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# Response of a salt marsh microbial community to antibiotic contamination

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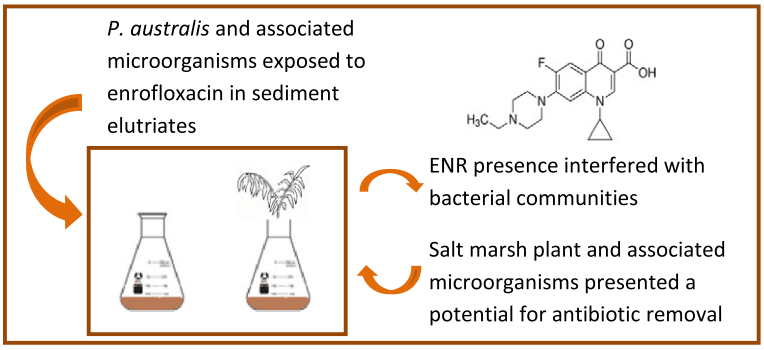
## HIGHLIGHTS

- Salt marsh plant and associated microorganisms exposed to ENR in sediment elutriate
- Significant effect of plant/nutritional conditions on microbial community structure
- ENR presence interfered with bacterial communities
- *P. australis* and associated microorganisms showed an antibiotic removal potential

## GRAPHICAL ABSTRACT

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## ABSTRACT

Salt marsh plants and associated microorganisms can have an important role in contaminant removal from estuaries, through bioremediation processes. Nevertheless, the interaction between emerging contaminants, namely antibiotics, and plant–microorganism associations in estuarine environment are still scarcely known. In this vein, the aim of the present study was to evaluate, in controlled conditions, the response of a salt marsh plant–microorganism association to a contamination with a veterinary antibiotic. For that a salt marsh plant (*Phragmites australis*) and its respective rhizosediment were collected in a temperate estuary (Lima estuary, NW Portugal) and exposed for 7 days to enrofloxacin (ENR) under different nutritional conditions in sediment elutriates. Response was evaluated in terms of ENR removal and changes in microbial community structure (evaluated by ARISA) and abundance (estimated by DAPI). In general, no significant changes were observed in microbial abundance. Changes in bacterial richness and diversity were observed but only in unplanted systems. However, multivariate analysis of ARISA profiles showed significant effect of both the presence of plant and type of treatment on the microbial community structure, with significant differences among all treatment groups. In addition, plants and associated microorganisms presented a potential for antibiotic removal that, although highly dependent on their nutritional status, can be a valuable asset to recover impacted areas such as estuarine ones.



## 1. Introduction

Estuaries are not only one of the most productive, but also one of the most sensitive and fragile ecosystems on Earth (Bouvy et al., 2010) and

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are consequently very difficult to recover (Mucha et al., 2011). Several estuaries are suffering from eutrophication and losing water quality due to high nutrient loads and pollutant inputs (Bouvy et al., 2010), and these areas are considered both sinks and sources of contaminants (Mucha et al., 2011). In fact, a wide variety of chemical contaminants, such as persistent organic pollutants (POPs), metals and emerging pollutants, can reach estuarine areas through industrial, urban and farmland discharges, storm drains and atmospheric deposition (Pan and Wang, 2012; Sun et al., 2012; Stewart et al., 2014).

Pharmaceuticals are emerging pollutants that include a varied range of substances with different chemical–physical properties, environmental behavior and biochemical activities (Bottoni et al., 2010; Caracciolo et al., 2014). Among these, veterinary antibiotics can be found as they are extensively used in veterinary medicine (Kümmerer, 2009). The use of veterinary antibiotics to treat diseases as well as growth promoters has increased in the last few years (Kemper, 2008; Kim et al., 2012). In Europe the use of antibiotics as growth promoters was banned in 2006 due to the emergence of microbes resistant to antibiotics, because these substances can cause a selective pressure for bacteria that are resistant to antibiotics compromising their continued use (Kümmerer, 2009; Huyghebaert et al., 2011). About 75% of the antibiotics are excreted as active metabolites being excretion the major source of antibiotic input into the environment (Pei et al., 2007). In fact, conventional methods of wastewater treatment are generally not equipped to remove these compounds and, consequently, veterinary antibiotics or their active compounds can enter directly in the water system through effluent discharges. Moreover, veterinary antibiotics can reach the environment indirectly through lixiviation of manure used as organic fertilizer in agriculture (Carvalho et al., 2014). Therefore, these contaminants can reach estuarine areas.

Antibiotics can affect different organisms in the environment, not only soil/sediment microbial communities, but also, for instance, algae and other organisms (Kümmerer, 2009 and references therein).

Microbial communities present in natural ecosystems can have an important role in the quality of ecosystems and in the fate of contaminants in the environment (Caracciolo et al., 2014). Microorganisms can degrade pollutants by metabolic and co-metabolic pathways, transforming them into less toxic and/or less bioavailable compounds (Ribeiro et al., 2011). In fact, biodegradation is one of the most important process in the elimination of contaminants from ecosystems (Caracciolo et al., 2014 and references therein).

