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Potential of autochthonous microorganisms from estuarine environments for bioremediation of pharmaceuticals

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ABSTRACT

The environmental fate of pharmaceuticals has received increasing attention, especially since the detection of their presence in wastewater treatment plant (WWTP) effluents, surface and groundwater. These compounds can be released into rivers and estuaries directly, by discharge or inadequate treatment of water, or indirectly, through groundwater contamination when contaminated manure is used as agriculture fertiliser. Within the veterinary pharmaceuticals, antibiotics are the group of most concern due to the risk of spread of antibiotic resistance in the environment. In addition to their use to treat diseases, antibiotics are extensively used as enhancer of feed efficiency to, between other things, promote growth. Microorganisms naturally occurring, mainly the ones present in areas that are usually exposed to several types of pollution, can be adapted to the presence of contaminants. Therefore, some of them can be used in bioremediation, a process that involves the use of microorganisms that catabolize specific molecules, destroy dangerous contaminants or transform them into less harmful forms.

The main objective of this study was to evaluate the potential of autochthonous microorganisms from estuarine environments for bioremediation of pharmaceuticals, namely veterinary antibiotics. This potential was studied in two experiments, one of them using these microorganisms in association with plants, in constructed wetland (CW) microcosms, and the other using them to produce microbial consortia with capacity to degrade antibiotics.

The first experiment aimed to evaluate the response of microorganisms from CW microcosms to the presence of veterinary antibiotics, both in terms of community structure and removal performance. Four sets of microcosms planted with *Phragmites australis* (3 replicates each) were run in parallel: a set was feed only with livestock wastewater and three sets were feed with the same wastewater doped with antibiotics, alone or combined (enrofloxacin or/and ceftiofur at 100 µg/L). Wastewater was treated during 18 one-week cycles. After each one-week cycle wastewater was removed and replaced by new one (doped or not). Water and sediment samples were collected at the end of week 1, 8, and 18. Antibiotics removal was evaluated by HPLC while microbial community was characterized by ARISA and by 454-pyrosequencing analyses. Results show that microbial communities were dominated by the phylum *Proteobacteria* (38 to 48%), *Firmicutes* (20 to 27%), *Bacteroidetes* (12 to 15%) and *Actinobacteria* (4 and 9%) but their relative abundance was clearly affected by the presence of the antibiotics. The study also shows that the systems were able to remove more than 90% of the added antibiotics,

pointing to the applicability of CWs for the removal of veterinary antibiotics from livestock wastewaters.

Further, the potential of bacteria from the rhizosphere of two saltmarsh plants (*P. australis* and *Juncus maritimus*) for the antibiotic biodegradation was investigated in a second experiment. In this, two set of flasks were prepared with sediment from the rhizosphere of each plant, collected in Lima estuary, and low nutrient medium doped with enrofloxacin. The mixture was constantly shaken during 52 weeks. Every three weeks period the mixture was diluted with new nutrient medium, to progressively eliminate the sediment, and doped again with the antibiotic to produce a microbial consortium with capacity to degrade enrofloxacin. The biodegradation of enrofloxacin, a fluorinated antibiotic, was evaluated by measuring, with a fluoride (F^-) ion-selective electrode, the concentration of F^- present in cultures supernatants along time. The presence of this anion in the culture solution is an indicator of defluorination of enrofloxacin and consequently the degradation of the compound. The obtained consortia were able to degrade between 20 and 80 % of the antibiotic, depending on its initial concentration. In addition, bacterial strains potentially involved in the biodegradation processes were isolated.

This work points for the applicability of the use of autochthonous microorganisms collected from estuarine environment, for bioremediation of pharmaceuticals, namely veterinary antibiotics, providing new knowledge about the bacteria potentially involved in the removal processes.

Key-words: Constructed wetlands; veterinary antibiotics; autochthonous microorganisms; microbial community; bioremediation.

RESUMO

O destino ambiental dos fármacos tem recebido uma atenção crescente, especialmente desde que a sua presença foi detectada em efluentes de estações de tratamento de águas residuais, nas águas superficiais e nas subterrâneas. Estes compostos podem atingir os rios e estuários directamente, através de descargas ou do tratamento inadequado da água, ou indirectamente, através da contaminação de águas subterrâneas quando excreções de animais contaminadas utilizadas como fertilizantes na agricultura. Entre os fármacos veterinários, os antibióticos são o grupo mais preocupante devido ao risco da dispersão de resistência a antibióticos no ambiente. A juntar ao seu uso no tratamento de doenças, os antibióticos são usados extensivamente no aumento da eficiência da alimentação e, entre outras aplicações, como promotores de crescimento. Os microrganismos presentes na natureza, principalmente os que existem em zonas que estão constantemente expostas a diferentes tipos de poluentes, podem adaptar-se à presença de contaminantes. Por tudo isto, alguns deles podem ser utilizados em técnicas de bioremediação, um processo que envolve o uso de microrganismos que catabolizam moléculas específicas, destroem contaminantes perigosos ou que os transformam em formas menos prejudiciais.

O principal objectivo deste estudo era avaliar o potencial dos microrganismos autóctones de ambientes estuarinos para a bioremediação de fármacos, nomeadamente antibióticos veterinários. Este potencial foi estudado em duas experiências, uma na qual se usaram estes microrganismos em associação com plantas, numa microcosmo de zonas húmidas construídas, e na outra utilizando-os para produzirem um consórcio microbiano com capacidade de degradar antibióticos.

A primeira experiência teve como objectivo avaliar a resposta dos microrganismos dos microcosmos de zonas húmidas construída à presença de antibióticos veterinários, quer em termos da estrutura da comunidade quer na performance de remoção. Quatro conjuntos de microcosmos plantados com *Phragmites australis* (três réplicas por conjunto) foram testados em paralelo: um conjunto em que apenas se adicionou água residual de pecuária e os outros três nos quais se adicionou a mesma água mas contaminada com antibióticos, em separado ou combinado (enrofloxacina or/and ceftiofur at 100 µg/L). As águas residuais foram tratadas durante 18 ciclos de uma semana. A seguir a cada ciclo de uma semana a água residual foi removida e substituída por nova (com ou sem a adição de antibiótico). Amostras de água e sedimento foram recolhidas no final das semanas 1, 8 e 18. A remoção dos antibióticos foi avaliada por HPLC enquanto a comunidade microbiana foi caracterizada por análises de ARISA e 454-

pirosequenciação. Os resultados obtidos demostram que a comunidade microbiana era dominada pelos filos *Proteobacteria* (38 para 48%), *Firmicutes* (20 para 27%), *Bacteroidetes* (12 para 15%) e *Actinobacteria* (4 e 9%) mas a sua abundância relativa foi claramente afectada na presença dos antibióticos. Este estudo também demostrou que os sistemas tinham a capacidade para remover mais de 90% da quantidade de antibiótico adicionada, indicando a aplicabilidade das zonas húmidas construídas para a remoção de antibióticos veterinários em águas residuais de pecuária.

Para além disto, o potencial das bactérias presentes na rizosfera de duas plantas de sapal (*P. australis* and *Juncus maritmus)* para a biodegradação de antibióticos foi investigado na segunda experiência. Nesta, dois conjuntos de frascos foram inoculados com sedimento proveniente na rizosfera de cada planta, recolhidas no estuário do Lima. A biodegradação da enrofloxacina, um antibiótico fluorado, foi estudada com base nos resultados obtidos com um eléctrodo selectivo de iões de fluoreto (F^-). A medição da concentração de aniões de fluor presentes no subrenadante de culturas. A presença destes iões na solução das culturas são um indicador da deflurinação da molécula e da consequente perda de actividade. O consórcio obtido demonstrou capacidade de degradação entre 20 e 80% dos antibióticos, dependendo do consórcio inicial. Em adição, as estirpes bacterianas potencialmente envolvidas no processo de biodegradação foram isoladas.

Este trabalho demostra a aplicabilidade dos microrganismos autóctones recolhidos num ambiente estuarino, para a bioremediação de fármacos, nomeadamente antibióticos veterinários, fornecendo um novo conhecimento sobre as bactérias potencialmente envolvidas nos processos de remoção.

Palavres-chave: Zonas húmidas construídas; antibióticos veterinários; microrganismos autóctones; comunidade microbiana; bioremediação.

ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of variance
ARISA	Automated rRNA intergenic spacer analysis
ARISA AFLs	ARISA fragment lengths
BOD	Biochemical oxygen demand
CEF	Ceftiofur
CIP	Ciprofloxacin
CNT	Control
COD	Chemical oxygen demand
CWs	Constructed wetlands
DAPI	4',6'-diamidino-2-phenylindole
DFC	Desfuroylceftiofur
DNA	Deoxyribonucleic acid
ENR	Enrofloxacin
EU	European Union
F^-	Fluoride
FEDESA	Federation of Animal Health
FQs	Fluoroquinolones
FWS-CW's	Free water surface constructed wetlands
HPLC	High-performance liquid chromatography
HRT	Hidraulic retention time
ITS	Internal transcribed spacer
JNC	*Juncus maritimus*
K_{oc}	Octanol-water partition coefficient
K_{ow}	Carbon and Water partition coefficient
LOD	Limits of detection
LOQ	Limits of quantification
MDS	Multidimensional scaling
min	Minutes
MIX	Mixture
MM	Minimal salt medium
N	Nitrogen
NH_3	Ammonia
NO_2^-	Nitrites
NO_3^-	nitrates

OTUs	Operational taxonomic units
PBPs	Penicillin-binding proteins
PCA	Plate count agar
PCR	Polymerase chain reaction
PHR	*Phragmites australis*
PO_4^-	Phosphorous
rpm	Rotations per minutes
s	Seconds
SSHF-CW's	Horizontal subsurface flow wastewater
TCC	Total cell acount
TISAB	Total ionic strength adjustment buffer
w	Week
WWTPs	Wastewater Treatment Plants

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CHAPTER 1

Introduction

1. INTRODUCTION

1.1. Presence of pharmaceuticals in the environment

In European market is possible to find more than 100,000 different chemicals. They were introduced in the attempt to improve life quality by utilization in dairy products, medicinal products and improvement of industrial processes. Despite all the benefits that these compounds represent, the features they have, especially the poor biodegradation, make their entrance in the environment a risk factor. Pollutants are xenobiotic compounds that when enter in the environment can have negative consequences. Pollutants, as result of spills (fuel, solvents), military activities (explosives, chemical weapons), agriculture (pesticides, herbicides) and industrial activities are released into the environment. Organic matter, suspended particles, micropollutants, nutrients (phosphorus and nitrogen) or heavy metals are examples of pollutants that can be released into environment. Their presence in the soil and in the aquatic environment is creating problems for environmental protection (Pilon-Smits, 2005; Schröder et al., 2007).

New chemicals compounds that appear and are not currently regulated are called emerging pollutant. Information about these compounds and possible treats they posed for environment and human health are still unknown. In this group we found compounds such as: pharmaceuticals, person-care products, steroids and hormones, industrial additives and agents. Besides these compounds it is also important to take into account their transformation products making even more difficult to predict the consequences of their present and to regulate them. The source of these products in the environment is diffused, and are related with their utilization mode, with domestic and industrial wastes having a great impact (Farré et al., 2008; Garcia-Rodríguez et al., 2014).

With respect to pharmaceuticals there are 3,000 different compounds that can be divided into different classes, among which can be distinguished: antibiotics, hormones, analgesics, anti-inflammatory drugs, chemical compounds used for disinfection and cleaning and endocrine-disrupting compounds. These compounds are identified as potential pollutants in diverse ecosystems. Antibiotics are one of the most commonly pharmaceuticals detected in wastewater treatment plants (WWTPs) effluents. Antibiotics can be defined as natural, semi-synthetic and synthetic compounds used in the treatment and prevention of diseases caused by microorganism, due their antimicrobial activity. They are also used as feed additives for animals and fishes, either to prevent or treat a variety of diseases, as well as growth promoter. According to data from the European Federation of Animal Health (FEDESA), 29% of the 13,288 tonnes of antibiotics used in

European Union (EU) in 1999 were used in veterinary medicine, 6% as growth promoters, and 65% applied in human medicine. The excessive application of these compounds and their inefficient removal in WWTP is a matter of concern since this can have as consequence environmental contamination, with special concern to the possible increase of antimicrobial resistance and impacts in human and wild life (Carvalho et al., 2014; Jjemba, 2002; Kemper, 2008; Kümmerer, 2004; Li et al., 2014; Rivera-Utrilla et al., 2013; Sarmah et al., 2006). The utilization of antibiotics as growth promoter in agriculture was banned from Europe in 2006 by Regulation No 1831/2003, in attempt to minimize the possible spread of microbial resistance.

After administration, pharmaceuticals are only partially metabolized being excrete from humans and animal bodies (in faeces and urine). These excretion products are a mixture between metabolites and parental forms with the possibility of being both still bioactive. The metabolization of these compounds varies with their structural formula and physical and chemical properties. In turn, excretion rates can vary depending of different parameters such as: the substance itself, the mode of application, the excreting species and time after administration (Carvalho et al., 2014; Jjemba, 2002; Kemper, 2008).

Pharmaceuticals compounds can be discharged into the nature as a result of direct discharged or through inadequate treatment of water (Fig. 1) and can have adverse effects on human health and undesirable changes in the composition of aquatic biota. The use of sewage sludge and manure as a fertilizer represents another way of organic pollutants entrance in soil, surface water and crops. Manure can be storage before being

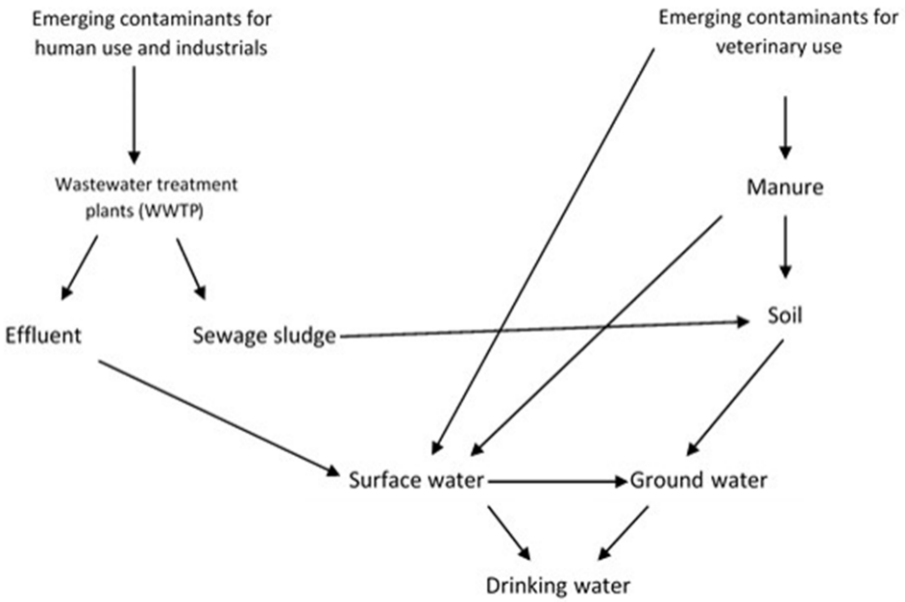

Figure 1: Pharmaceuticals fate in different environmental compartments (Adapted from: Farré et al. 2008).

used and when this is made without any treatment performed, may lead to a biochemical oxygen demand increase, under which pharmaceuticals are less likely to be degraded. After this entry into the environment, metabolites of some compounds are converted back to the parent compound (Carvalho et al., 2014; Jjemba, 2002; Schröder et al., 2007). Pharmaceutical have already been detected in surface water in different concentrations (Babić et al., 2010; Grujić et al., 2009; Verlicchi et al., 2012). In the European Union over 37% of all the sewage sludge generated annually (more than 6.5 million tons) are applied in agriculture. Although the pharmaceuticals concentration found in waters are relatively low the fact that they, and their degradation products, are continually released, make them 'pseudo-persistent'. The presence of antimicrobials in the environment leads to a repeated low-dose exposure of bacteria to sub-lethal dosage, which can cause the development of resistance. Transmission of these resistant strains can occur in two ways: via direct contact or via food chain and can have as outcome the decrease of pharmacotherapeutic effects (Carvalho et al., 2014; Jjemba, 2002; Schröder et al., 2007).

Pharmaceuticals fate, behaviour and effects and their potential impacts in the environment are not completely understood, which is influenced by the physical and chemical properties of the compound. The possible entrance of compounds in water or their adsorption to the organic components (manure or soil) are determinate by the log K_{oc} and K_{ow}. Compounds with low log K_{oc} and K_{ow} represents risk to aquatic compartments once, due to their characteristic, it is likely for them to enter the groundwater. In the case of substance that presents high log K_{oc} and K_{ow} the risk is higher for the terrestrial ambient due to their strong adsorption (Kemper, 2008; Slana and Dolenc, 2013).

In order to restrict the risk to which humans are exposed as consequence of antibiotics used in veterinary medicine, risk management strategies are required. With that propose, World Health Organization has ranked these compounds according the importance they have for human therapy. Each antimicrobial agent was assigned to 1 of the 3 categories of importance (critically important, highly important and important) on the basis of 2 criteria: "the agent or class is the sole therapy or one of few alternatives to treat serious human disease" and "the antimicrobial agent or class is used to treat diseases caused by organisms that may be transmitted via nonhuman sources or diseases caused by organisms that may acquire resistance genes from nonhuman sources" (Collignon et al., 2009).

1.1.1. ENROFLOXACIN

In enrofloxacin (ENR) (Fig. 2) the presence of fluorine, an atom belonging to halogenated compounds, is of great importance, since this is the main responsible for their antibiotics properties. These properties result of the biological courses that fluorine affect, such as the inhibition of enzymes, cell-cell communication, membrane transport and processes for energy generation. Among the halogens found in earth, fluorine is the most abundant and the most electronegative, conferring a strong polarity and energy to carbon-fluorine bond (Key et al., 1997).

