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Biodegradation of the veterinary antibiotics enrofloxacin and ceftiofur and associated microbial community dynamics

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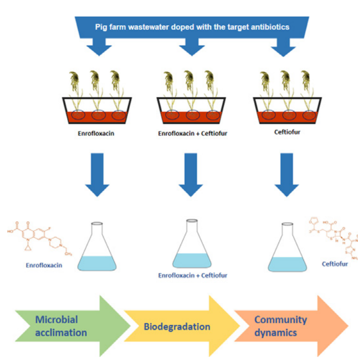
HIGHLIGHTS

- Rhizosediment of plants from constructed wetlands were used as microbial inocula.
- The acclimation with ENR and CEF led to changes on microbial communities.
- Biodegradation of ENR was never complete and defluorination was the limiting step.
- CEF was always completely removed by a combination of biotic and abiotic degradation.
- Concomitant presence of the two antibiotics did not influence their biodegradation.

GRAPHICAL ABSTRACT



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ABSTRACT

Fluoroquinolones and cephalosporins are two classes of veterinary antibiotics arising as pollutants of emerging concern. In this work, the microbial degradation of two representative antibiotics of both these classes, enrofloxacin (ENR) and ceftiofur (CEF), is reported. Biodegradation of the target antibiotics was investigated by supplementing the culture medium with ENR and CEF, individually and in mixture. Microbial inocula were obtained from rhizosphere sediments of plants derived from experimental constructed wetlands designed for the treatment of livestock wastewaters contaminated with trace amounts of these antibiotics. Selected microbial inocula were acclimated during a period of 5 months, where the antibiotics were supplemented every three weeks at the concentration of 1 mg L^{-1}, using acetate as a co-substrate. After this period, the acclimated consortia were investigated for their capacity to biodegrade 2 and 3 mg L^{-1} of ENR and CEF. Complete removal of CEF from the inoculated culture medium was always observed within 21 days, independently of its concentration or the concomitant presence of ENR. Biodegradation of ENR decreased with the increase in its concentration in the culture medium, with defluorination percentages decreasing from ca. 65 to 4%. Ciprofloxacin and norfloxacin were detected as biodegradation intermediates of ENR in the microbial cultures supplemented with this antibiotic,

Abbreviations: CEF, ceftiofur; CIP, ciprofloxacin; CP, cephalosporin; ENR, enrofloxacin; FQ, fluoroquinolones; MM, minimal medium; NOR, norfloxacin; OD, optical density; OTU, Operational Taxonomic Unit; QIIME, Quantitative Insights into Microbial Ecology; TISAB, total ionic strength adjusting buffer.

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indicating that defluorination of at least part of ENR in these cultures is not an immediate catabolic step. Abiotic mechanisms showed high influence in the removal of CEF, affecting less ENR degradation. The acclimation process with the target antibiotics led to significant shifts in the structure and diversity of the microbial communities, predominantly selecting microorganisms belonging to the phyla *Proteobacteria* (e.g. *Achromobacter*, *Variovorax* and *Stenotrophomonas* genera) and *Bacteroidetes* (e.g. *Dysgonomonas, Flavobacterium* and *Chryseobacterium* genera). The results presented in this study indicate that biodegradation can be an important mechanism for the environmental removal of the tested compounds.

1. Introduction

Veterinary drugs are commonly used to treat numerous animal diseases. Antibiotics constitute one of the most broadly applied groups of these pharmaceuticals, being used not only for the treatment and prevention of diseases, but also for the promotion of animal growth and improvement of the nutritional value of animal-based foodstuffs, despite the legal restrictions concerning these latter applications (Cromwell, 2002; Li et al., 2011).

The overuse of veterinary drugs has contributed to the emergence of these products in several environmental compartments, essentially as a result of the employment of contaminated livestock waste as natural fertilizers (Loke et al., 2000; Tasho and Cho, 2016). In addition, these drugs are also released to the environment through wastewater treatment plants (WWTP) effluents, because WWTP are, in most cases, not capable of dealing with this type of contaminants, resulting in incomplete or even no removal of these compounds from agro-industrial effluents (Corcoran et al., 2010).