The interactions between microorganisms and plants can be a determinant in contaminant degradation. Sediment and plant rhizosphere present in estuarine ecosystems are very rich in microorganisms that can be stimulated by plant root exudates (Bais et al., 2006; Prosser et al., 2006; Salvato et al., 2012). On the other hand, plants can play an important role in the bioremediation of organic pollutants by enhancing microbial degradation through creation of specific microenvironments for pollutant-degrading microorganism (Johnson et al., 2004).

Salt marsh plants and associated microorganisms can have an important role, through bioremediation processes, in the removal of contaminants from estuaries, namely for hydrocarbons (Ribeiro et al., 2011). Bioremediation, the use of natural biological processes for contaminant removal and ecosystem recovery, can arise as a less damaging and more cost effective method when compared with traditional techniques such as soil washing, incineration or disposal landfills (Mucha et al., 2011). Nevertheless, knowledge regarding the interaction between emerging contaminants, namely antibiotics, and salt marsh plant–microorganism associations in the estuarine environment is still lacking. Research on this topic is pertinent to evaluate the bioremediation potential of these associations for the removal of these contaminants from estuarine areas.

The aim of this study was to evaluate, in controlled conditions, the response of a salt marsh plant–microorganisms association to a contamination with a veterinary antibiotic, enrofloxacin (ENR), and assess its potential for the bioremediation of this emerging contaminant. For that a salt marsh plant (*Phragmites australis*) and respective rhizosediment were exposed to ENR under different nutritional conditions in sediment elutriates, a simplified but realistic medium. ENR removal and changes in terms of microbial community structure and abundance were then determined.

## 2. Material and methods

### 2.1. Sampling

Plants (*P. australis*) with the respective rhizosediment (sediment in contact with plant roots) and nearby estuarine water were collected in Lima River estuary (North of Portugal) at low tide in October 2013. In the laboratory, sediment was separated from plant roots and kept aside for the experiments that were assembled immediately, as described in Section 2.2. A fraction of this sediment was stored at −20 °C for posterior microbial analysis (initial sediment).

### 2.2. Elutriate experiments

Elutriates were prepared according to Environmental Protection Agency protocols (USEPA, 1991, EPA 503/8-91/001), by mixing in individual flasks 50 g of sediment with 200 mL of estuarine water. Flasks were manually shaken, to break soil clods, and placed on a shaker for 30 min, at room temperature, in a reciprocal movement (100 rpm). In total, 32 flasks were prepared. Flasks were left to settle for 24 h before the beginning of the experiments at room temperature.

The systems were set up in glass flasks and divided into 4 treatments (all in triplicate), all flasks containing sediment soaked in its elutriate: (1) control (C), without antibiotic addition; (2) ENR (100 μg $L^{-1}$) treatment (E); (3) ENR + Nutrients (1008 μg $L^{-1}$ $KH_2PO_4$; 3790 μg $L^{-1}$ $KNO_3$) treatment (EN) (4) ENR + Nutrients + Glucose (180 μg $L^{-1}$ $C_6H_{12}O_6$) treatment (ENG) (see Fig. S1 in Supplementary material). ENR has been found at low μg $L^{-1}$ concentrations in river waters and in insufficiently treated wastewaters (Yan et al., 2012). The tested concentration was selected to allow comparisons with previous studies (Carvalho et al., 2012, 2013a; Fernandes et al., 2015). Also the concentrations of nutrients and glucose were in accordance with authors' previous studies and aimed to have a suitable C:N:P (carbon:nitrogen:phosphorus) proportion. For each treatment, planted (with *P. australis*) (SP) and unplanted (S) systems were prepared. In the planted ones, plant roots were completely submerged. For ENR treatment, an additional set of flasks was prepared with elutriate solution but without sediment, both without (E W) and with plants (E WP). To prepare the flasks without sediment (elutriate flasks), before dosing with ENR, elutriate solutions from 8 of the 32 prepared flasks were centrifuged and filtrated sequentially through 0.8 μm and 0.45 μm pore size filters (cellulose nitrate membrane, Millipore), to remove particulate suspended matter (except colloids) and to reduce the presence of microorganisms.

Each flask was wrapped in aluminum foil to avoid photodegradation of ENR due to light penetration into the substrate and plant root exposure to light. Flasks were kept in an open indoor environment, subject to indoor environmental temperature variations (15–18 °C) and natural day:night light exposure for 7 days. At day 4, a second dosing of 100 μg $L^{-1}$ of ENR was performed.

At the end of the experiment, all elutriate solutions were collected directly from each flask and stored at −20 °C for further quantification of ENR. Portions of each sediment samples were collected (after sediment homogenization) and stored at −20 °C for DNA extraction and ENR analysis. Another portion of sediment was immediately fixed for microbial abundance estimation, as described in Section 2.4.

### 2.3. Enrofloxacin determination

Solid-phase extraction (SPE) was performed to concentrate ENR present in elutriate solutions collected directly from the flasks of the experiment and to clean the matrix as described in Cavenati et al. (2012).