With the insertion of a fluorine atom at the 6-position and substitution of a carbon atom for nitrogen at 8 in the basic quinolone ring structure, a rise in this antibiotic action occurs leading to a broader spectrum of activity. These synthetic compounds resulting from this quinolone family modification are named as fluoroquinolones (FQs), been flumequine the first described (Redgrave et al., 2014). Quinolones are, their self, a modification of compound isolated from production of the antimalarial drug chloroquine, with the first one in the class, nalidixic acid, first used in 1965. FQs started to be used in 1980 after the first fluoroquinolones were approved for clinical medicine (norfloxacin and ciprofloxacin (CIP)). After these, many other compounds were also approved for use including ENR (1989), the most important for veterinary use in this class (Ball, 2000; Jia et al., 2012; Pallo-zimmerman et al., 2010). Since then, and in order to produce a range of more efficient compounds capable to provide better therapeutical effects, alterations in R_1, R_7 and R_8 groups (variable one) of FQs have been tested. These new developed compounds are categorized according to their structures, and divided in different generations. Besides structure, these generations vary from each other in terms of spectrum activity, with the most recent ones having an improved activity when compared with the previous generations (Redgrave et al., 2014).

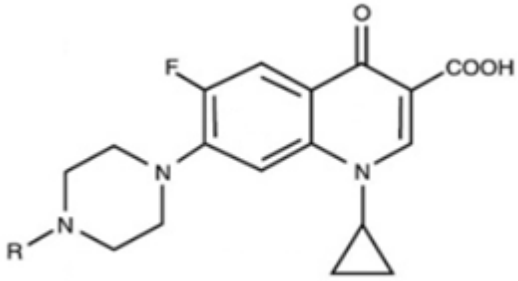

Figure 2: Chemical structure of Enrofloxacin and Ciprofloxacin (Adapted from: Pallo-zimmerman et al., 2010).

Their mechanism of action is related with the inhibition of enzymes involved in several key cellular processes, as the DNA replication. In response to environmental stress, growth stage or cellular process, large amount of DNA have to be packed into the cell. For this to be possible, DNA molecule of bacteria have to suffer a supercoiling process, consisting in the twist of the double-helix strutted over itself. In this process several enzymes are involved. FQ act on topoisomerases type II and IV inhibiting their

action of controlling supercoiling in cells alteration, changing, in this way, the DNA topology and, consequently impairing DNA replication. Maintain the balance of supercoiling is extremely important since the state in what DNA is found are correlated with the expression of many gene including the ones involved in the responses to changes in the environment. The bacterial species and the type of fluoroquinolones used will define which of the enzymes (DNA gyrase or topoisomerase IV) will be affected. DNA gyrase are the preferential target in gram-negative bacteria, while in gram-positive bacterial is topoisomerase IV the usual target. The result of this inhibition are depending of antibiotics concentration, at lower concentration an impairment of DNA replication can be seen and at higher concentration cell death can occur (lethal concentrations). The difference between affinity for bacterial DNA gyrase and mammalian DNA gyrase, exhibited by fluoroquinolones, with a higher affinity for the first one, allows their rapid activity against bacterial without having adverse effects on the host (Drlica et al., 2009; Hooper, 2001; Pallo-zimmerman et al., 2010; Redgrave et al., 2014).

Nowadays, FQs represent the third largest group of antibiotics with an increased used in hospitals, households and veterinary (Van Doorslaer et al., 2014). Humans are potentially exposed to antimicrobial-resistant bacteria via food chain. The development and spread of bacteria resistant to FQs can be a consequence of antibiotic use in food animals. Besides the risk for public heath, this will also have impacts on the ecosystem. In order to restrict the risk to which human are exposed as consequence of antibiotics used in veterinary medicine, risk management strategies are required. With that propose, World Health Organization has ranked these compounds according the importance they have for human therapy. FQs have been establish as ‘critically important’, fill in the 2 established criteria mentioned above (Collignon et al., 2009). FQ were then divided into two categories, the one applied in human medicine and the one used in veterinary (Redgrave et al., 2014).

Presence of FQs in nature are a result of their extensively use in both human and veterinary medicines and lack of appropriate treatment for removing such compounds. After administration, only part of FQ are metabolized, being the rest excreted as unmetabolized parental compound. Even the metabolized portion excreted pose problems once they are usually constituted by break down products that show the same or similar toxicity as primary compound, as in the ENR case. Prior application, as fertilizer, sludge as to be properly treated, however when it comes to removal of FQ by treatments before application, studies shows that they are not very efficient, with FQ still found in digested sludge demonstrating their resistance for the treatment applied to sludges. After this, sludges are usually stored, a process that did not affect positively degradation either. This

shows how sewage sludge can be seen as a FQ reservoir and the vital advance that management of this have to suffer (Chenxi et al., 2008; Golet et al., 2003; Lindberg et al., 2006; Van Doorslaer et al., 2014).

Once in the environment, FQs are submitted to different processes of degradation as photolysis, adsorption and biodegradation. In addition to their use in veterinary medicine, ENR has as primary degradation product CIP, the most widely prescribed FQs, making their fate in environmental systems of great interest. Importance of photolysis for ENR degradation in water was already analysed. Photodegration represents for ENR an important degradation pathway. Presence of ENR last longer in deepest waters when compared with superficial waters. Consequences for sediment can be more significant once exposures are prolonged. If, in one hand, photolysis represents, in water, the primordial process of degradation, that is not the case in sediment. In water, and despite its importance, photolysis is a slow process with FQs being a long period of time in environment before degradation take place. In sediment, this process can only take place in surface or in the first millimetres of depth being therefore its role less important. Pseudo-persistence in the environment is due to lack of biodegradation and high adsorption affinity with half-life times varying between water and sediment. Even if biodegradation or photolysis occurs in sediments the process is not complete with residual traces of FQ remaining (Ball, 2000; Knapp et al., 2005; Pallo-zimmerman et al., 2010; Van Doorslaer et al., 2014).

The tendency that a chemical has for volatilization and for adsorb to soil are defined by Henry's law constant and the octanol-water partition, respectively. Henry's law constants for FQs compounds are $<10^{-15}$ atm.m^3 mol $^{-1}$, a very low value which means that the volatilization is a minor process. The values found for K_{ow} have a large variability, and the main value found for most of compounds are less than 2.5. With this value, it was expected a low adsorption potential by FQs, however the affinity that they show for sludge, soil and sediments is closer to what happens for higher K_{ow} values (Van Doorslaer et al., 2014). The move from water to soil occurs rapidly. FQs can, therefore, reach soil from different point as the, aforementioned, use as fertilizer of both digested sewage sludge and manure, excretion of livestock on fields or through the use of treated wastewater or already polluted river water for irrigation fields. For all of the variables, there are several aspects that have to be taken into account when persistence of FQs in soil are analysed as their photostability, adsorption capability, rate of degradation and leaching into water (Picó and Andreu, 2007). FQs spread through diverse environment compartments pose a risk for non-target animal that somehow end up being in contact with these compounds. Presence of them have been already detected in several animals

in wildlife, as molluscus, shrimps and river snails (Li et al., 2012a, 2012b). Another route that can be seen as potential risk for public health is the uptake of these compounds by plants used for human consumption. When humans ingest products that have been in contact with FQs there is the possibility of accumulation. Tests for the assessment of the impacts in crop plants (carrot and lettuce) of soil contaminated with ENR already conducted showed a significantly reduction in growth of plants, with ENR being detected both in carrot root and lettuce leaves. Besides that, the time to achieve 90% dissipation was calculated as >152 days, showing the capability of ENR to persist in soil environment and subsequently making more likely the continuous exposure of plants (Boxall et al., 2006). In another study with crop plants exposed to ENR, in the plants exposed to the higher concentration, CIP was found at the end of experiment. This demonstrates the capability of plants for ENR transformation, as demonstrate for animals. Therefore antimicrobials can be introduce in food nets through this process (Migliore et al., 2003).

1.1.2. CEFTIOFUR

Ceftiofur (CEF) belongs to the family of semi-synthetic antibiotics cephalosporins, a derived from the product of fungus *Cephalosporium acremonium,* cephalosporin C. Cephalosporins structure contains a β-lactam fused with a six-membered dihydrothiazine ring forming the 7-aminocephalosporanic acid nucleus. The β-lactam ring can also be found in other molecules, as penicillins and β-lactamase inhibitors. These last ones are used in conjunction with β-lactams to extend their spectrum activity. The presence of the β-lactam ring is essential for the antibacterial activity of cephalosporins and, therefore, the cleavage of this at any point will result in the complete loss of activity. Besides the β-lactam ring cephalosporins have a cepham nucleus, alterations at R_7 and R_3, of this, lead to creation of a large diversity of compounds that differed from each other. This difference is in terms of spectrum of activity, stability to hydrolysis by β-lactamases, protein binding affinities and pharmacokinetic characteristics. The alteration in the R_7 will affect essentially antibacterial characteristics, while changes on R_3 affects the absorption, protein binding, metabolism and drug half-life. The basic mechanism of action of cephalosporins is analogous to all β-lactam antibiotics. They target a class of proteins located in the inner portion of bacterial cell wall, the penicillin-binding proteins (PBPs). These are involved in various stages of cell wall synthesis and interferences in their functions leading to a disruption in cell wall synthesis. The distinctive alterations in the R_7 and R_3 groups in the different antibiotics will affect their affinity for the different PBPs on bacterial species. In the case of cephalosporins alterations that can occur will change the ability to penetrate

the outer membrane and so target PBP. Due to all the possible substitutions that can take place the spectrum of activity of each molecule is unique (El-Shaboury et al., 2007; Hornish and Kotarski, 2002; Prober, 1998).

Classification of cephalosporins are based in their spectrum of activity (in vitro) and structural similarities, being divided in four different generations (El-Shaboury et al., 2007; Hornish and Kotarski, 2002; Prober, 1998):

- First-generation: usually active against gram-positive but with a very restricted activity against gram-negative bacteria.
- Second-generation: maintain the active against the same strains of the first-generation but with a more active action against gram-negative.
- Third-generation: raise of the activity for gram-negative were achieved being the cephalosporins more potent against this type of bacteria, while the activity against gram-positive bacteria already see in the other two were maintained. The use of first and second-generation created a selective pressure and in order to respond to that certain β-lactamases emerged. The presence of an oxyimino side chains in the most of cephalosporins in this class confers stability against them.
- Fourth-generation: these have potency against a broader range of organisms then the third-generation.

Ceftiofur (Fig. 3) is a third-generation broad-spectrum cephalosporin used exclusively in veterinary, for the treatment of several conditions as respiratory diseases and metritis, introduced in 1988. Its structure in position 3 contains a furoic acid thioester, a unique substitution for 3rd generation cephalosporins. Its metabolization consist in the hydrolytic cleavage of the thioester bond which release furoic acid and the primary metabolite desfuroylceftiofur (DFC). The structure of DFC still keeps intact the β-lactam ring and, consequently retains the activity associated to the Ceftiofur. This makes possible that part of the effects associated to Ceftiofur being in fact a result of DFC action. The further metabolization of DFC can occur by the hydrolysis of the β-lactam ring and parallel loss of antimicrobial activity. DFC can also form several conjugates with glutathione and cysteine through reversible bind to plasma proteins and tissues. The fact that the bounds

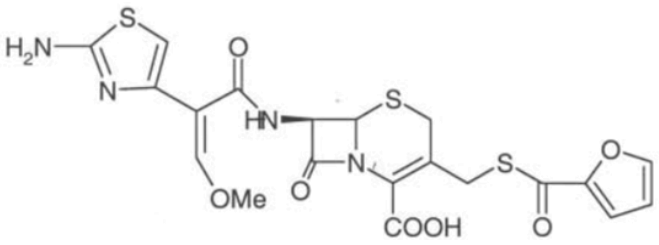

Figure 3: Ceftiofur chemical structure (Source: Hornish and Kotarski, 2002).

are reversible implies that DFC-conjugates may still have microbiological activity. In the environmental hydrolytic, photolytic and biological mechanisms, contributes to CEF degradation (Hornish and Kotarski, 2002; Li et al., 2011). Studies to evaluated distribution and excretion of CEF, in both type of CEF salts (hydrochloride and sodium), were already conducted in rats, cattle and pigs. Presence of CEF and several of their metabolites were detected in urine, faeces and plasma of all the mentioned groups of animals. The highest amount of CEF and derivatives are normally found in the kidneys as a result of the excretion by urine. The second tissue with highest concentration is the lung which is consistent with the fact that this is the primary target for this antibiotic. The amount of metabolites found that still have microbiological activity is proportional to the existent total residues. Investigation on the presence of CEF on effluent of WWTP from different sites (urban, livestock and slaughterhouse) are scarce (Beconi-barker et al., 1996; Beconi-Barker et al., 1996; Gilbertson et al., 1995; Jaglan et al., 1989).

1.2. Wastewater treatment plants

Conventional WWTPs are designed to remove easily or moderately biodegradable compounds. Influent (urban or industrial) of a WWTP can undergo a serial of processes that aims the elimination or at least the reduction of all the undesirable compounds, including pharmaceuticals residues. For EU the relevant parameters for design are defined in the EU Urban Wastewater Directive 91/271/EEC. Treatments usually consist in a primary, a secondary and occasionally a tertiary stage. They differ in terms of biological and physicochemical processes and in their main objective. In the first step, reduction of wastewater suspend sediments content, through filtration and sedimentation, is attempt. This is the most used step in all WWTPs. In the secondary treatment, organic matter and nutrients are removed by biological routes (aerobic or anaerobic). Treatment with activated sludge is generally used in these facilities, and represents the crucial step in biological component of WWTPs. Sorption process and biodegradation are the two most important routes for the elimination of pharmaceuticals. Other mechanisms can be applied, however they are negligible or non-effective. Besides these mechanisms, ultraviolet can be used as a disinfection step, with the intention of eliminating pathogens. However this method is not a routine procedure, being applied only in some WWTPs and even in the ones that applied it, effluents do not always receive this treatment. For antibiotics in WWTPs there are various biological degradation pathways as: mineralization to carbon dioxide or transformation to more hydrophobic/hydrophilic compounds (Batt et al., 2007; Kim and Aga, 2007; Michael et al., 2013).

Presence of different pharmaceuticals has already been detected in effluent of WWTP with different operational designs (Cavenati et al., 2012). Choosing a treatment system capable of remove all of contaminats is usually difficult due the different processes that exist and the variances in WWTP influent. The different types of treatment have their advantages and disadvantages. The total antibiotics removal is result of their sorption on the sewage sludge and degradation/transformation during the course of the treatments. The properties of these compounds will define their route of elimination, with hydrophobic residues predictably appearing in higher concentration in primary and secondary sludge due their affinity to solids. On the other hand, the most hydrophilic are expect to remain in the aqueous phase. The implementation of advanced treatments, such as advanced oxidation process, activated carbon adsorption, membrane separation and membrane reactor will allow the achievement of greater removal efficiencies. Yet the cost that they represent, as higher level of energy consumptions and expensive maintenance makes them a less attractive choice. These treatments do not guarantee the full removal of the total pharmaceuticals received. Therefore, it is of great importance the selection of low-cost and efficient alternatives. Currently, assessments of the degradations does not take into account the metabolites but these also represents a risks once they can still show the antimicrobial activity of parental compound. Thus, searching only for parental compounds is not enough to evaluate the possible impacts of effluents into the environment. Industrial wastewater represents a most challenging for treatment due to their high concentration of pollutant matter. In the particular case of livestock industry, suspended solids, organic matter and nutrient load in their wastewater can be responsible for deterioration of water bodies into which they are discharged. The traditional wastewater treatment systems have already demonstrated their inefficiency in the removal of pharmaceuticals, and up to 40% of residues are released together with the effluents. In an attempt to reduce this release, different complementary treatments can be applied (Batt et al., 2007; Garcia-Rodríguez et al., 2014; Li et al., 2014; Michael et al., 2013).

1.3. Constructed wetlands

Wetlands occur in nature as a transitional environment between the dry land (terrestrial ecosystem) and open water (aquatic ecosystem). Due to their localization, these systems exhibit characteristics from both systems at the same time. The benefits provide from these systems goes from the habitat for wildlife to the removing of contaminants. The capability demonstrated for the removal of contaminants drew attention for these systems. Their characteristics started to be seen as potential applicable for

alternative or possible additional treatment for the conventional WWTPs. Constructed wetlands (CWs) are the artificial man-made systems developed with the objective of imitate the natural systems and their removal ability. This, thus, permits control the variable in the attempt of improving either further contaminants removal. Wetland vegetation, soil and associated microbial community in CWs offer the possibility for carbon sequestration in biomass, as well as the recycling of materials and matter. Besides this efficiency in the degradation, their high sustainability associated with low operating cost, relatively easy maintaining and operation make them an attractive choice. In a CWs design there are several parameters, such as organic matter (expressed as biochemical oxygen demand – BOD – and chemical oxygen demand – COD) and nutrients (nitrogen and phosphorous), that have to be taken into consideration. Limit levels of these parameters are defined by the EU Urban Wastewater Directive 91/271/EEC. The low-cost of construction, operation and maintenance that they demand associated with their benefits for environment makes them a low-cost green technology alternative (Li et al., 2014; D. Zhang et al., 2014; D. Q. Zhang et al., 2014; Zhou et al., 2009). In CWs, the global nitrogen transformation is a complex process that includes various mechanisms, which can remove nitrogen from wastewater or convert this nitrogen (N) among its various forms (Fig. 4).

The N cycle involves several processes as ammonification, nitrification and denitrification. The first process corresponds to the conversion of N in ammonia. Nitrification is a two-step process where ammonium is converted in nitrate. The first step only occurs in strict aerobic conditions where ammonia is converted to nitrite and, this

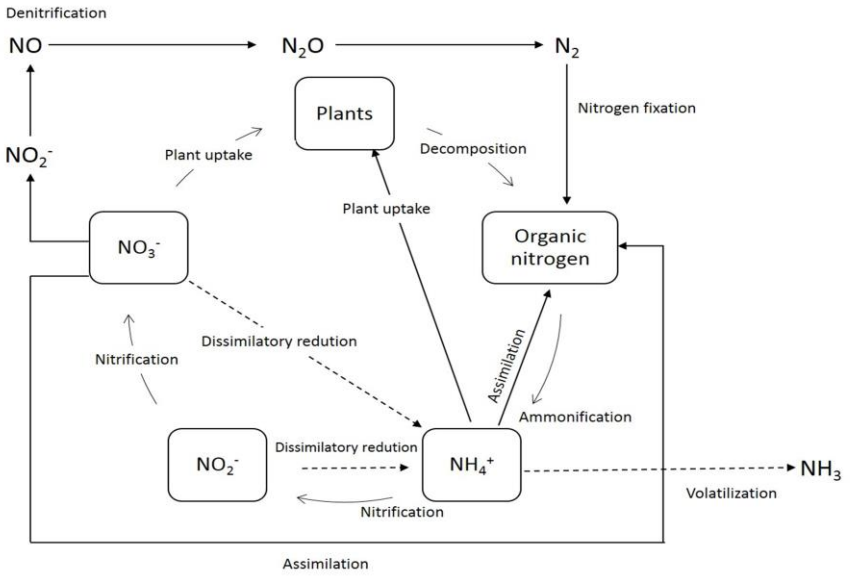

Figure 4: Mechanism of nitrogen transformation in CW's (Saeed and Sun, 2012).

one, in the second step is transformed in nitrate. Denitrification process is responsible for the reduction of inorganic forms of nitrogen, transforming them into nitrogen gas, under anaerobic/anoxic conditions. As described, the N cycle contains processes with different oxygen demands, making challenging the design of a CWs capable of promoting efficiency for all of them (Lee et al., 2009; Saeed and Sun, 2012).