Pharmaceuticals may be released to the environment in their parental form or as metabolites, which may still hold biological activity. As these compounds are designed to induce specific physiological and biochemical effects on target organisms, their environmental presence can cause a wide range of toxic effects (Sarmah et al., 2006). For the particular case of antibiotics, their environmental presence may also promote the selection of antibiotic-resistant microorganisms (Martinez, 2009). Fluoroquinolones (FQ) and cephalosporins (CP) are two of the most widely used antibacterial pharmaceuticals worldwide. In 2012, the consumption in Europe of both FQ and CP accounted for over 20% of the total antibiotics consumption (Weist et al., 2014). FQ are piperazinyl derivatives of the N-heterocyclic antibacterial compounds designated as quinolones (Felczak et al., 2014). Their mode of action relies on the ability to inhibit the activity of topoisomerases type II and IV, key enzymes in DNA replication, which leads to the blockage of microbial cell multiplication (Hu et al., 2007). CP are semi-synthetic analogous of the naturally-produced cephalosporin-C (Rex and Susan, 2002). Being a class of β-lactam antibiotics, their antibacterial activity resides in their capability to disrupt peptidoglycan biosynthesis, affecting bacterial-cell integrity (Rex and Susan, 2002). Both classes of antibiotics have a broad-spectrum activity towards several aerobic and anaerobic pathogens. FQ have been widely reported to occur in both terrestrial and aquatic ecosystems in trace concentrations, typically ranging from ng L^{-1} to μg L^{-1}, though concentrations of several mgL^{-1} have also been reported (Picó and Andreu, 2006; Larsson et al., 2007; Zhang and Li, 2011). Physical-chemical properties of CP promote a faster environmental dissipation of these antibiotics, leading to lower residence times of these pharmaceuticals in the environment (Junker et al., 2006) and lower detections. As a consequence of the environmental release of these two classes of antibiotics, an increasing number of microorganisms resistant to these drugs has been reported in the literature (Miranda and Castillo, 1998; Walsh, 2000; Ho et al., 2001; Hooper, 2002; Su et al., 2008), highlighting the importance of studying their biodegradation potential.

Microorganisms play a central role in the biodegradation of environmental pollutants, including pharmaceutical compounds. The presence of environmental contaminants frequently leads to shifts on functional diversity, abundance and organization of microbial communities colonizing affected areas, which may compromise ecosystems equilibrium. Metagenomics approaches have been employed as a powerful tool to unveil how microbial communities adapt and respond to environmental contaminants, allowing better understanding their impact on microbial ecology (Röling and van Bodegom, 2014).

The main objective of this work was to investigate the capacity of microbial communities from experimental constructed wetlands to biodegrade two antibiotics representative of the FQ and CP groups, enrofloxacin (ENR) and ceftiofur (CEF), respectively. ENR has been reported to occur in wastewaters, agricultural soils and animal manure, while several metabolites of CEF resultant from animal detoxification have been detected in manure and soils (Rex and Susan, 2002; Zhao et al., 2010; Sim et al., 2011; Li et al., 2014). Also, ENR and CEF may occur simultaneously in the environment, due to their similar prophylaxis and its frequent use in veterinary applications. Degradation of these compounds mainly focuses in physical-chemical processes (Sturini et al., 2012; He et al., 2014; Zamanpour and Mehrabani-Zeinabad, 2014; Yang et al., 2016), while less studies are found in the literature concerning their biodegradation (Martens et al., 1996; Wetzstein et al., 1997; Rafii et al., 2009; Erickson et al., 2014). In the present work, biodegradation of ENR and CEF was investigated individually and in mixture in order to understand if their biological removal was affected by their concomitant presence. Moreover, the effect of these antibiotics in the microbial dynamics of the degrading cultures was studied through metagenomics analysis.

2. Materials and methods

2.1. Reagents

The quality of all chemicals used was *pro analisis* or equivalent. ENR, CEF, ciprofloxacin (CIP) and norfloxacin (NOR) were purchased from Sigma-Aldrich® (Barcelona, Spain). Working solutions of the antibiotics were prepared in methanol at a concentration of 1 g L^{-1} and stored at −20 °C. Methanol and formic acid were acquired from Sigma-Aldrich® (Barcelona, Spain).

2.2. Acclimation of microbial degrading cultures

Microbial cultures capable of degrading ENR and CEF were obtained by acclimation of inoculated cultures with the target antibiotics, supplemented either individually or in mixture, and using acetate as a co-substrate. Rhizosphere sediment samples obtained from experimental constructed wetlands were used as inocula. These experimental systems were planted with *Phragmites australis* (in a layered substrate composed of gravel, lava rock and plants roots bed substrate) and designed for the treatment of pig farm wastewaters, which were artificially contaminated with ENR or CEF (100 μg L^{-1}) either individually or in a mixture (Almeida et al., 2017). The rhizosphere sediment samples used as inocula were collected from each of these systems after 20 weeks of operation. The process of acclimation was conducted in duplicate, in batch mode and under aerobic conditions, along a period of 5 months. For that, 250 mL flasks containing 50 mL of sterile minimal salts medium (MM) were inoculated with 5 g of rhizosphere sediment, which was obtained from one of the three experimental constructed wetlands systems, i.e., constructed wetlands supplemented with pig farm wastewaters doped with ENR, doped with CEF or doped with a mixture

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