



Antibiotic resistance profiles of representative wetland bacteria and faecal indicators following ciprofloxacin exposure in lab-scale constructed mesocosms

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ABSTRACT

In this study, the effect of the antibiotic ciprofloxacin on the presentation and potential development of antibiotic resistance among selected faecal indicators (*Escherichia coli* and *Enterococcus* spp.) and the intrinsic bacterial community for a freshwater model ecosystem was investigated. Four parallel mesocosm wetlands planted with *Phragmites australis* were initially seeded with an activated sludge inoculum from a wastewater treatment plant and monitored over the course of 63 days. Two of the four mesocosms (CIP1 and CIP2) were initially exposed to a ciprofloxacin concentration of $2 \mu\text{g mL}^{-1}$ for a 5 day period, and two were considered controls (CON1 and CON2). Minimum inhibitory concentrations were determined for the culturable interstitial microbial community using ciprofloxacin concentrations ranging from 0.5 to $16 \mu\text{g mL}^{-1}$ and compared with total heterotrophic plate counts (HPCs). *E. coli* and *Enterococcus* spp. were enumerated and screened for antimicrobial resistance to twelve different antibiotics both before and after mesocosm ciprofloxacin exposure. Concentrations for total heterotrophs within the interstitial water were in the order of 10^6 – 10^8 CFU 100 mL^{-1} , while *E. coli* and *Enterococcus* spp. were present in lower concentrations, at 10^2 – 10^3 CFU 100 mL^{-1} . All four mesocosms had the same minimum inhibitory concentrations (MICs) of $2 \mu\text{g mL}^{-1}$ for total heterotrophic community during the first week of start-up (before ciprofloxacin exposure), followed by a sharp increase in ciprofloxacin exposed mesocosms on day 14 (after ciprofloxacin exposure), reaching a maximum MIC of $16 \mu\text{g mL}^{-1}$. The resistance levels of interstitial heterotrophic counts determined by the agar dilution method peaked 7 days following the ciprofloxacin exposure, and decreased thereafter. A significant increase ($p < 0.05$) to the following antibiotics was observed among *E. coli* following ciprofloxacin exposure within mesocosm CIP2: ciprofloxacin (CIP), chloramphenicol (C), cefotaxime (CTX), ceftriaxone (CRO), and doxycycline (D). These results illustrate the potential for a single antibiotic exposure event to create a noticeable increase in the antibiotic resistance profile, not only to the imposed antibiotic, but to other classes of antibiotics. The initial background levels of resistance among *Enterococcus* spp. were significantly higher than those observed among *E. coli*, exhibiting a combined average of 96%, 96%, and 100% resistance to ciprofloxacin (CIP), linezolid (LZD), and streptomycin (S), respectively. Following the addition of ciprofloxacin, *Enterococcus* spp. retained high levels of resistance to ciprofloxacin, linezolid, and streptomycin, with a significant increase ($p < 0.05$) to vancomycin (VA), reaching 100% on Day 63. The findings of this study suggest that the transient presence of an isolated exposure event of ciprofloxacin can impact the antibiotic resistance profiles among faecal indicators in lab-scale constructed mesocosms and has the potential to impact constructed wetland treatment systems.

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1. Introduction

Bacterial resistance to antibiotics has become a serious problem among pathogenic bacteria, which has led to an increased concern surrounding environmental risks and potential spread of antibiotic resistance among microorganisms. Resistance is typically common where antibiotics are heavily used (hospitals, long term care centers, and large livestock operations), although antibiotic resistant

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bacteria are also shown to be present in wastewater, surface water, ground water, sediments and soils, and increasingly in surface aquatic environments (Baquero et al., 2008; Martinez, 2008; Zhang et al., 2009a). Due to the high amounts of microbial biomass and the abundance of nutrients, wastewater treatment plants serve as potential hot spots for horizontal gene transfer, frequently involving the passage of plasmids and transposons encoding antibiotic resistance (Guardabassi et al., 2002). The combination of municipal sewage and hospital waste effluents, with surface waters, may facilitate the spread of antibiotics, antibiotic resistance genes, and antibiotic resistant bacteria within wastewater treatment systems (Schluter et al., 2007; Zhang et al., 2009b). The widespread use of antibiotics in animal husbandry and agriculture for treating or preventing infection and promoting growth, can also play an important role in the development and spread of antibiotic resistance (Witte, 2001). It is known, for example, that long-term exposure to low doses of an antibiotic over time is likely to contribute to the selection of resistant bacteria (Gellin et al., 1989).