Classification of CWs can be made in different ways depending of the parameters take into account. Thus, they can be classified by its hydrology (open water-surface flow and subsurface flow), macrophyte type used (emergent, submerged or free-floating), and flow path (horizontal or vertical). In what regards to the systems type of flow, it can distinguish the CWs with surface flow from the ones with a subsurface flow. The first one is also recognised as *free water surface constructed wetlands* (FWS CW, Fig. 5). The removal pathway occurring in FWS CWs in photodegradation once this process is enchained in water directly exposed to sunlight (Li et al., 2014; White et al., 2006; D.

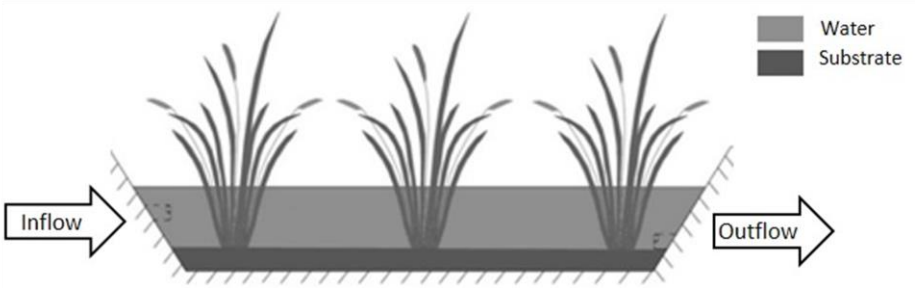

Figure 5: Schematic representation of a free water surface constructed wetland (FWS CW). (Adapted: Li et al. 2014).

Zhang et al., 2014; D. Q. Zhang et al., 2014).

Subsurface flow CWs (SSF CWs) can be divided into horizontal and vertical flow. Comparing these two types of CWs it is possible to notice their differences in the processes that are favoured by each one. In SSF CWs rhizosphere, root zone, have a more pronounced effect and adsorption surface is superior in FWS CWs. In the horizontal subsurface flow wastewater (SSHF CWs, Fig.6) pass from the inlet, under the surface, to the outlet. The systematic passage of flow in SSHF CWs causes the permanent saturation of bed and most zones become anoxic/anaerobic with the aerobic zone confined to the area around roots. Since most of the bed is in an anoxic/anaerobic state this type of system has conditions for occurrence of denitrification.Contrasting with this is what happens in SSVF CWs (Fig. 7), where the intermitting feed allows the full drainage of the system and consequently the refill of bed with air. The mostly aerobic state favours the

nitrification with denitrification being a minor process. All these differences have impact in the system performance and have to be taken into account when CWs are being design.

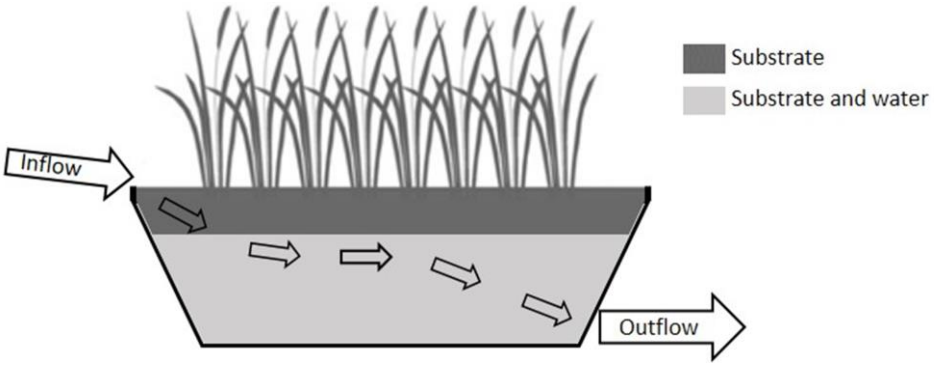

Figure 6: Schematic representation of subsurface horizontal flow constructed wetland (SSHF CWs) (Adapted from: Li et al. 2014).

Alterations in degradation rates of a compound in a CW in different zones, occurs, as a result of the conditions alterations (e. g.: redox conditions alteration with depth) in the system (Li et al., 2014; White et al., 2006; D. Zhang et al., 2014; D. Q. Zhang et al., 2014).

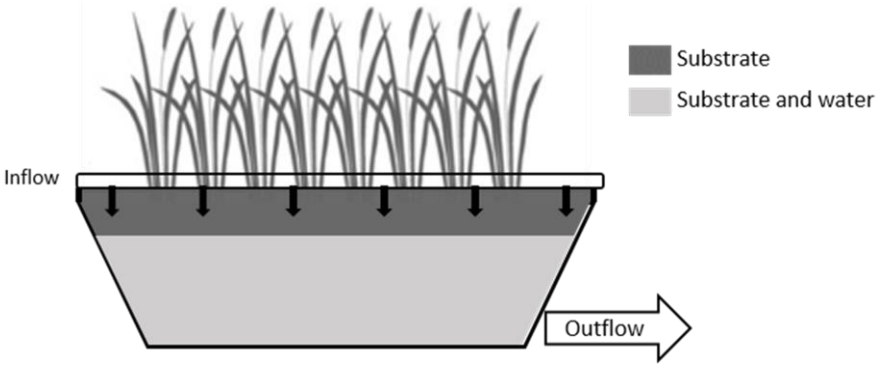

Figure 7: Schematic representation of a subsurface Vertical flow constructed wetland (SSVF CWs) (Adapted from Li et al., 2014).

Different CWs designs present several advantages and disadvantages. To exploit the advantages of the different CWs types, diverse systems can be combined and jointly used. These are called hybrid CWs, and have as goal to achieve the highest removal efficiency possible by using different CWs with the intention of using their best capabilities. Another critical factor affecting the removal efficiency is the length of time during which the pollutants are in contact with the substrate and the rhizosphere, known as hydraulic

retention time (HRT). In systems with higher HRT, the time of contact between wastewater, rhizosphere and microorganisms will be higher. Otherwise the increase of hydraulic loading rate (HLR), will have the opposite effect since the amount of wastewater entering the system will be higher and consequently a reduction of time contact will happen (Carvalho et al., 2014; D. Zhang et al., 2014).

The substrate in this type of systems have a relevant importance once they provide support for the growth of plants and microorganism and interact directly with contaminants by sorption process. The sorption of pollutants is dependent on some soil characteristics like the amount of organic matter, pH, mineral concentration, clay composition and soil temperature. Grain-size distribution can influence the treatment efficiency. Small size particle (clay) in the soil have the capacity to hold more water than sandy soils, and have more binding sites. The binding of hydrophobic organic pollutants to soil are positively correlated with the organic matter concentration. Evapotranspiration is another very important aspect to take in account in the construction of a CW, once this can lead to a raise of water salinity through a decrease on water volume, affecting negatively the system and the treatment. The decrease in water volume also means a rise in dissolved compounds concentration, as the pollutants. This is calculated through the sum of physical evaporation from water surface and plant transpiration. To minimize this effect, in areas with high temperatures, it will be better to select a shorter hydraulic retention time, avoiding the potential to salinity increasing (Carvalho et al., 2014; Pilon-Smits, 2005; Stottmeister et al., 2003).

Another factor that has influence in extend of the treatment is the microbial community. Microbial communities in these systems have a primordial role in the performance at the level of antibiotics degradation. These communities have in their constitution autochthonous (indigenous) and allochthonous (foreign) species (Fig. 8). The

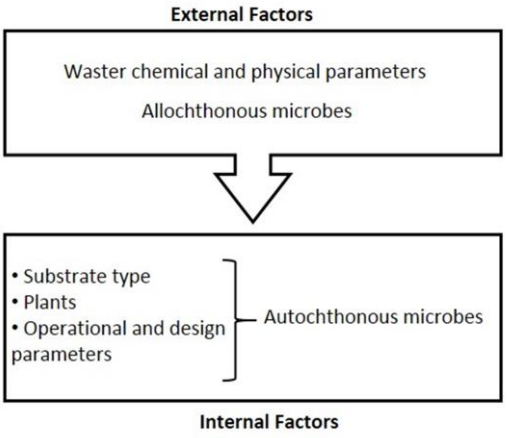

Figure 8: Factors affecting the microbial community in CW. (Adapted from: Truu et al., 2009).

first ones have adaptive features that allow their survival and growth, participating in purification processes. Allochthonous microbes, which come with water, usually can not survive in these systems ending to not have any functional importance (Truu et al., 2009).

In CWs several physical, chemical and biological mechanisms can contribute for the elimination of the pollutant (Fig. 9). In the most dominant processes, there are 2 biological (biodegradation and phytoremediation) and 2 physical (photodegradation and sorption) processes. Biodegradation correspond to the decomposition, by microorganisms, of organic substances, resulting in simpler and less harmful chemical substances. This process can occur in aerobic, anaerobic or anoxic compartments. Phytoremediation, as it will be described in more detail, is a removal process that involves the use of higher plants. The photodegradation pathway is the compounds degradation, by direct or indirect mechanism, when exposed to sunlight. Directly, the sunlight could be absorbed resulting in a chemical reaction and alteration of the compound. Besides that the oxidation species generated by natural photosensitizers can cause degradation by themselves. The sorption process can be the physical adherence of the pollutant onto the surface of a sorbent (adsorption) or the assimilation of the pollutant into the sorbent (absorption) (Garcia-Rodríguez et al., 2014; H. Jones et al., 2005; Truu et al., 2009).

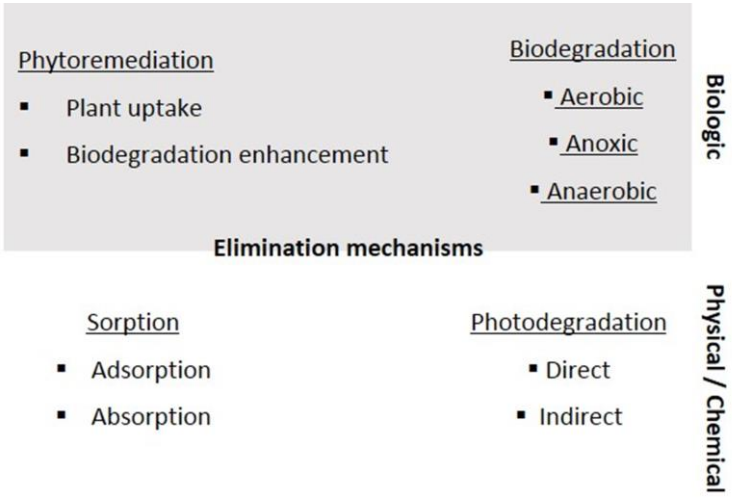

Figure 9: Elimination mechanisms of emerging organic compounds (ECOs) in CW's (Source: Garcia-Rodríguez, 2014)

Analyses of efficiency of CWs in the removal of veterinary pharmaceuticals, in contrary to what happens relative to human medicines, is still scarce (Carvalho et al., 2013a; Hussain et al., 2012; Xian et al., 2010).

1.4. Phytoremediation

In CWs the behaviour of plants and their interaction with the substrate and the associated ecological community play an important role. Plants and their associated microbes can be used in phytoremediation, a process for environmental clean-up, use for the removal and transformation of possible toxic chemicals. Plants will be subject to adverse conditions that they have to cope. Some of the compounds present in wastewaters, including pharmaceuticals are designed to be chemicals with a high biological activity and can have negative effects in the plants by inducing phytotoxicity or by changing the microbial communities, affecting the microorganism-plant interaction and making plants more susceptible to diseases and stress condition and reducing, therefore, the efficiency of the phytoremediation process (Boxall et al., 2006; Carvalho et al., 2014, 2012; Pilon-Smits, 2005; Schröder et al., 2007).

Although, due to concentrations found in the environment, the presence of pharmaceuticals should not be enough by itself to lead to plant death. However, some alteration as deficient growth or reduction crop production can be seen. The type of agents, dosage, adsorption kinetics and mobility in soil affect the impact of toxics in plants. The continued exposure to pharmaceutical compounds can lead to the bioaccumulation of these agents in plants, as already been demonstrated. The most concerning about this, are the potential threats to human health that this accumulation in crop, used in alimentation, may represent (Carvalho et al., 2014; Dolliver et al., 2007; Jjemba, 2002; Kumar et al., 2005; Migliore et al., 2003).

Several characteristics, as fast growing, high biomass, competitive, tolerance to pollution and dense root systems, make certain type of plants more suitable to be used in CWs. There are a diversity of plant species that can be used in this type of treatment. The capacity that halophytes exhibit to tolerate adverse conditions (salinity, ph, water toxicity) that occurs in CWs, and that other plants can't handle, makes them the main choice for these systems. For exemple, *Phragmites australis* is defined as a perennial and flood-tolerant grass with an extensive rhizome system capable to penetrate to depths up to 1m, frequently used in wastewater treatments in CWs (Vymazal, 2013). Phytoremediation may be limited by the bioavailability of the pollutants and their possible toxic effects, and the lack of nutrients in the soil. The plants that mediate the clean have to be able to act on the pollutant but for that they have to be where the pollutants are (Carvalho et al., 2014; Pilon-Smits, 2005; Stottmeister et al., 2003).

The presence of plants in CWs has an important role for different physics factors, helping in the sedimentation of suspend soils, and reducing the erosion and re-suspension. The roots systems are important for the stability of the soil. On the other hand their presence contributes for the treatment itself, both directly through uptake, adsorption, decomposition and phytovolatilization, and indirectly. This indirect way relates to the importance of roots in the biofilm growth, with the release of several compounds (roots exudates) from plants that can serve as carbon sources to stimulate microbial growth, and may also assist in the compounds degradation. The degradation of the pollutants depends strongly on the chemistry within the rhizosphere and the retention time. The alteration of the rhizosphere redox conditions resultant of oxygen release. The release of oxigen fixed in a plant by roots results in a higher microbial density in rhizosphere than in bulk sediments. Root system must obtain oxygen coming from the aerial organs. Oxygen reaching the roots can be release to the rhizosphere creating the oxidize conditions for aerobic decomposition of organic matter. The growth of nitrifying bacteria benefits with this oxygen release since without this it would be an anaerobic environment. The amount of oxygen release from the roots is dependent on different factors as: the internal oxygen concentration, the oxygen demand of the surrounding medium and the permeability of the root-walls. Investigations on the amount of oxygen released by roots have showed widely different concentrations. For *P. australis* the estimated amount of oxygen release varies between different authors mainly because the distinct techniques applied, and it goes from 5-12 g m-2 day -1 to 0,02 g m^{-2} day $^{-1}$ (Białowiec et al., 2014; Brix, 1997, 1994; Carvalho et al., 2012; Saeed and Sun, 2012; Vymazal, 2013).

Not only plants can have a positive effect in microbial communities, but also these communities can promote plant health by stimulating root growth, enhancing water and mineral uptake, and inhibiting growth of other plants. The use of a particular type of plant can lead to the increase of the number of microorganisms responsible for remediation. Alternatively, the raise of this number is also possible through the addition of microorganisms grown in laboratory, a process defined as bioaugmentation. Uptake by plants depends on the physicochemical properties (k_{ow}, pka, concentration) of compounds. The impact that the presence of plants can have in the removal /degradation of pollutants are affected by the HRT. The movement of compounds in the soil are affect by hydrophobicity (log K_{ow}) and volatility (henry's law constant). Molecules with high log K_{ow} do not dissolve in the soil's pore water since they, due to their hydrophobicity, are tightly bound to soil organic matter. These compounds can, therefore, be classified as recalcitrant pollutants since this lack of bioavailability results is a limitation of the

phytoremediation process (Li et al., 2014; Pilon-Smits, 2005; Stottmeister et al., 2003; D. Zhang et al., 2014).

Phytoremediation comprise several processes (Fig. 10), with different characteristic. This can occur in the rhizosphere or involve the uptake of compounds into the plant. In this, can be distinguished (Carvalho et al., 2014; Pilon-Smits, 2005; Schröder et al., 2007; Stottmeister et al., 2003):

- Phytostimulation: microbial biodegradation of organic pollutants in rhizosphere is promoted by plants.
- Phytostabilization: plants will stabilize and immobilize contaminants in soil, through the preventing erosion or runoff.
- Phytoextraction: used mainly for metals and other inorganic pollutants comprises their uptake by plants and subsequent accumulation in tissues.
- Phytovolatilization: compounds present in soil and water can be transported across plant membranes and after being uptake by plant tissue, pollutant can leave the plant to atmosphere in volatile form.
- Phytodegradation: plants can also degrade organic pollutants itself through their enzymatic activity. The compounds can be catabolized by mineralization or partial degraded to stable intermediates that can be store.

The main physico-chemical and biological processes in CW's occurs in rhizosphere. This is where the interaction between plants, microorganisms, soil and pollutants occurs. The rhizosphere extends approximately 1 mm around the root and is under the influence of the plant. It is expected that most of the reactions of these systems occurs in this contact area, referred as rhizoplane, between the roots and the surrounding soil. This zone is located between two: other the endorhiosphere, zone in roots, and ectorhizosphere, zone that surrounds the roots. Organic and inorganic compounds differ in the way that they are phytoremediated. In opposed to what happens in organic compounds, which can be degraded in the root zone of plants either by complete mineralization to inorganic compounds or partially degradation to a stable intermediate, inorganics cannot be degraded, but can undergo stabilization or sequestration. The accumulation of inorganic pollutants in tissues may result in toxicity both directly, by damaging cell structure, and indirectly via replacement of other essential nutrients. Inorganic elements are undegradable and can only be stabilized or moved and stored. Higher temperatures accelerate physical, chemical, and biological processes in general (Pilon-Smits, 2005; Stottmeister et al., 2003).

