacteria. Anthropogenic release of antibiotics in the environment may facilitate both proliferation and dissemination of ARGs (Baquero et al., 2008). Studies investigating antibiotics in rivers, wastewaters, etc. (Lindberg et al., 2005; Abuin et al., 2006; Segura et al., 2009; Verlicchi et al., 2012; Hughes et al., 2013) as well as studies investigating ARGs (Pei et al., 2006; Xi et al., 2009; Börjesson et al., 2010; Knapp et al., 2010) show that both antibiotics and ARGs are prevalent in many different environments. Environments polluted with high levels of antibiotics have also been shown to contain high levels of ARGs (Kristiansson et al., 2011; Khan et al., 2013). Additionally, class 1 integrons, a common genetic assembly platform capable of capturing and disseminating ARGs (Mazel, 2006), have been found to be positively correlated to polluted environments (Wright et al., 2008; Gaze et al., 2011).

While low levels of antibiotics are frequently encountered in the environment, it is unknown what effect such concentrations have on resistance gene development and dissemination (Kümmerer, 2004). It has been shown that sub-inhibitory concentrations of antibiotics can encourage the selection of antibiotic resistant bacteria in vitro (Gullberg et al., 2011), suggesting that low concentrations in the environment could have the same effect. To be able to properly evaluate the risks of environmental antibiotic contamination it is important to elucidate the effect of low, environmentally relevant concentrations (i.e. ng $\times$ l^{-1} to low μg $\times$ l^{-1} levels, which are commonly encountered in wastewater) of antibiotics on larger scale microbial ecosystems.

The aim of this study was to investigate the removal efficiency of antibiotics for surface-flow CWs and to evaluate the short-term effect of environmentally relevant concentrations of antibiotics on the diversity and evenness of a CW bacterial community, and ARG and integron abundance and expression. A replicate series of surface-flow CWs was exposed to low levels of antibiotics (ranging from 100–2000 ng $\times$ l^{-1}) for 25 days and concentrations of antibiotics were measured with liquid chromatography–mass spectrometry (LC–MS/MS). Nucleic acids were extracted and concentrations of ARGs, ARG cDNA and integrons were measured with real-time PCR. Furthermore, the effect on the diversity and evenness of the eubacterial community was analysed by denaturing gradient gel electrophoresis (DGGE).

2. Materials and methods

2.1. Sampling site and experimental set-up

The experiment was carried out in an experimental wetland park near Halmstad, Sweden, consisting of 18 similarly shaped, small (water surface 29 m^2) surface-flow wetlands (Weisner and Thiere, 2010; Bodin et al., 2012). The wetlands are not connected to any wastewater outlets and had not previously been exposed to any anthropogenic source of antibiotics. In-flowing groundwater with pH 6.5 was distributed through individual pipes to each wetland. Water flows were adjusted using gate valves fitted on each inlet pipe. The mean water depth was 0.6 m with a slide slope of 1:1 or 45° and the inflows were set to 2 l $\times$ min^{-1}. The corresponding hydraulic loads were 0.1 m $\times$ day^{-1} giving a theoretical residence time of 5.7 days. The individual wetlands were located 2–3 m apart and heavy clay constituted the soil in the area, minimising water exchange between the wetlands and the surrounding ground.

Eight of the wetlands in the wetland park were randomly selected to be used in the experiment. Vegetation in these wetlands was dominated by *Phragmites australis* and *Typha latifolia*. In four wetlands (designated A, B, C, and D) a mixture of 12 antibiotics (Table 1) was continuously added for 25 days. The antibiotics and concentrations were Azithromycin (AZI) (0.1 μg $\times$ l^{-1}), Ciprofloxacin (CIP) (0.2 μg $\times$ l^{-1}), Clarithromycin (CLA) (0.4 μg $\times$ l^{-1}), Clindamycin (CLI) (0.2 μg $\times$ l^{-1}), Doxycycline (DOX) (0.1 μg $\times$ l^{-1}), Erythromycin (ERY) (2.0 μg $\times$ l^{-1}), Norfloxacin (NOR) (1.0 μg $\times$ l^{-1}), Oxytetracycline (OXY) (0.4 μg $\times$ l^{-1}), Sulfamethoxazole (SUL) (1.0 μg $\times$ l^{-1}), Tetracycline (TET) (1.0 μg $\times$ l^{-1}), Trimethoprim (TRI) (1.0 μg $\times$ l^{-1}) and Vancomycin (VAN) (0.1 μg $\times$ l^{-1}). The antibiotic concentrations corresponded to ten times the average measured environmental levels in treated effluent (Segura et al., 2009; Verlicchi et al., 2012). Solutions of antibiotics were prepared by dissolving the antibiotics in 10 ml of methanol and adding it to 25 l of groundwater taken from the same source as the in-flow water. The solutions were subsequently added to the individual inlet pipes using pumps (iksVario Blue II, iksKarlsbad, Germany) at a flow rate of 2.0 ml $\times$ min^{-1}. The antibiotic solutions were replaced once a week and all solutions were analysed prior to addition. Four additional wetlands were used as controls (designated E, F, G and H), with no antibiotics added. The experiment started at 1 July 2009. Temperatures for in-going and outgoing water were measured between 8.6–9.8 °C and 10.7–17.1 °C respectively.

Samples were taken from all 8 wetlands immediately before antibiotic addition (i.e. day '0'), and on days '7', '14', '21', '25', '50', and '100' after the experiment started. Samples were additionally taken at days '220' and '460' to evaluate long term effects after the antibiotic treatment had stopped. Each sample consisted of water and stirred up sediment gathered from five different points along a transect running across the wetland. 200 ml of water was gathered from each point and pooled to a final volume of 1 l per sample. The water phase of the sample was used for chemical analysis and the sediment phase was used for molecular biological analyses.

2.2. Chemicals

All antibiotics used were purchased from Sigma Aldrich (Steinheim, Germany) and were of HPLC grade purity (>98%). Methanol, acetonitrile and water (Lichrosolv, Hypergrade) were obtained from Merck (Darmstadt, Germany) and formic acid (puriss pa) from Fluka