Faecal indicator organisms are typically used to demonstrate the potential presence or absence of groups of pathogens associated with wastewater or sewage sludge. *Escherichia coli* and *Enterococcus* spp. are two representative organisms that have been used as indicators for faecal contamination (Kator and Rhodes, 2003). *Escherichia coli* is a useful enteric bacterium for the study of water-borne transfer of antibiotic resistance because it has adapted to human and other warm-blooded animal gastrointestinal tracts, and is exposed to a variety of medical and veterinary antibiotic treatments (Edge and Hill, 2005). Due to their intrinsic ability to acquire vancomycin resistance genes (Panesso et al., 2002; Shaghghi et al., 2007), *Enterococcus* spp. have become a common cause of nosocomial infections and also represents an important factor in the emergence of vancomycin resistant strains (CDC NNIS System, 2003). Enterococci are intrinsically “rugged” bacteria, and as such are able to survive under unusually wide ranges of temperature, pH, and salinity, as well as resisting the bactericidal effects of detergents such as bile salts and sodium dodecyl sulfate (Flahaut et al., 1996). The most common cause of the vast majority of clinical enterococcal infections in humans is associated with *Enterococcus faecalis* and *E. faecium* (Mundy et al., 2000). The emergence of *E. faecalis* and *E. faecium* as leading nosocomial pathogens has paralleled the appearance of resistant strains within both species to most antimicrobial drugs used to treat human infections. The range of antimicrobial agents to which enterococci have acquired resistance is quite broad and appears to be escalating at a rate that closely approximates the introduction of new agents to the pharmaceutical market (Gonzales et al., 2001; Shepard and Gilmore, 2002).

Constructed wetlands (CWs) are engineered systems which are designed and constructed to utilize natural processes involving wetland vegetation, solids and associated microbial assemblages to assist in treating wastewaters (Vymazal, 2005a). Constructed wetlands (CWs) are designed to take advantage of many processes that occur in natural wetlands, but with a more controlled approach offering relatively low investment and operation costs, while producing high quality effluent with less dissipation of energy (Song et al., 2008). Recently, attention has focused on the ability of constructed wetlands to reduce pathogenic bacteria in wastewater (Karim et al., 2004; Weber and Legge, 2008). The removal rate of microorganisms in constructed wetlands has shown varying degrees of effectiveness; however, several studies illustrate the improvement of microbial water quality using constructed wetlands (Vymazal, 2005b; Song et al., 2006). Several mesocosm studies have recently been used for undertaking a quantitative approach to the study of constructed wetlands (CWs) (Stein et al., 2006; Werker et al., 2004; Weber et al., 2008, 2010). The

mesocosm approach has been shown to suitably represent interactions between microorganisms, differing substrates, and contaminants within a complex rhizosphere system (Stein et al., 2006; Werker et al., 2007; Weissner et al., 2008). CW mesocosms cannot be said to completely represent full-scale CWs, as full-scale CWs can contain many different hydrological, biological and geochemical sub-environments (or “units”) within the same system.

The occurrence of antibiotics in aquatic environments is of ecotoxicological concern because of potential ecosystem alteration. Antibiotic residues have been detected in the final effluents of wastewater treatment plants (WWTPs) worldwide (Batt and Aga, 2005; Carballa et al., 2004; Costanzo et al., 2005). Contamination of surface waters by antibiotics and other pharmaceutical compounds has been reported in recent studies through discharge from domestic sewer systems (Metcalf et al., 2003). Antibiotics used in human treatment have the potential to enter the environment by excretion or by disposal of surplus drugs into sewage systems, which are eventually released into the local aquatic surroundings from the effluent of WWTPs (Jorgensen and Halling-Sorensen, 2000). Ciprofloxacin is a second generation fluoroquinolone antibiotic, and is commonly used to treat bacterial infections. Although quinolones are prescribed less often than macrolides, these compounds are still the fourth most prescribed class of antimicrobials in Canada (Miao et al., 2004). In particular, ciprofloxacin has dominated the Canadian and global quinolone markets since its entry in the late 1980s. Ciprofloxacin was also chosen as the antibiotic of choice because it is a broad-spectrum antibiotic, effective against both Gram-negative (*E. coli*) and Gram-positive (*Enterococcus* spp.) bacteria.

Little information is available on the prevalence of antibiotic resistance among indicator and pathogenic microorganisms in wetlands designed for wastewater treatment or in natural wetland systems. Some researchers have used mesocosm models to study the effects of antibiotics on removal rates of faecal indicators (Atoyan et al., 2007) or the effect of antibiotic administration modes on the development of antibiotic resistance (Yu et al., 2009). However, to the best of our knowledge, this is the first study that examines and monitors antibiotic resistance profiles among both faecal indicators (*E. coli* and *Enterococcus* spp.) and total heterotrophs before and after the addition of an antibiotic exposure, within a bench-scale system. The objective of this study was to better understand the effect an influent antibiotic has on both the intrinsic microbial community and the resulting impact on the level of antibiotic resistance among representative faecal indicators (*Escherichia coli* and *Enterococcus* spp.) in a model wetland system.

2. Materials and methods

2.1. Wetland mesocosms

Four mesocosm wetlands used in this study (labelled as CON1, CON2, CIP1, and CIP2) were planted with *Phragmites australis* and seeded with activated sludge from a wastewater treatment plant in south-western Ontario. The mesocosms in this study were set up in parallel duplicates, and of the same design as those used previously by Weber and Legge (2010) and Weber et al. (2011). Water entered approximately 65 cm from the bottom of the mesocosm resulting in an oxidative reduction potential (ORP) of ~–125 mV at the bottom. Each mesocosm was comprised of schedule 80 (wall thickness of 1.5 cm), polyvinylchloride (PVC) columns (90 cm by 25 cm diameter) filled to ~80 cm with pea gravel (average equivalent spherical diameter of 2 cm, 80% limestone) and operated to 70 cm with tap water. Water was circulated using a 1/200 HP, 3200 rpm, March (Glenview, Illinois) series 1 (1A-MD 1/2)

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