1.5. Objectives

In this work is attempted to explore and understand the potentialities of the autochthonous microorganisms of estuarine environments in the bioremediation of veterinary antibiotics. With this propose two separated experiments were conducted.

The first experiment was carried out with CW microcosms planted with *Phragmites australis*, in the presence of two different antibiotics (ENR and CEF) and their mixture (MIX). This work aimed to evaluate the changes in microbial community structure caused by the presence of this type of compounds, and to understand the contribution of microorganisms for the antibiotics removal.

The second work consists in an enrichment degraders experiment, for ENR, from rhizosphere of two different saltmarsh plants (*P. australis and Juncus maritimus*) with the aim of investigate the biodegradation of ENR and the isolation of strains possible involved in ENR degradation process.

This work is structured in 4 different chapters. The first consisted in a general introduction with information overview for both works. Second and third chapters, correspond to the first and second experiment, respectively. In each of them a short introduction of the theme is made, followed by the material and methods applied, results obtain, discussion and main conclusions. In the last chapter an overview for both works is made, emphasizing the new knowledge obtained from the work done.

CHAPTER 2

Removal of Veterinary Antibiotics in constructed wetlands microcosms

2. Removal of Veterinary Antibiotics in constructed wetlands microcosms - Response of bacterial community

2.1. Introduction

Pharmaceutical compounds have been used with the aim of improving life quality, medicine, food production and for industrial processes. These compounds are mostly xenobiotic and can have negative impacts on environment (Schröder et al., 2007). Their extensive use can represent severe risks to public health, since they are not totally removed on wastewater treatment plants (WWTPs) and, consequently, are released into nature. As a result, their presence has been reported in WWTPs effluents, surface and ground water. Within the veterinary pharmaceutical, antibiotics are one of the most used and, in addition, their capability to treat diseases, they are used as an enhancer of feed efficiency and, until the prohibition in 2006, as growth promoters (Dordio et al., 2010; Kemper, 2008; Sarmah et al., 2006). These are designed to act at low doses and to be completely excreted from the body after a short time of residence (Thiele-Bruhn, 2003). Part of these, are not completely metabolized resulting in their excretion as parental compound to the environment. In addition, metabolites have, themselves, activity or can be transformed back to the parental compounds after excretion. As a result, a significant percentage of the administered antibiotics that are released into the environment still have activity (Kemper, 2008; Sarmah et al., 2006). All of these parental/metabolite compounds will suffer different unknown transformation and will be capable to appear in different compartments. Besides this, the use of manure and sewage sludge as agriculture fertiliser represents another route for soil contamination. Surface runoff and leaching of the contaminated soil makes possible the unmetabolized antibiotics and their metabolites reach the aquatic medium (Kim and Aga, 2007; Thiele-Bruhn, 2003). The knowledge about the ecotoxicity of antibiotics is still scarce, the long term exposure to low doses and mixtures of compounds, with the possibility of synergistic effects, are two concerning factors (Dordio and Carvalho, 2013). This constant presence of antibiotics in the environment, in sub-therapeutically concentration, set a selective pressure under the bacteria leading to the selection of antibiotic resistant strains. This antibiotic resistance can be spread, through the gene transference to others (Kümmerer, 2004).

Due to all this, there was the need to develop systems with the capability to remove these compounds, and therefore, preventing their entry into the environment. Constructed wetlands (CWs) are artificial complexes, designed to simulate the capacity

for removal of pollutants, from water, shown by natural wetlands, that can be used as alternative or additive low-cost wastewater treatments (Białowiec et al., 2014; Carvalho et al., 2013a). Based on the different characteristics presented by CW's it can be distinguish different types. The characteristic choose to produce the classification can be either the type of plant used or the hydrology of the system. It has already been proved the capacity of these systems in the removal of various pharmaceutical compounds. However, little attention has been given to the veterinary drugs (Carvalho et al., 2013a; Hussain et al., 2012; Xian et al., 2010).

The presence of plant can bring several benefits, helping improving these systems, having the selection of the appropriated plant a great influence in the treatment. Plants are capable of establishing different interactions with the rhizosphere bacteria providing support for the growth and influencing their composition (Segura and Ramos, 2013; Vymazal, 2013). They can also, by several mechanisms as adsorption, uptake and/or degradation, promote the removal of pollutants (Dordio and Carvalho, 2013). The presence of the rhizosphere bacteria also has a positive impact into plants. This bacteria have an important role in the system functionality, being involved in the carbon and nitrogen cycles and in the biodegradation of the different organic substances (Garcia-Rodríguez et al., 2014; Kümmerer, 2004).

The physicochemical and biological processes results of the interaction between plants, microorganisms, soil and pollutants, therefore, the root zone is the most active reaction zone on a CW's (Pilon-Smits, 2005; Stottmeister et al., 2003). Macrophytes represents the plants that occur naturally in the wetlands, in this type of plant it is possible to identified several adaptation that allow them to survive in such environments. Their adaptations to this natural conditions, identical to the ones observed in the CW's, make them a viable option for these systems (Brix, 1997, 1994). Another important aspect to take into account is the length of time that water and soil will be in contact (hydraulic retention time), once these influences the removal efficiency of contaminants and influent pollutants concentration (Akratos and Tsihrintzis, 2007; Kipasika et al., 2004). For this work *P. australis,* a plant that already demonstrated capacity for removal drugs from livestock and slaughterhouse industries wastewater, was chosen (Carvalho et al., 2012).

Considering that bacteria, as have already been described, can have a key role in CWs and that the bacterial community can be affected by a diversity of contaminants, the present study aimed to evaluate the response of bacteria from CW microcosms to the presence of veterinary antibiotics, both in terms of community structure and removal performance.

2.2. Methods

2.2.1. Chemicals and reagents

Enrofloxacin and ceftiofur were purchased from Sigma-Aldrich® (Spain). Stock solutions were prepared in methanol with a concentration of 1g/L and stored at -20ºC. Methanol, acetonitrile and formic acid (98%) were acquired from Sigma-Aldrich® (Spain). Quality of all the used reagents was *pro analisis* or equivalent.

2.2.2. Collection and preparation of plants, soil and wastewater

P. australis, and sediment around their roots (rhizosediment), were collected in the margins of Lima estuary (North of Portugal) in May 2014. Sand was collected simultaneously, in the river basin.

At laboratory, sediments were separated from plant roots, and mixed with the collected sand (2:1 proportion). The utilization of the sediments that involves the roots was chosen with the intent to preserve the autochthonous microorganisms. The sediment/sand mixture was then homogenized to prepare the roots bed substrate for CW microcosms. The mixture of sand with sediment provides a more porous substrate permitting the water passaged.

Wastewater was collected weekly in a pig farm.

2.2.3. Microcosm's setup

Twelve microcosms were set up in plastic containers (0.4 x 0.3 x 0.3 m), filled with 3 different layers, in a total depth of 16 cm (Fig. 10). The first layer was composed by 4 cm of gravel, the second by 2 cm of lava rock and the third by 10 cm of roots bed substrate. To simulate what happen in real systems, all microcosms were wrapped in aluminium foil, preventing this way the penetration of light in substrate and the possible photodegradation of the compounds. All twelve microcosms were planted with *P. australis* (80 plants per

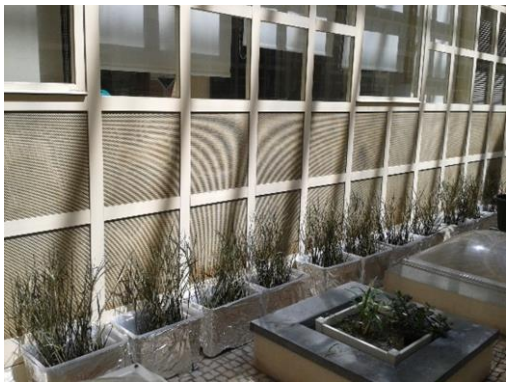

Figure 10: Constructed wetland – microcosm's setup.

microcosm). The set of microcosms was kept in an open indoor environment, subject to environmental temperature variations and environmental light exposure.

2.2.4. Microcosms operation and sampling

In the first ten days, 1,2 L of a nutritive solution (Hoagland and Arnon, 1950) was added and renewed daily to maintain plants at optimum nutritional conditions and allow microcosms acclimation. Water/solution level was maintained just above the substrate surface (flooding rate 100%) with only a tap on the base used for water exit. Four sets (3 microcosms each) were run in parallel, a set only with 1,2 L of wastewater, serving as control (CNT), and the rest with the same volume of wastewater but with the addition of the corresponding antibiotic. The division of the three contaminated sets were made in: two sets only with one of the drugs (ENR or CEF) and another with a mixture (MIX) of both compounds (Fig. 11).

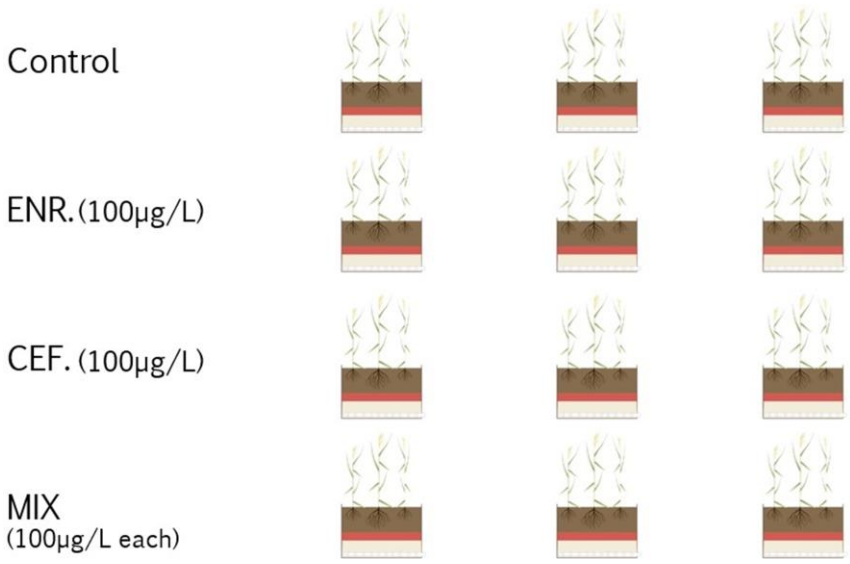

Figure 11: Schematic representation of the experiment for the different treatments (ENR – enrofloxacin; CEF – ceftiofur and MIX – mixture).

The concentration used (100 µg/L) have already been found in the environment (Babić et al., 2010). The used concentration was prepared by adding the necessary quantity of the stock solution at the wastewater. The wastewater was treated in 18-one week cycles. The time that wastewater was maintained in the systems (7 days) was chosen based in HRT normally used in full scale CW's (Carvalho et al., 2012). To prevent the development of anoxic areas, a daily recycle were manually operated, being the systems operated in batch mode. Water evaporation was daily controlled by addition of

deionized water, when necessary. In the end of each week, the microcosms were completely drained and refilled with new wastewater doped or not with the antibiotics. For the evaluation of different parameters, water and soil samples were collected at different weeks. Samples were used to evaluate the presence of veterinary drugs, microbial abundance and community characterization. For each microcosm, soil samples were collected in three different points to obtain a composite sample. Filtered (cellulose nitrate filters, 0,45 µm of porosity) wastewater samples, before and after treatment, were also collected for antibiotic and nutrient analyses. Samples for antibiotics and nutrient analyses and community characterization were stored at -20ºC. For microbial abundance, soil sample were immediately fixed with 2,5ml of distillate water with formaldehyde at 4% (0.2 µm filtered) and storage at 4ºC.

2.2.5. Nutrient analyses

Dissolved orthophosphate, ammonia, nitrites and nitrates were analyzed following the methods described in Grasshoff and Ehrhardt (1983). The dissolved orthophosphate (PO_4^{3-}) is typically measured by colorimetric method molybdenum blue. This method presupposes that in acidic solution, the orthophosphate reacts with ammonium molybdate and antimony potassium tartrate forming a heteropolar-phosphomolybdic acid, which is reduced by ascorbic acid to an intense blue complex.

For quantification of the concentration of ammonia (NH_3 and NH_4^+), the method is based on the fact that ammonium, in moderately alkaline solutions, reacts with the hypochlorite forming the monochloramine compound. This, in turn, in the presence of a catalyst (nitroprusside), phenol and excess of hypochlorite gives rise to an intense blue complex (indophenol).

Nitrites (NO_2^-) were quantified by the method of reaction of nitrite with an aromatic amine (sulfanilamide) to give a diazotized compound, which binds to a second aromatic amine (N- (1-naphthyl) ethylenediamine) resulting in a pink complex, whose intensity is proportional to the amount of nitrite in the solution. Nitrate (NO_3^-) was measured by an adaptation of the spongy cadmium reduction technique described in Jones (1984), subtracting nitrite value from the total. All the analyses were performed in triplicate.

2.2.6. Veterinary antibiotics analyses in soil samples

To evaluate if any of the added antibiotic still remains in the system, soil samples were collected during the microcosm experience at week 1 (w1), 2 (w2), 4 (w4), 8 (w8), 14 (w14) and 18 (w18). Analyses were performed according to the process developed by (Carvalho et al., 2013b). For that 2 g of each sediment samples, previously lyophilized, were extracted with 10 ml methanol-acetone (95:5, v/v) using USE (period of 15 min each). This procedure was repeated twice for each composed samples. After each extraction the soil sample was centrifuged and the supernatants were collected and combined. Combined supernatants were then evaporated to dryness under a nitrogen stream at 35 ºC. The residues were dissolved in 1.0 ml methanol-mobile phase (1:3, v/v) and analysed by HPLC. In some of the samples, a standard solution (known concentration) of which one of the antibiotics was added, to see possible interference of the matrix and calculated the recovery rate. Compounds separation was proceeded with a high-performance liquid chromatography Beckman Coulter equipment (HPLC system gold) provided with a DAD (diode array detector) detector (module 128) and an automatic sampler (module 508). A 100 mm × 4.6 mm Kinetex 2.m C18 column (Phenomenex, UK) using a linear gradient program. The mobile phase was composed by two eluents: (A) filtered water–formic acid (99:1, v/v; filter at 0.45 µm of porosity) and (B) acetonitrile. Before used, the eluents were degassed in an ultrasonic device for 15 minutes. The linear gradient was composed by: 100% of the eluent A, staying this isocratic conditions for 2 min, then 70% of eluent A (30% eluent B) of being this condition maintained for 10 min. The initial conditions (100% of eluent A) were reached 10 min later, with a re-equilibration rime of 2 min to restore the column. 50 µL of samples was injected and the detector signal was monitored at λ = 280 nm.

2.2.7. Microbial abundance

To estimate microbial abundance in sediments, Total Cell Counts (TCC) was obtained by DAPI (4',6'-diamidino-2-phenylindole) direct count method (Kepner and Pratt, 1994; Porter and Feig, 1980). After collected, samples were immediately fixed with 2,5 mL of distillate water with formaldehyde at 4% (0.2 µm filtered). Two drops of Tween 80 (0,2 µm-filtered, 12,5% (v/v)) were added to samples and then stirred at 150 rpm for 15 min followed by 15 min of resting. Samples were, then, sonicated for 10 min and stirred for 1min, stirred samples were left to rest overnight at 4º C. From the previously fixed samples, 150µl were taken to test-tubes. To this was added 2,5 ml of saline solution (0,2 µm-filtered, 9g L^{-1} NaCl) and 2 drops of Tween 80. To stain the samples, DAPI was

added, and incubated in the dark for 12-15 minutes. The tube content was then filtered through black nucleopore polycarbonate filters (0.2 µm pore size, 25 mm diameter, Whatman, UK) under vacuum and washed with 5 mL of autoclaved 0,2 µm-filtered distilled water. Membranes were set up in glass slides and cells count in an epifluorescence microscope (Leica DM6000B).

2.2.8. Microbial community structure using automated rRNA intergenic spacer analyses (ARISA)

To evaluate the microbial community structure, ARISA (automated rRNA intergenic spacer analyses) was performed, for triplicate samples of w1, w8 and w18. This technique allows amplification of the 16S-23S intergenic spacer region in the rRNA operon. Total DNA was extracted from 0,5 g wet weight of homogenized sediment samples, using an Ultra Clean Soil DNA Isolation Kit (MoBio, Carlsbad, CA, USA), according to the included protocol. A 1.5% electrophoresis agarose gel was used to evaluate the quality of extracted DNA. For ARISA, extracted DNA was amplified using ITSF (5-GTCGTAACAAGGTAGCCGTA-3) and ITSReub (5-GCCAAGGCATCCACC-3) primers set (Cardinale et al., 2004). PCRs were performed in duplicated 25µl volumes. The PCR mixture was held at 94 ºC for 2 min, followed by 30 cycles as 94 ºC for 45s, 55 ºC for 30s, 72 ºC for 2 min, and a final extension at 72 ºC for 7 min. PCR products were visualized on 1.5% agarose gel. For samples purification was used a purification kit (UltraClean 15 DNA Purificatio Kit from MO BIO). Once purified, products were quantified using the Quant-it HsDNA assay kit and Qubit fluorometer (Invitrogen). In STABVIDA Sequencing Facilities samples fragments were run on an ABI3730 XL genetic analyser.

2.2.9. 454-pyrosequencing analysis

To complement the information retrieved from ARISA, samples from week 8 were also analysed by 454-pyrosequencing. For that composite, DNA sample prepared. This composite samples was send to Biocant facilities for analysis. Briefly, the samples V3/V4 hypervariable region of the 16S rDNA was amplified. This amplification was made using the forward primer 5'–ACTCCTACGGGAGGCAG-3' and the reverse primer 5'–TACNVRRGTHTCTAATYC–3, containing also an upstream 454 Life Science's titanium sequencing adaptor (5'- CTATGCGCCTTGCCAGCCCGCTCAG -3'), in the following the PCR program: initial denaturation at 94ºC for 4 min followed by 25 cycles of denaturation at 94ºC for 30 s; annealing at 44ºC for 45 s and extension at 68ºC for 60s,

and a final extension step at 68ºC for 10 min. All the amplifications were carried out in a MyCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, California, USA). The amplicons are clonally amplified by emulsion PCR. After obtain the amplified products their purification was made with AMPure XP beads (Agencourt, Beckman Coulter, USA) and the quality assessment visualized on 1.2% (w/v) agarose gel and quantification by fluorescence using the PicoGreen dsDNA quantitation kit (Invitrogen, Life Technologies, Carlsbad, California, USA). Resulting DNA library beads are loaded into the wells of a PicoTiterPlate (PTP) device. Once in the Genome Sequencer FLX Instrument (454 Life Sciences, Roche) addition of one (or more) nucleotide(s) generates a light signal that is recorded by the CCD camera in the instrument, signal strength being proportional to the number of incorporated nucleotides. Proprietary software converts the light signals into nucleotide information generating the final sequencer reads. The microbes present in each sample were identified with a bioinformatics pipeline developed at Biocant.

2.2.10. Statistical analyses

Triplicated samples, of each treatment, were analysed and treated in separate, being the mean and standard deviation values calculated to obtain a value for treatment at each week. To evaluate the possible statistically significant differences ($p < 0.05$) in TCC, bacterial richness and diversity a parametric one-way analysis of variance (ANOVA) was applied. If any significant difference was detected in ANOVA a multiple Tukey comparison test was performed to detect where the differences were. The overall statistical test were performed using commercial software STATISTICA, version 12, StatSoft, Inc. ARISA fragment lengths were analysed by Peak Scanner version 1.0 Software (Applied Biosystems) and data were transferred to an excel sheet and transformed in a matrix of aligned fragments. In this matrix, fragments with fluorescence units below 50 were considered “background noise” and not take into account, the same happens to fragments with less than 200 bp, that were removed since were considered to be too short ITS for bacteria. The matrix was imported to the PRIMER 6 software package (version 6.1.11) (Clarke and Gorley, 2006). First, based on ARISA profiles, and for better address the ecological description of the bacterial community with samples, bacterial richness and diversity index were calculated. For this, peaks number was considered to represent species number and peak height was considered to represent the relative abundance of each bacterial species. After, for the evaluation of community structure, the imported matrix was normalized using the presence/absence pre-treatment function. Samples were analysed using the Bray-Curtis similarity method and a hierarchical cluster, with the

default parameter and SIMPROF test. A multidimensional scaling (MDS) plot was also generated using the default parameters with a minimum stress of 0.01 to generate a configuration plot based on percentage similarity. Similarity of bacterial community composition was assess through an analyses of similarities (two-way crossed ANOSIM, based on Bray-Curtis similarity) performed using the PRIMER 6 software (Clarke and Gorley, 2006). This analyse (ANOSIM) is a permutation-based hypothesis statistical test, equivalent to univariate ANOVA, which test for differences between groups (multivariate) samples for different factors or experimental treatments.

2.3. Results

2.3.1. Evaluation of the systems functionality

To evaluate the systems functionality over time, analyses for water quality parameters before and after treatment were conducted. Results chemical and biochemical oxygen demand (COD and BOD) were obtained by (Ferreira, 2014) and are summarised in Table 1. High removal rates occurs for both COD and BOD, with the first having a percentage of removal rate ranging from 71% to 93% and the second oscillating from 85% to 96%.

The results obtained in the analyses of different nutrients (ammonia – NH_3; nitrates - NO_3^- nitrites – NO_2^- and phosphorous- PO_4^{3-}) can be found in Table 2. In this, it is possible to see a decrease in the concentration of NH_3 and PO_4^- in water, after the treatment, when compared with the influent. In the case of NH_3 for all of the weeks the removal rates were higher than 88%. These high removal rates were similar between the treatments and through time. Regarding PO_4^-, removal rates ranged between 81 and 92% in week 1 and 8. However, a decrease in the removal efficiency, for values between 5 and 43% can be noticed in week 18. For both, NO_3^- and NO_2^- concentrations present in the water at the end of the treatment increased with time. This increase lead to higher concentrations in the water after treatment, when compared with the concentrations found in the initial water.

Table 1: Water quality parameters (chemical oxygen demand - COD and biochemical oxygen demand – BOD) initial concentration and removal rates for the three selected weeks (adapted from Ferreira, 2014)

Parameter	*Treatment*	*Week*		
		W1	*W8*	*W18*
COD	Initial (mg/L O_2)	2210	3420	1431
	Control (Removal rates %)	83% (±10%)	87% (±2%)	83% (±5%)
	ENR (Removal rates %)	93% (±2%)	90% (±3%)	71% (±8%)
	CEF (Removal rates %)	89% (±2%)	84% (±3%)	77% (±8%)
	MIX (Removal rates %)	91% (±6%)	87% (±1%)	78% (±7%)
BOD	Initial(mg/L O_2)	223	242	516
	Control (Removal rates %)	88% (±2,4%)	85% (±1%)	96% (±1,1%)
	ENR (Removal rates %)	91% (±3%)	91% (±1,7%)	96% (±1,6%)
	CEF (Removal rates %)	90% (±1,4%)	85% (±0,6%)	96% (±1,8%)
	MIX (Removal rates %)	88% (±2,3%)	89% (±1%)	96% (±0,9%)

Table 2: Nutrients parameters - initial and final (average and standard deviation) and concentration of the three selected weeks.

Parameter (µM)	***Treatment***	***Week***		
		W1	***W8***	***W18***
NH_3	Initial	69.7	57.1	45
	Control	3.6 (±2.7)	4.3 (±4.7)	1.6 (±0.3)
	ENR	2.4 (±0.4)	4.3 (±3.4)	1.4 (±0.3)
	CEF	6 (±1)	6.6 (±2.7)	1.3 (±0.5)
	MIX	4.6 (±1.6)	4.3 (±1.6)	2.3 (±0.6)
NO_3^-	Initial	1.1	9.6	51.5
	Control	15.4 (±7.6)	189.6 (±94.5)	396.8 (±118.5)
	ENR	31.2 (±37.2)	32.4 (±17.4)	101.1 (±39.3)
	CEF	38.7 (14.4)	61.4 (±13.2)	258.8 (±174.6)
	MIX	9.0 (5.9)	49.4 (±52.5)	344.5 (±104)
NO_2^-	Initial	6.0	9.9	9.7
	Control	1.3 (±0.7)	66.4 (±9.3)	70 (±61)
	ENR	3.5 (±2.6)	85.0 (± 109.6)	43.3 (±32.9)
	CEF	15.3 (±8.4)	137.4 (±96.3)	86.6 (±28.2)
	MIX	1.2 (±1.3)	87.2 (±84.8)	51 (±7.2)
PO_4^-	Initial	436	712	130.9
	Control	47.9 (±15.2)	54.1 (±16.8)	89.8 (±10.5)
	ENR	36.3 (±2.9)	84.5 (± 51)	123.9 (±38.8)
	CEF	58.6 (±17)	138.8 (±37.1)	91.6 (±30.2)
	MIX	52 (±52)	109.9 (±5.7)	74.6 (±1.1)

Parameter (µM)	***Treatment***	***Week***		
		W1	***W8***	***W18***
	Initial	69,7	57,1	45
	Control	3,6 (±2,7)	4,3 (±4,7)	1,6 (±0,3)

2.3.2. Veterinary antibiotics removal

The recovery percentages obtained for the sediments extraction processes demonstrated that the method chosen was valid. The analysed sediment samples did not present any peaks that could indicate the antibiotics presence. Limits of detection (LODs) and quantification (LOQs), were already calculated considering the extraction of 2 g of soil sample (Carvalho et al., 2013b). Values of ENR and CEF were 0.09 µg/g to 0.2 µg/g for LOD and from 0.2 to 0.6 µg/g for LOQ, respectively. The analyses of contaminated samples shows no interference of matrix and any peak resulting from the presence of drugs should appear well define.

In the data obtained from the water analyses it was not possible to detect the added compounds. This result, as already happened in the soil samples, signified that antibiotics concentrations present are lower than the LODs calculated (0.2µg/L for ENR and 0.6µg/L for CEF). This decrease in concentration signifies a removal rate above 90%.

2.3.3. Microbial abundance and bacterial richness and diversity

Microbial abundance was evaluated for the different treatments at week 8. The averageabundance, estimated by TCC, for all the treatments were 7 $\log_{10}$ g^{-1}. No significant differences ($p > 0.05$) were observed, between treatments.

Bacterial richness and diversity index were estimated from ARISA profiles, for samples of different treatments for week 1, 8 and 18. Results obtained for richness and diversity are present in Figure 12 and Figure 13, respectively. For each week, no significant differences ($p > 0.05$) were observed between treatments, both in terms of bacterial richness or diversity. Regarding evolution of bacterial richness through the time, it was observed a tendency to decrease, but differences were not significant ($p > 0.05$). Also, it was observed a decrease in bacterial diversity through the time, with significant differences ($p < 0.05$) between week 1 and week 8 for all treatments. For MIX this difference was also significant between weeks 1 and 8.

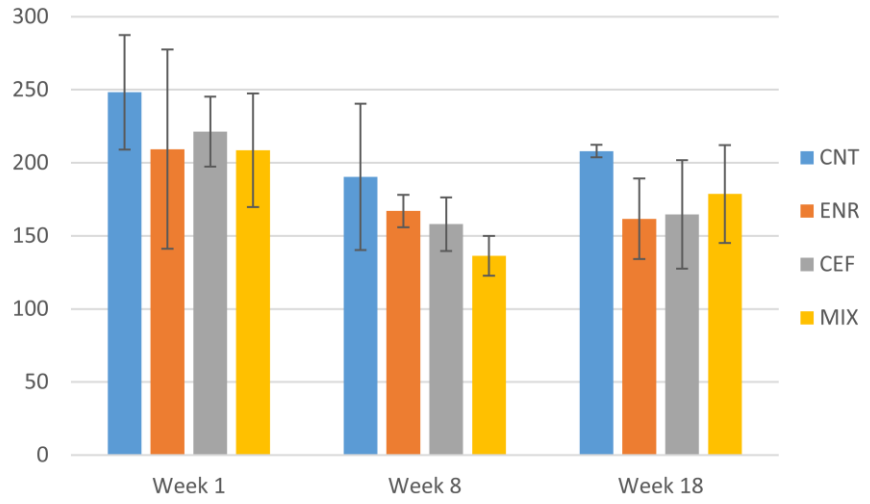

Figure 12: Bacterial richness in different treatments along experiment.

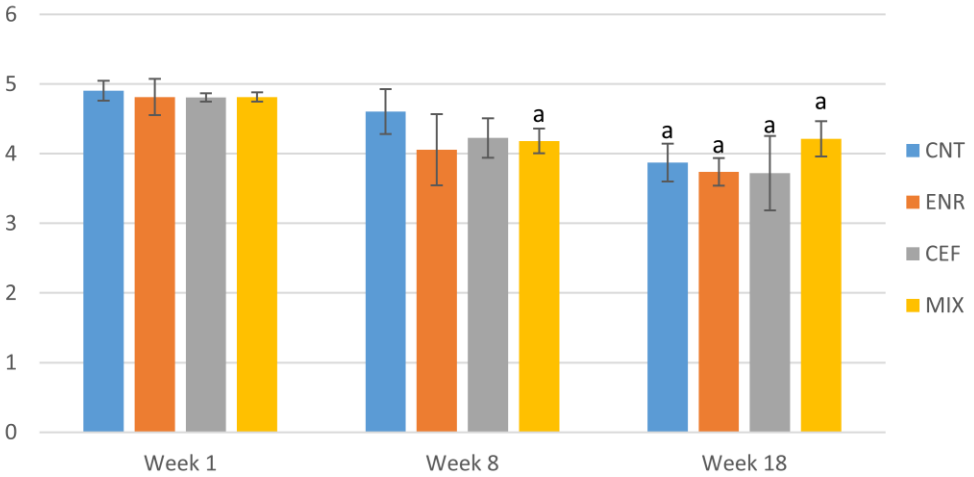

Figure 13: Bacterial diversity in different treatments along experiment; a-significant differences ($p < 0.05$) when compared with the same treatment for week 1.

2.3.4. Bacterial community structure

Bacterial community structure was obtained from ARISA profiles, were each peak representing a different ARISA fragment length (ALF) corresponds to a different bacteria phylotypes. More important than the total number of peaks (total phenotypes) it is the evaluation of their distribution. With that information it is possible to assess differences in samples community structure. These differences were analysed in terms of treatment and over time. In these analyses a good experimental replication, exhibited in the hierarchical cluster analyses (Fig. 14A), was obtained. Replicates from the same treatments (CNT, ENT, CEF and MIX) are grouped together being, at the same week, more similar between each other than with the others treatments, with only one exception. Also, samples from

the same week (w1, w8 and w18) are more similar between each other than with samples for another week, independently of the treatment. However, it is possible to see that week 8 and 18 community showed a higher similarity between each other, than with week 1. MDS plot at 50% of similarity from three separate groups, corresponding to the three different weeks, representing the time effect on the community (Fig. 14B).

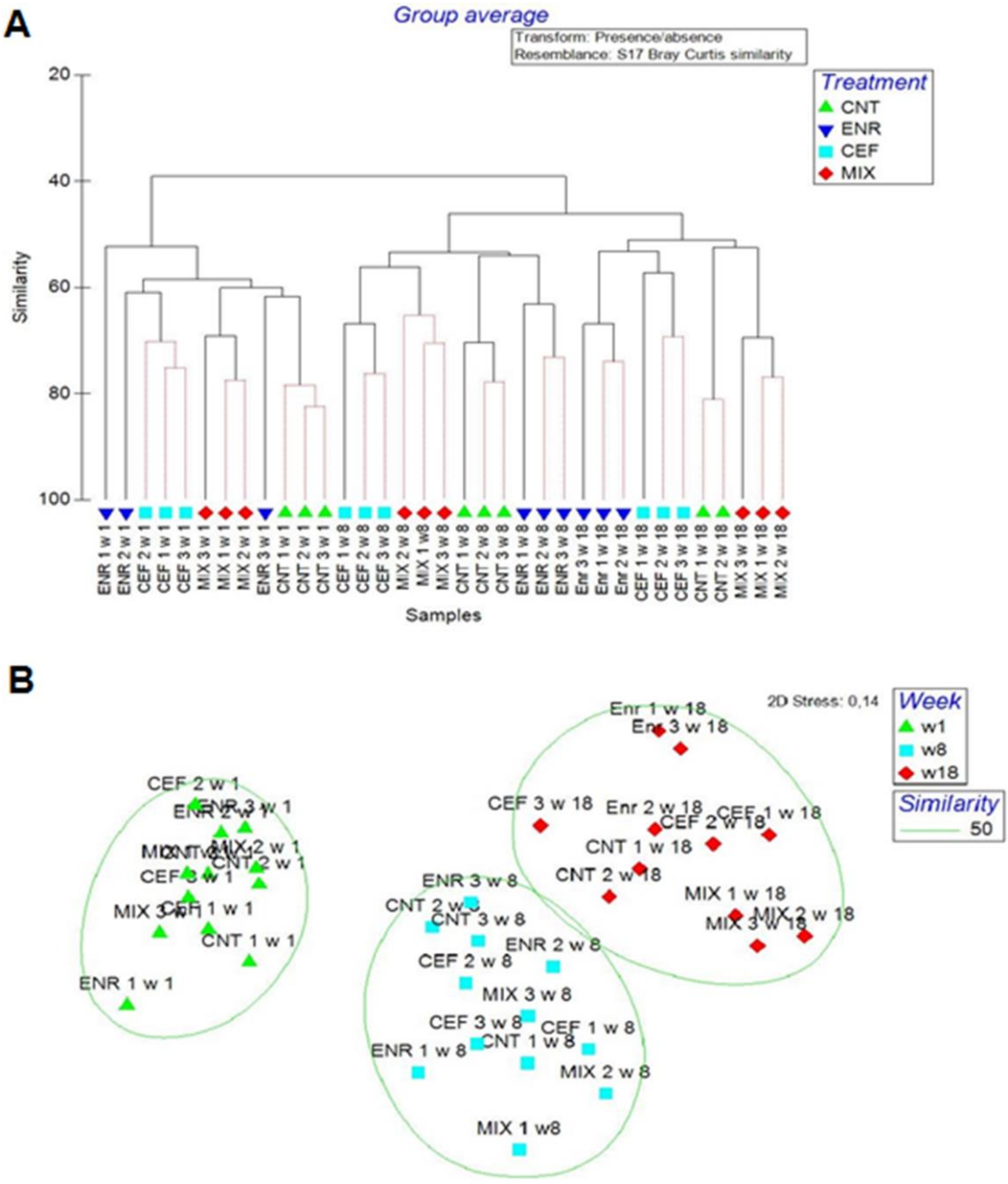

Figure 14: Hierarchical clustering (A) and multidimensional scaling (MDS) ordination (B) based on Braey-Curtis similarities from ARISA fingerprints of bacterial communities at week 1, 8 and 18 for the different treatments (CNT – control, ENR – enrofloxacina, CEF – ceftiofur and MIX – mixture).

Analyses of similarity (two-way crosses ANOSIM) confirmed a significant effect of both factors (treatment and time) in community structure (Table 3).

Table 3: Global and pairwise values for two-way crossed ANOSIM test based on ARISA results for the different weeks and treatments.

Differences between	Statistic value (R)	Significance level (%)
Treatment		
Global test	0,841	0,1
Pairwise tests		
CNT, ENR	0,708	0,2
CNT, CEF	0,986	0,1
CNT, MX	1	0,2
ENR, CEF	0,704	0,2
ENR, MIX	0,728	0,3
CEF, MIX	0,914	0,5
Week		
Global test	0,993	0,1
Pairwise tests		0,1
w1, w8	1	0,1
w1, w18	1	0,1
w8, w18	1	0,1

2.3.5. 454-pyrosequencing analysis

The sequences obtained from the 454-pyrosequencing analyse were transformed in percentage to allows a better comparison between treatments. Once this analyse was only possible to be done for week 8, it is not possible to see the differences through the time. Therefore, there will be only analyse the differences between treatments and consequently the changes caused by the presence of the antibiotics. Results are presented in term of most abundant phylum, class and orders. In addition, the taxa with more than 2% in at least one of the systems are represented in Table 4. In terms of phylum there are clearly four that stand out from the 26 phyla present: *Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria*. Together they represent between 83.25% and 89.47% of all sequences present. In addition to these ones, another four (*Chloroflexi, Acidobacteria, Gemmatimonadetes and Deinococcus-Thermus*) contributes, at least in one of the microcosms, for more than 1% of the total (Fig. 15).

For the eigth most representative phyla, above mentioned, an illustration of the classes was made, except for *Actinobacteria* that is represented by only one class (*Actinobacteria class*) and in which orders will be represent. The six classes that represent the phylum *Proteobacteria* are shown in the Fig. 16. Besides these classes, sequences

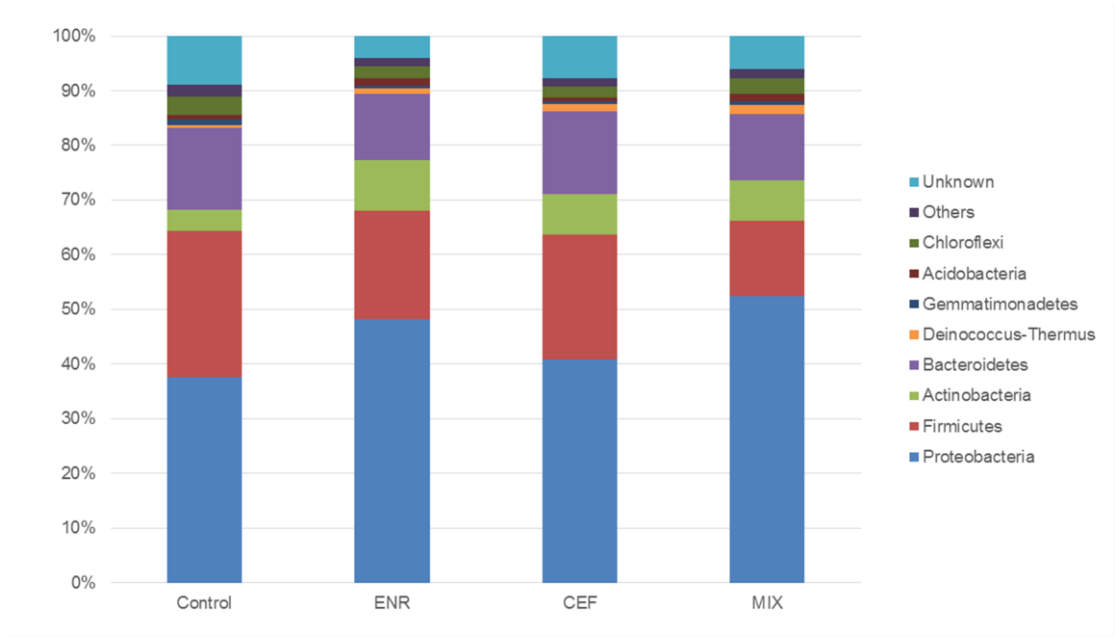

Figure 15: Relative abundance of differente bacterial phyla within the different systems. The "Others" refers to the other 18 phyla not showed. "Unknown" are the sequences that were not classified in any phylum.

classified as "unknown" are represent, which includes the sequences associated to *Proteobacteria* for each it was not possible to identify the classes. The most representative classes, in this phylum, are *Alphaproteobacteria* and *Betaproteobacteria*. Due to that, a closer attention to each one of them was given. Besides these classes, sequences classified as "unknown" are represent, which includes the sequences associated to *Proteobacteria* for each it was not possible to identify the classes. Due to that, a closer attention to each one of them was given. *Gammaproteobacteria* will also be

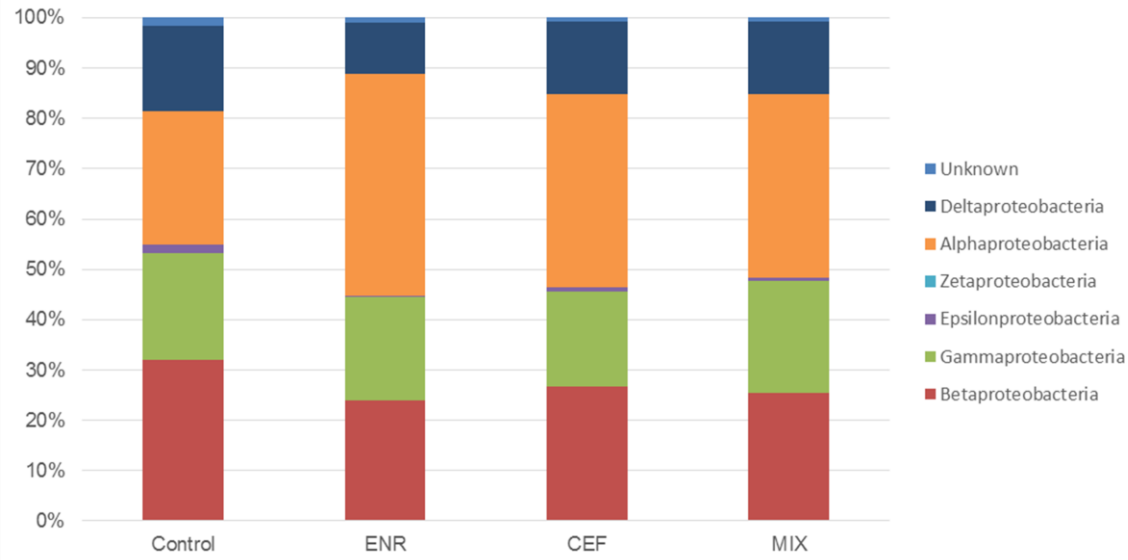

Figure 16: Relative abundance of different bacterial classes within *Proteobacteria* phylum.

focused. Comparing with control it was observed an increase in the class *Alphaproteobacteria* and a decrease in the class *Betaproteobacteria*, in all systems exposed to antibiotics.

To evaluate better these changes a representation of the orders, in this specific class, were also put in chart (Fig. 17 and 18). Inside *Alphaproteobacteria* it is possible to see an increase in *Rhizobiales* order in the systems with antibiotics addition. This increase is, in part, a result of the representative presence of the family *Phyllobacteriaceae,* that alone, account for at least 1.5% of the total of sequences presents in that systems (Table 4). *Rhodobacterales* is another order with representative presence in this class, the percentage of sequences, in this case, decrease in the systems treated with antibiotics. However, when *Rhodabacteraceae* family, present in this order, is compared with the total sequences in the systems there is an increased. The sequences belonging to this family, without a more specific classification, suffers an increase in the systems with antibiotic addition. In the ones attributed to the *Rhodobacter* genus, this increase is only existent in the systems treated with ENR. In the case of order *Sphingomonadales* there are two

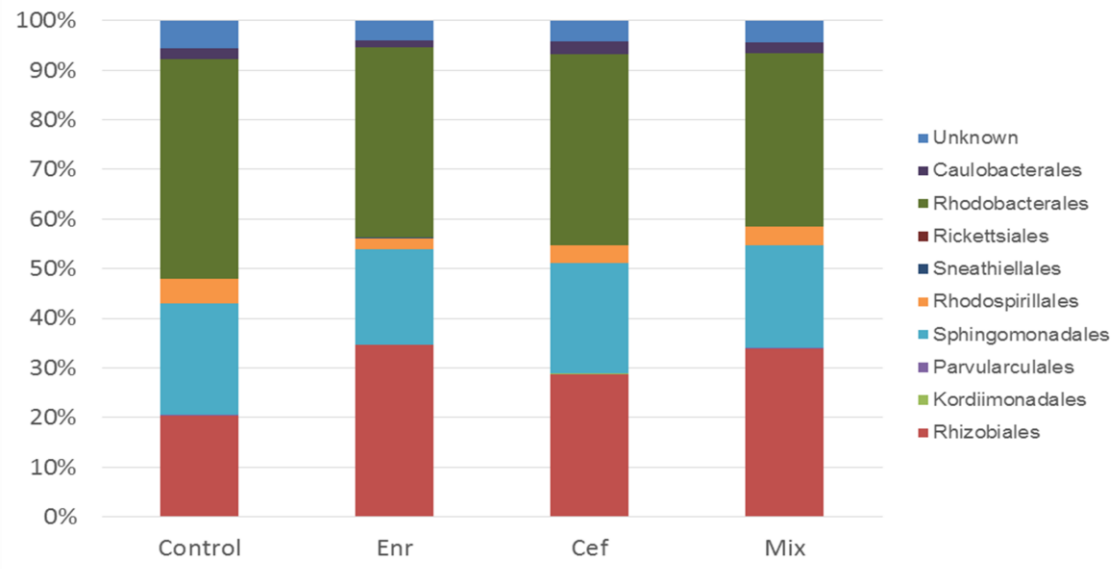

Fig. 17: Relative abundance of different bacterial order within *Alphaproteobacteria* class.

Figure 17: Relative abundance of different bacterial order within *Alphaproteobacteria* class.

different genus (*Erythrobacter* and *Novosphingobium*) present in the table with the total sequences of the most abundant taxa (Table 4).

In *Betaproteobacteria* classes (Fig. 18) the two dominants orders (*Burkholderiales* and *Rhodocyclales*) follows opposite trends. In *Burkholderiales,* the percentage of sequences is higher in the contaminated systems, than in the control one. Actually, this

order represents, it self, 4.2% in systems with antibiotic and for control 2.8%, in the total of sequences for that systems. In *Rhodocyclales,* its the opposite with the percentage of sequences being higher in the control system. This is also what happens in the ones present in the overall sequences percentage. *Thauera,* a genus in the order of *Rhodocyclales*, represent, for control, 3.3%, with almost only half of this for the rest of the systems. *Gammaproteobacteria* class sequences appear in the table of most representative taxa (Table 4). These sequences are either only associated with the phylum or classified in *Pseudoxanthomonas* genus. For the latter it is possible to see an

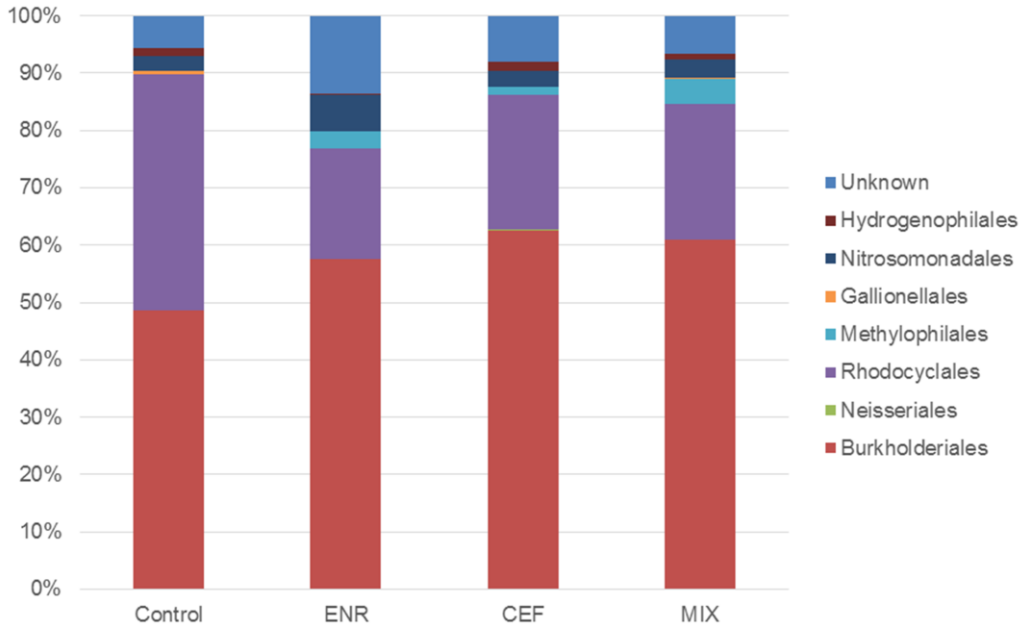

Figure 18: Relative abundance of different bacterial order within Betaproteobacteria class.

increase in the antibiotic treatment systems. The sequences only associated with the class the tendency is for the maintenance of sequence percentage, with only an increase in the MIX system.

The *Firmicutes* phylum is represented by four different classes (Fig. 19), being dominated by *acidomicrobiales*

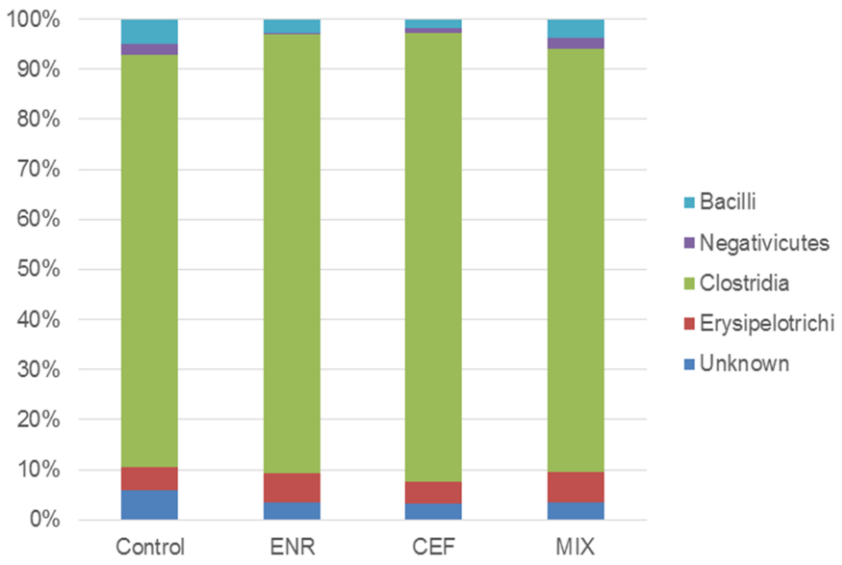

Figure 19: Relative abundance of different bacterial classes within *Firmicutes* phylum.

The *Clostridia* class that stands from the others with over 80% of all the with in this phylum. Although this expression, only one order was found, *Clostridiales*. The sequences associated only to the *Clostridiales* order represents, in the total of the sequences found in the systems, between 7.9% and 13.3%, which was the higher percentage found for a single taxon. In addition, two of the families identified within *Clostridiales order, Ruminococcaceae* and *Peptostreptococcaceae* are also among the most representative taxa families, than have in this order the higher percentage, are also englobed in the taxa with more total percentage.

In the *Bacteroidetes* phylum, sequences can be grouped in four different classes or be only associated with the phylum (Fig. 20). The latter, includes a large percentage representing more than 3.9% of the total sequences in all systems (Table 4). In the case of the sequences grouped in orders, it is possible to see an increase in the order *Bacteroidia* within ENR systems. This increase is mainly a consequence of the presence of the genus *Alkaliflexus*. In the table of the total sequences is possible to see that this genus alone represent 2.6% of the total sequences for ENR systems. Another order worth of noticed is the *Flavobacteriia* that clearly increased in the systems with antibiotic.

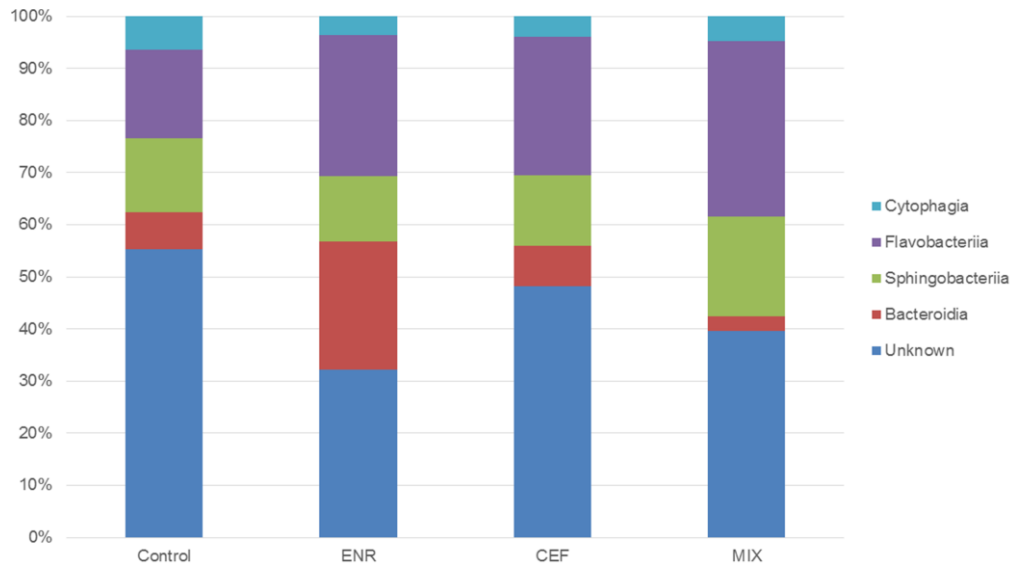

Figure 20: Relative abundance of different bacterial classes within Bacteroidetes phylum

In the *Actinobacteria* phylum, and due to the existence of only one class (*Actinobacteria* class) the different orders were analysed (Fig. 21). In this, there is a clear abundance of *Acidimicrobial* sequences that increase in the systems that contains antibiotics, particular in the ENR systems. This have such an influence that is also demonstrate for the total number of sequences with *Acidomicrobiales* accounting for 3.9% of the total sequences in systems containing ENR. The sequences that was only classified in terms of phylum are also present in this table with a percentage ranging between 0.7% and 2.4%. The *Coriobacteriales* order present an opposite trend decreasing, notouriously,

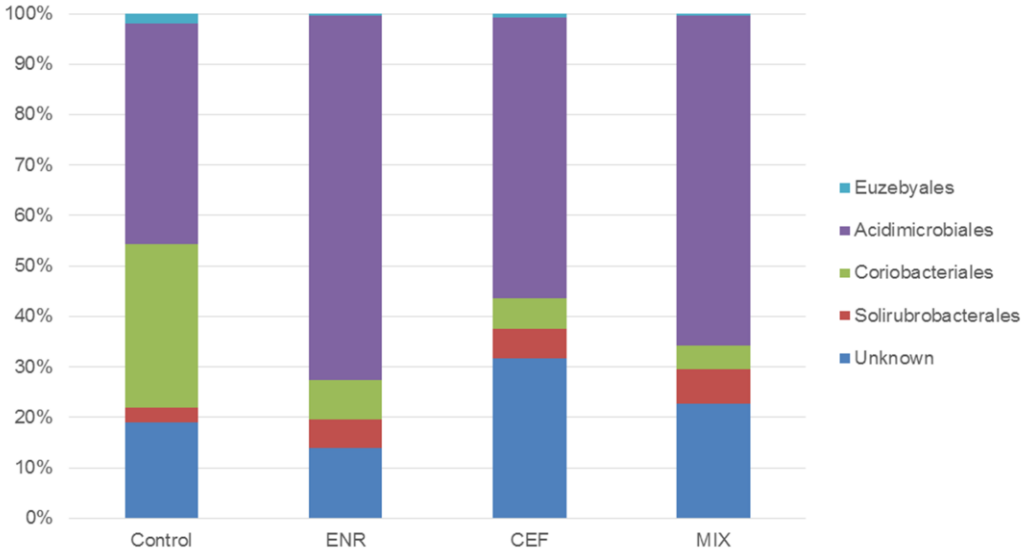

Figure 21: Relative abundance of differente bacterial orders within the class Actinobacteria that is the only class from the Actinobacteria phylum.

in the presence of antibiotics.

Table 4: Percentage of sequences of the most abundant taxa (abundance >2% at least one of the systems).

Phylum	Class	Order	Family	Genus	Control	ENR	CEF	MIX
Bacteroidetes					8.3%	3.9%	7.3%	4.8%
	Bacteroidia	Bacteroidales	Marinilabiaceae	Alkaliflexus	0.1%	2.6%	0.1%	0.0%
Actinobacteria					0.7%	1.3%	2.4%	1.7%
	Actinobacteria_(class)	Acidimicrobiales			0.4%	3.9%	1.9%	2.0%
Firmicutes	Clostridia	Clostridiales			12.9%	12.1%	13.3%	7.9%
			Ruminococcaceae		3.6%	1.8%	2.6%	1.1%
			Peptostreptococcaceae		2.0%	2.4%	1.7%	1.6%
Proteobacteria	Betaproteobacteria	Burkholderiales			2.8%	4.6%	4.2%	5.8%
		Rhodocyclales	Rhodocyclaceae	Thauera	3.3%	0.8%	1.8%	1.7%
	Gammaproteobacteria				3.9%	3.0%	3.1%	5.1%
		Xanthomonadales	Xanthomonadaceae	Pseudoxanthomonas	0.8%	2.4%	1.5%	1.9%
	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae		0.6%	3.0%	1.5%	2.4%
		Sphingomonadales	Erythrobacteraceae	Erythrobacter	1.4%	0.0%	0.0%	2.7%
			Sphingomonadaceae	Novosphingobium	0.1%	3.1%	2.4%	0.3%
		Rhodobacterales	Rhodobacteraceae		2.3%	4.2%	3.7%	4.1%
				Rhodobacter	1.9%	3.1%	1.6%	1.7%

2.4. Discussion

Utilization of CW's in the last 25 years became more frequent in an effort to improve the quality of effluent discharged into the environment and, consequently, decrease their potential impacts on the ecosystems (Lee et al., 2009). In these systems, bacteria play a key role in the processes. For this, the evaluation of the responses of the bacterial community to the presence of veterinary antibiotics is important, in order to better understand the alterations in the communities.

In order to assess the functionality of the system, in terms of water purification, throughout the experiment, water parameters were analysed. For COD's and BOD's high removal rates were achieved. These removal percentages are in agreement with high removal rates already described for different CW's systems (Hsueh et al., 2014; Ong et al., 2010; Saeed and Sun, 2012; Xian et al., 2010).

The results obtained in the nutrients analyses shows that between the influent water and the effluent water exists a great decrease in the ammonia concentrations, whereas for NO_3^- and NO_2^- their concentrations increased over the time of the experience. These results are a direct consequence of the chosen system design. The use of a SSVF CW's with recirculation leads to more efficient aeration of the systems, benefiting the aerobic processes. The presence of plants also has an important role for this, once they can release oxygen into the rhizosphere. In CW's the nitrification process, is one of the first steps in the nitrogen removal mechanism. This process transformed the ammonia in nitrate, with nitrite as intermediary, in the presence of oxygen. This is, in agreement with the results obtained, where a decrease in ammonium concentrations are a result of its transformation either in nitrate or nitrite. This last two nitrogen forms can be transformed in large scale through the denitrification process that occur mainly in low oxygen zones (Saeed and Sun, 2012; Vymazal, 2007). This high ammonium removal for a vertical system as already been described (Pelissari et al., 2014; Yalcuk and Ugurlu, 2009). The results obtained for phosphorous demonstrated the capability of CW's for its transformation. However, this capability seems to be lost over time, with a decrease in removal efficiency being noticed in the last week of the study. The amount of phosphorous present in *P. australis* of a CW's have showed a decrease over time (Bragato et al., 2006), this and the fact that plants in our study demonstrate a visible decline, can help to explain the decrease in removal rates. Another hypothesis that can help to explain this, is the fact that one of the most important mechanism of phosphorus retention in wetlands is the adsorption to soil. The fact that this process is saturable could implied a decrease over time (Vymazal, 2007; Zhang et al., 2012).

For COD, BOD and nutrients no alterations were notorious between the treatments showing that the presence of antibiotics do not have an impact in the system capability for transforming these compounds.

The fact that concentration of the antibiotics after treatment are below the detection limit for the method demonstrate the applicability of CW's for the treatment of livestock effluents. High rates of removal of diverse compounds in CW's are already documented in other works (Carvalho et al., 2013a; Hussain et al., 2012; Xian et al., 2010; Zhao et al., 2015).

Another factor analysed in this work was the presence of a system were the two selected antibiotics were add together. In the environment, organism are exposed simultaneously to a mixture of chemicals from different classes, which can interacted with each other. The result of this interaction it is not always equal to the results obtained for the compound in separate (Backhaus et al., 2000; González-Pleiter et al., 2013). The results obtained in the present study, in terms of water quality, show no differences between the systems exposed to the mixture of antibiotics (MIX) and the other systems. The alterations that the presence of the compounds simultaneously make in the microbial community structure did not influence the system depuration capacity.

The absence of differences between the control and different treatments, shows that the presence of the selected antibiotics, in the concentration tested, have no interference in the both bacterial diversity and richness. Comparing different treatments along the time it was observed a significant decrease ($p < 0.05$) for diversity but not for richness. The decrease in soil community diversity exposed to different chemicals have been already described (Kong et al., 2006; Kraigher et al., 2008; Zhao et al., 2015). Estimation of bacterial richness and diversity are very important once they help to predict the possible alteration in community composition and consequently systems functionalities. In this case, and despite changes in the diversity over time, systems seem to maintain their ability for water treatment, discuss above. The decrease in bacterial diversity, but not in bacterial richness, can indicated an alteration of relative abundance of species. The maintenance of water quality, despite this alteration, can indicate that systems functionality is played for specific species that did not suffer relevant alteration (Hooper et al., 2005), or species were replaced by others with the same functions revealing functional redundancy (Mucha et al., 2013).

In order to understand the variations that occurred in terms of community structure, a molecular fingerprinting techniques PCR-based (ARISA - automated ribosomal intergenic spacer analyses) were performed. In this method, the 16S-23S ribosomal

intergenic spacer region (ITS) is amplified. This spacer is characterized for being a highly variable section of the gene region, making this heterogeneity possible the distinction of bacterial phylotypes. This technique, and the specific pair of primers chosen, have already being compared with others techniques (even advanced ones) and other set of primers with good results obtained (Cardinale et al., 2004; Gobet et al., 2013; Purahong et al., 2015). This, and the fact that ARISA enables a rapid, reproducible and cost-effective evaluation, even for complex communities, demonstrate the suitability of select technique/primers for studies of changes in community over time (Cardinale et al., 2004; Gobet et al., 2013; Purahong et al., 2015). After the ARISA profiles obtained were analysed, it was possible to see differences in the microbial community. These differences were noticed for both time of exposure and type of treatment applied. These differences were confirmed by the ANOSIM analyses that revealed that time of exposure was the most significant factor affecting the microbial community structure. It seems to be an evolution of the community involving all the systems over time. This alteration caused by time of exposure can signify an adaptation of community to the systems and to the new conditions that they were exposed. The differences in similarity are more notorious between week 1 and the other two week (8 and 18). The initial community suffers an adapting process changing their composition in order to respond to the environmental conditions and the presence of the wastewater. Alterations verified in the controls systems shows that this adaptation process occurred independently of the presence of antibiotics. For each week differences between the different treatments were detected. There are several reports about the impacts of veterinary compounds on structure and function on microbial communities. In Fernandes et al., (2015) the response of a CW microbial community, to the presence of enrofloxacin and another veterinary antibiotic, tetracycline, was evaluated. The result obtained showed that the community suffers an adaptation process. This process occurs over the time, independent of the presence of antibiotics, being the time the most influent factor. The effect of triclosan was studied by Zhao et al., (2015) by pyrosequencing. The presence of this antimicrobial agent lead to difference between the control systems and the ones with their presence, with a great part of the OTU's being unique for the control. Microbial community shifts, in response to contaminated manure with sulfadiazine, was reported in soils. Over the time more delayed and prolonged effects in the microbial structures were noticed (Hammesfahr et al., 2008).

Samples from week 8 of the experiment were select to be analysed by 454-pyrosequencing analyses. This week was selected based on the results in terms of water purification and in terms of microbial structure evolution, revealed by ARISA. The comparison between present results and those from other authors is difficult, once the

community composition is a result of different factors. For this, differences in the type of flow, operation mode or the bed media, that change the soil properties, could lead to differences in the community. Beside these, design factors and the technique used for the analyses can be a factor of discrepancy in the results. The introduction of bacteria from wastewater that enter the system is also an factor important. Most of the phyla present have already been described in others CW's systems, however they are not all always present and even when they are, their relative percentage, fluctuate (Adrados et al., 2014; Ansola et al., 2014). In this work, at phylum level, there are 4 phyla more represented in the community. The most representative is *Proteobacteria*, this phylum is known to include a diversity of bacteria that contributes for the carbon and nitrogen cycling. This is, most of the time referred as the predominate phylum present in soil (Ansola et al., 2014; Sklarz et al., 2011; Truu et al., 2009). In this phylum the *Alphaproteobacteria* class represents one of the more dominant. In the *Rhizobiales* order, one of the most recovered in this class, a strain (F11) capable of degrade *Fluorobenzene* have already been described (Carvalho et al., 2006). Once the most of *Phyllobacteriaceae* sequences are only associated with the family, it is not possible to establish a further relation between their increase, in the doped systems, and the potentials responsibles. The same happens with the sequence in the *Rhodabacteraceae* family. In the Rhodobacter genus, the increase is only seen in the systems doped with ENR. Some species of this genus have been identified in enriched cultures capable of degrade several aromatic hydrocarbons (Oberoi et al., 2015). In the *Sphingomonadales,* two genus with different patterns appears. The first, *Erythrobacter,* appear in the control and MIX systems, but not in the CEF and only with a very low percentage in ENR (0.03%). Their presence in the control system, but not in the ones with the addiction of only one antibiotic, demonstrate their sensibility to this. However, the fact that they achieved the maximum of sequences percentage in the presence of the pharmaceutical simultaneous shows that the conjugation of this can lead to alterations that possibilities the resistance of this family. In the *Novosphingobium* genus, several strains have already been described as capable of degraded several compounds, as bisphenol A in the rhizosediment of *P. australis* (Lyu et al., 2014; Toyama et al., 2009). The fact that they have a much higher presence in the systems treated with only one of the antibiotics, shows that their elements are capable to adapt to these conditions. The fact that the number of sequence suffers a decrease in the MIX systems demonstrate that, in this case, the interaction of both antibiotics results in a negative effect on the bacteria.

In the *Betaproteobacteria*, the *Rhodocyclales* order suffers a decrease in the treated systems. This decrease also happens in their most represent genus, *Thuera*, in

the total sequences. This decrease shows the impact of antibiotics in this type of bacteria. A specie of this genus have already been describe in a CW's with a vertical flow used in the treatment of domestic wastewater (Adrados et al., 2014). In the case of the *Brukholderiales* an increase is seen both when it is compared to class level, as when it is compared with the total sequences present in the systems. Due the majority of the sequences being only classified in terms of order, it is not possible to attribute this increase to a particular type of bacteria. In a test with soil contaminated with CIP, the isolation and classification of species present shows that the majority of them was classified as belonging to *Brukholderiales.* Due to the fact that CIP is the primary degradation product of ENR it is possible that this degradation capability could be also extend to ENR (Dantas et al., 2008).

The *Gammaproteobacteria* phylum have a representative presence in all the systems, but specially in the one with the mixture of the selected compounds. The fact that no further classification was made for the sequences makes impossible to determinate which group or groups of bacteria are the main responsible of this increase. The *Pseudoxanthomonas* genus have already been described as possible degrader of ampicillin (Shen et al., 2010). However, this could be species dependent as there are others species from this group described as sensitive (Kumari et al., 2011). Besides this, no mention about ENR or any related compound could be found in the literature.

In the *Firmicutes* phylum there is a class (*Clostridia*) that clearly stands out from the rest. In this class only sequences belonging to one order (*Clostridiales*) were found. This class of bacteria is present in gut environment. Therefore, the presence of this order must be related with the effluent used in the experiment, despite it is described that allochthones microorganism that came with water, usually can not survive in the CW's systems (Truu et al., 2009). In fact, in present study, the sequences associated to *Clostridiales* order appear as the most dominant among the entire community (Table 4). Also in this order is possible to distinguish two different families that are part of the most representative taxa. The *Ruminococcaceae* family presents a decrease in their sequences number in the systems with antibiotics, showing their incapacity to adapted. The *Peptostreptococcaceae* is the other family belonging to this order that is represented among the most representative taxa (table 4).

Bacteroidetes is a well spread phylum with members present either in gastrointestinal tract (mainly *Bacteroidia* class) and in the environment (*Flavobacteriia* class) (Thomas et al., 2011). The *Bacteroidia* class shows an increase in the ENR systems, which is, mainly, a consequence of the presence of the genus *Alkaliflexus,* that

alone represents 3% of the total sequences for these systems. The association between *Alkaliflexus* and ENR were never made before.

Actinobateria phylum are present in terrestrial and aquatic (marine) ecosystem. In soil, they are important in decomposition and humus formation (Ventura et al., 2007). This phylum is known for having some types capable of produce secondary metabolites like antibiotics (Mahajan and Balachandran, 2012). In the only class found, the *Acidimicrobiales* order are the most represent. In both cases, an increase of percentage occured in the presence of antibiotics.

The enchain, that makes the sequences percentage of same groups being higher in the presence of antibiotics, can be the result of not only their possible resistance, but also of the fact that the presence of antibiotics lead to a inhibition on other types of bacteria that in normal condition (control) will be the predominant ones. Without this dominant bacteria, the ones that normally appears with a lower abundance, and that have conditions to survey in these adverse conditions, have the opportunity to survive and reproduce (Huang et al., 2014).

The analyses of community structure and the shifts that occur in these systems are of great importance once they are responsible for the final effluent quality. Therefore, the design of these systems should be directed to provide a higher diversity and consequently enhance the processes.

2.5. Conclusion

The results obtained in this work, with high removal rates for the veterinary antibiotics tested (Enrofloxacin, Ceftiofur and Mixture), organic matter and nutrients demonstrate the applicability of CWs for the removal of veterinary antibiotics from livestock wastewaters. The method applied make possible the assessment of community at different complexity levels.

The evaluation of community structure demonstrates that both time of exposure and the presence of antibiotics influenced significantly the microbial community of the CW's. However, this alteration did not affect the capability demonstrated for the removal of pollutants. Information supplied by pyrosequencing provided new knowledge about the bacteria potentially involved in the removal processes.

CHAPTER 3

Biodegradation of Enrofloxacin by bacterial consortia obtained from estuarine environment

3. BIODEGRADATION OF ENROFLOXACIN BY BACTERIAL CONSORTIA OBTAINED FROM ESTUARINE ENVIRONMENT

3.1. Introduction

The amount of pharmaceutical compounds utilized in human and veterinary medicine account for more than 5000. These compounds are divide in different classes, as: analgesics and anti-inflammatory drugs, antibiotics, antidepressants, hormones, between others (Van Doorslaer et al., 2014). Among the antibiotics compounds, the FQ, the class where ENR is insert, are the third largest group. The FQ are a result of the alteration of the quinolone structure, existing the insertion of fluorine element. This element is the most abundant of all the existent halogen elements, and the 13th of all the existing element in earth crust. The substitution of the hydrogen atom for the fluorine one leads to an alteration in several physic-chemical characteristics of the molecule (Murphy et al., 2009; Van Doorslaer et al., 2014). This fluoroaromatic structure of the FQ can not be found in natural products. This insertion of the fluorine in compounds for medical use has suffered an increase representation. In 1970 this type of compounds only represented 2% with an increase up to 18% in 2006 (Isanbor and O'Hagan, 2006). According with the World Health Organization FQ are ranked as *"critical important"* (Collignon et al., 2009). In this class of antibiotics can be distinguished four generation representing a total of twenty different chemicals. This division is made based in the structural differences of the compounds that are responsible for alterations in their activity spectrum (Jia et al., 2012; Pallo-zimmerman et al., 2010).

The FQ antibiotics can be use in either human or veterinary medicine. ENR is an antibiotic used in veterinary medicine, including in the second-generation according to the already described division (Pallo-zimmerman et al., 2010). The fact that this is an antibiotic for veterinary use leads to concern about their entrance in the environment. In the animal body, these compounds suffers an incomplete metabolization being excrete as the unmetabolized parental compound. Even the metabolized portion, excrete pose problems once they are usually constituted by break down products that show the same or similar toxicity as primary compound (Thiele-Bruhn, 2003). In the ENR case, this is particularly visible, once their primary metabolite, CIP, is an antibiotic use in human medicine. Therefore, either by the inefficient of their removal in WWTPs or by the utilization of manure as fertilizer, this parental or metabolites active molecules, can enter in the environment. The presence of antibiotics in the natural systems poses several risks

for the human and wild life health. The long term exposure to low (sub-lethal) concentrations can promote alterations in the microbial community. In addition to these alterations this repeated exposition of bacteria to this type of compounds can lead to the appearance, transfer and/or spread of antibiotic resistance. The appearance or propagation of bacteria with the capability to resist to the known treatments is of great concern. This resistance can lead to the decrease in the treatments usually applied (Jjemba, 2002; Kemper, 2008; Sarmah et al., 2006).

In this present study, it was investigated the capacity that bacteria from the rhizosphere of two different type of plants (*P. australis* and *J. maritimus*), present in natural wetlands, have for the biodegradation of ENR. The degradation of ENR have already been described for fungi (Karl et al., 2006; Martens et al., 1996; Murphy et al., 2009). For bacteria there is a lack of this information. In this work, the option of using bacteria originating from natural wetland systems, and more specific from the rhizosphere of the two above mention plants, are related with the fact that these systems and, in particular, these plants and their associated bacteria, have already demonstrate capability for contaminants removal (Carvalho et al., 2013a; Fernandes et al., 2015; Ribeiro et al., 2013).

The biodegradation of ENR was studied based on the liberation of the fluorine anion. The presence of this anion in the culture solution is an indicator of defluorination of the molecule and consequently their loss of activity.

3.2. Materials and methods

3.2.1. Soil collection

Rhizosphere samples from two different types of macrophyte plants (*P. australis* – PHR- and *J. maritimus* - JNC) were collected in the margins Lima estuary (North of Portugal) in July 2014. Rhizosphere samples were transported from the collection site until the laboratory, where the inoculation was made, in plastic containers and maintained in a cooler bag.

3.2.2. Experiment design

Two sets, in triplicate, were run in parallel, each one for different rhizosphere plants. Approximately 5 g of rhizosphere were weighted and used to inoculate 250 mL

flaks containing 50 ml of sterile minimal salts medium (MM) (Fig. 22). Initial setups were fed with 200 µL of ENR, at a final concentration of 1 mg/L. In order to prevent the

Figure 22: Inoculation and culture setup and maintenance.

occurrence of photodegradation all the flasks were maintained in dark environment and to ensure proper oxygenation were constantly agitated.

The MM used was made with (per liter of ultra-pure water): Na_2HPO_4. $2H_2O$ 2,03 g, KH_2PO_4 1.4 g, $(NH_4)_2SO_4$ 0.5 g and $MgSO_4.7H_2O$ 0.2 g, and 10 mL of a trace elements solution with the following composition, per liter: NaOH 2.0 g, $Na_2EDTA.2H_2O$ 12.0 g, $FeSO_4.7H_2O$ 2.0 g, $CaCl_2$ 1.0 g, Na_2SO_4 10.0 g, $ZnSO_4 \cdot 7H_2O$ 0.4 g, $MnSO_4.4H_2O$ 0.4 g, $CuSO_4.5H_2O$ 0.1 g; $Na_2MoO_4.2H_2O$ 0.1 g and H_2SO_4 1.5 mL. The medium was sterilized before use. Stock solution of ENR, at 200 mg/L, was made by adding 0,015 g of the compound in 15ml of methanol. This solution was then filtered to a sterilized flask and storage at -20ºC.

Sodium acetate stock solution, at a concentration of 100.000 mg/L, was made by diluting 7 g in 70 ml of distilled water, and sterilized.

3.2.3. Cultures maintenance and sampling

In the first part of the experiment 200 µl of sodium acetate were added, daily, as a secondary source of carbon, at 400 mg/L. Every three carbon alimentation the culture was transferred to a new sterilized flask to ensure the oxygenation. Twelve days after the beginning, half of the suspension (25 ml) was removed and passed to a new flask

containing 25 ml of fresh medium. To analysed the fluoride (F^-) content immediately after the addition of the new medium 4 ml was collected and centrifuges at 4000 rpm, 10ºC for 10 min and supernatants were frozen. The suspension remained, in each of the triplicates, was joined. This was then centrifuged, as described before, and from the supernatant obtain 3 ml were collect for the F^- liberation analyse and the remained was kept for HPLC analyses of ENR. To avoid interference caused by the presence of soil particles, used in the inoculum, cultures growth monitorization, by spectrophotometry (600 nm), only started after the turbidity disappeared. This procedure was maintained for two and half months.

After that, the dilution of the culture passed from twelve to twenty one days, giving more time to possible degradation occurrence (Fig. 23), the volume passed for half to one quarter (12,5ml) once the growth registered was high and carbon addition started to be done three days per week. Transference of culture into a new flask, sampling (for

Figure 23: Culture maintenance.

liberation of F^- and HPLC analysis) and monitorization of growth were made as described before.

Four months after the beginning of the experiment, and based on the results obtained until that time, bacterial community present was studied (Fig. 24). For that, and in the attempt to recover every strain that could be involved in the degradation processes, triplicates were joined. A flask per plant of the initial consortia was also maintained, in the same condition as described before, to continue the F^- analysis. From this joined culture, samples were taken to prepare several dilution in eppendorfs. These diluted cultures were, then, spread onto plate count agar and MM (with 1 mg/L of ENR) plates and incubated in, at 30 ºC. After the recovery of bacterial strains, they were purified by

repetitive streaking into the corresponding medium. The differentiation of bacteria was made based on their morphology. Isolated strains were preserved in glycerol (85%) and kept at -80 ºC for future identification through DNA extraction and sequencing.

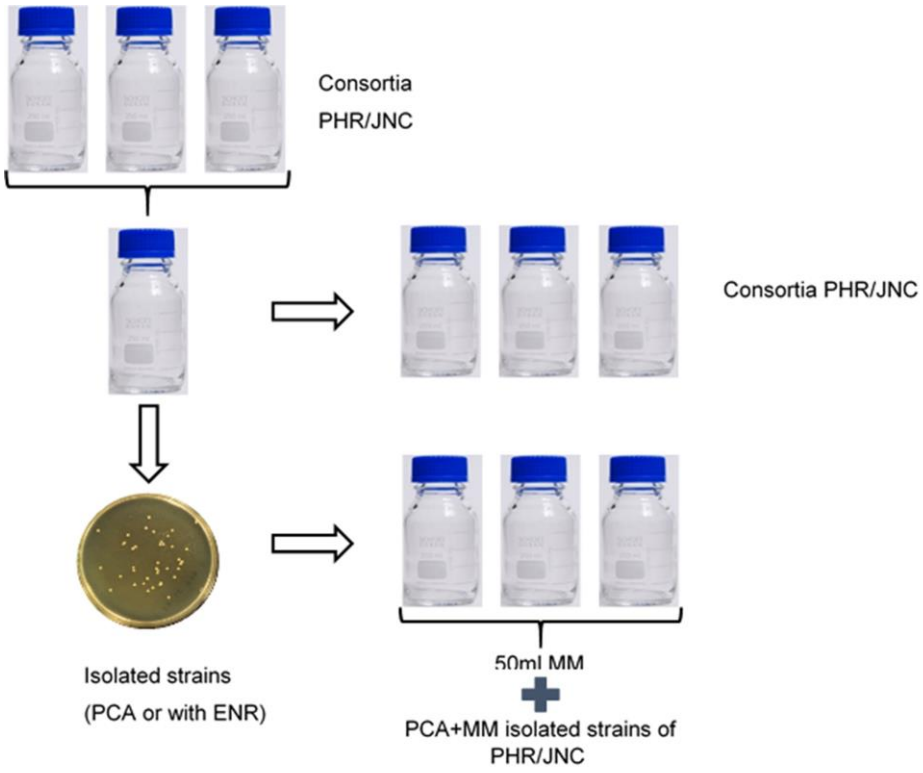

Figure 24: PHR/JNC culture maintained, isolation of strains and inoculation of new consortia from the isolated strains.

Degradation of ENR by these pure strains obtained from the initial consortia was evaluated. For these analyses, isolates from each plant were reinoculated together, in triplicate, into MM. The maintenance of these cultures was performed in the same way as the initial ones.

Due the low concentration of ENR initially used in this work, the maximum concentration of F^- released was close to the detection limite of the electrode. Oscillations in the results could be a consequence of that, so in the attempted to increase the accuracy of the F^- measurement an increase from 1 to 3 mg/L of ENR, in both initial culture and the one created from the isolated strains, were made. This concentration was maintained for 2 cycles. At this point, only a flask from the initial culture existed. To obtain a more realistic data, this flask passed from one to three. This was not made at once due the lack of culture volume to create them. For that, at the end of the first 3 mg/L cycle a duplicate was created from the one existent. At the end of the second cycle, a third flask was created with a mixture (half of each) of the other two flasks.

After two cycles at 3 mg/L, and with the results obtained a change in the ENR added concentration was made passing to 2 mg/L, a concentration that allows to work above the detection limit but without posing an excessive pressure on cultures.

3.2.4. Fluoride liberation

The measurement of the concentration of F^- anions present in cultures supernatants gives the information about defluorination of ENR. This measurement was made in a fluoride ion-selective electrode. For that, and before every samples measurement, a calibration curve was prepared. The concentration of the sodium fluoride (NaF) standards utilized in the curve were 0,001; 0,0025; 0,005; 0,01; 0,02; 0,1; 0,2 and 1 mM. These concentrations were chosen based on the predictable maximum F^- liberation. Standards were made from a 100 mM NaF stock solution dilution. The stock solution was prepared by adding 0,20995 g of NaF to 50 ml of ultra-pure water. The standards dilutions were made in the culture medium present in the samples. To minimize the interference, 100 µl of a total ionic strength adjustment buffer (TISAB) solution was added per ml of sample.

3.2.5. Isolation of strains

The isolation of strains from the different cultures was made in two different types of medium plates. Plate count agar (PCA) medium, a non-selective one, was prepared following the manufacturing instructions, in this medium all the culturable bacteria present is expected to grow. The other medium was prepared with the MM, equal to the one utilized in the cultures, with the addiction of agar and ENR (1 mg/L concentration). For each liter of medium, 12 g of agar was added to solidify this liquid medium. To prevent degradation, ENR was only added after the sterilization of the medium. The addition of ENR allows to select only the cultures capable of use ENR as carbon source.

3.3. Results

3.3.1. Initial consortia

The percentage of F^- release, defluorination, was calculated by subtracting the F^- present in the end of each cycle from the one present in the beginning. In the first 60 days of the experiment the measure of defluorination was not possible to be made due the interference of the remaining sediment. From day 60 until the day 114, F^- release was measured (Fig. 25). In this, was possible to see some F^- liberation for both consortia, with similar pattern. However, the percentage of defluorination was not stable along the time, showing some variations.

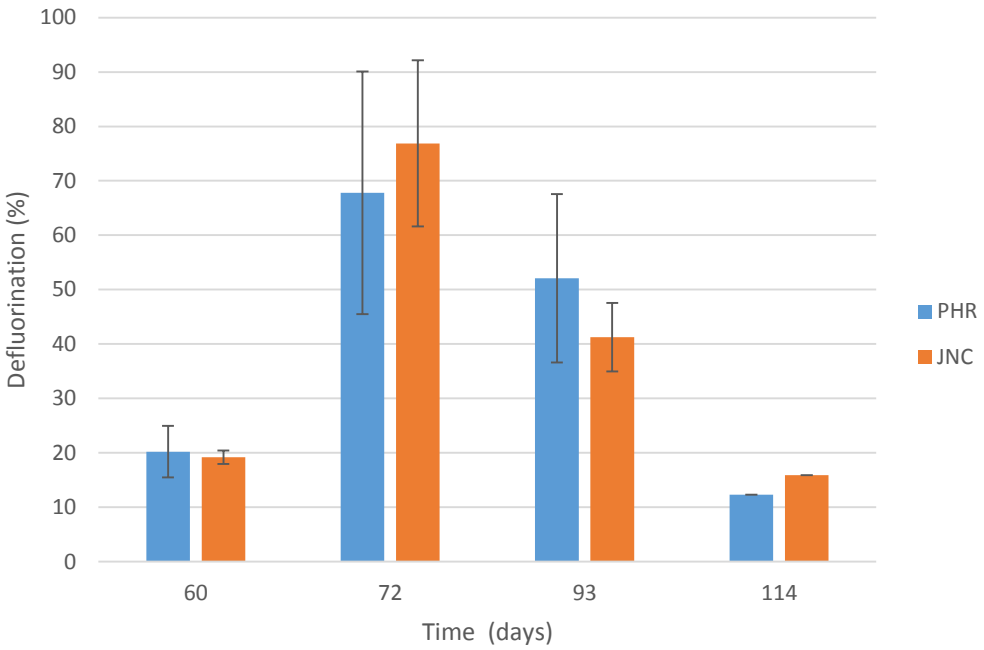

Figure 25: Defluorination percentage (average and standard deviation) for the two consortia from day 60 to 144 (all with 1 mg/L)

After the procedure for strain isolation, the initials consortia cultures were maintained in only one flask between days 135 and 261 (Fig. 26). The oscillations seen before were also seen here. This oscillation can be a result of the, already described, low concentration used. Besides this, the fact that at this point only one flask existed does not allows to see if this oscillation is a result of the conditions or of some alterations in this particular one. After this, the flaks passed to two and the concentration was increased for 3 mg/L. In the two cycles of 3 mg/L a decrease in defluorination percentage was detected. In the end of these two cycles, and once the volume of the culture permitted, the flasks passed to three in order to do triplicates and, therefore, to better understand the possible

variations. The ENR concentration was also adjusted to 2 mg/L, once the pattern seen in the cultures with 3 mg/L showed that the capability of the culture to biodegrade decreased and, therefore, this concentration appears to exceed the degradation capability of cultures. Nevertheless, the same was observed with the 2 mg/L concentration. Thus, percentage of defluorination ranged between 5 and 95% in the presence of 1 mg/L of ENR, while in the presence of 2 or 3 mg/L it ranged between 10 and 20% or 10 and 30%, respectively.

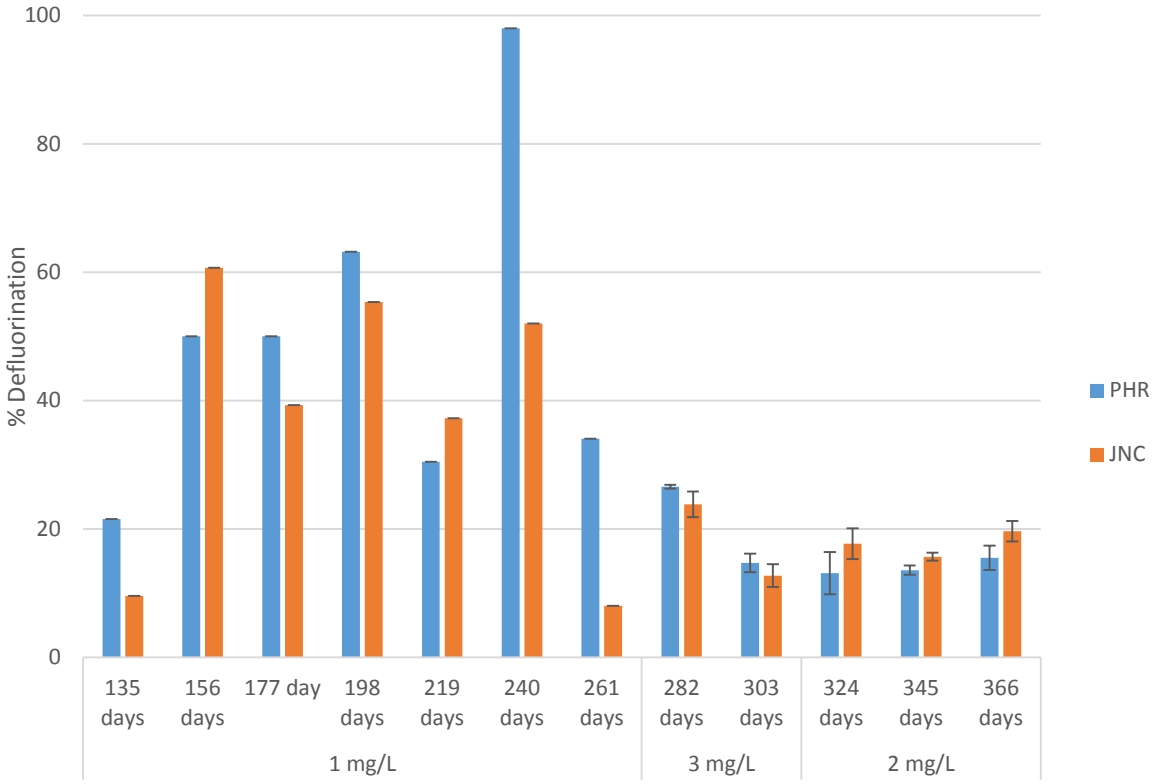

Figure 26: Defluorination percentage for the two consortia. From day 135 to day 261 data from one flask is available. In day 282 two flasks per consortia exist, with data from de medium degradation and standard deviation being represented. From day 303 until the end, the data represent the degradation and standard deviation of triplicates. Alteration in the ENR concentration added is also represent in horizontal axis (1mg/L, 2 mg/L and 3 mg/L).

3.3.2. Consortia produced with the isolated strains

After these 114 days of enrichment and looking at the deflurination results, the microbial community present was studied. The culture streaking in the different media result in the obtantion of 14 strains distributed among 11 different morphologies. From these 14 strains, 11 were recovered from the PCA (4 from PHR and 7 from JNC) medium and the 3 remaining were recovered from the plates with ENR addition (1 from PHR and 2 from JNC). Due to similar morphology, the same number was attribute to bacteria

provenient from different consortium, with the addition of the letter P (for the ones isolated from PHR consortium) or J (for the ones isolated from JNC consortium).

Table 5: Identification of the different isolated strains in the consortia. (x) Indicates the presence; (-) indicates the absence. Without shade- recovery from PCA; with shade recovery from plates with ENR.

	Identification number										
	1	2	3	4	5	6	7	8	9	10	11
PHR	x	x	x	x	x	-	-	-	-	-	-
JNC	-	x	x	x	-	x	x	x	x	x	x

The result of defluorination obtained from the consortium made with the isolated strains (Fig. 28) showed, as occur in the initial ones, variation in the percentage of degradation along time, with no clear differences observed between the two consortia. This variation was seen in all the concentration tested. In general, higher percentages of defluorination were observed in these consortia, in comparison with the ones observed in the initial consortia. Also, it was observed a tendency for a decrease of defluorination with the increase of ENR concentrations, as values range from 40 to 80% for 1 mg/L, from 30 to 60% for 2 mg/L and from 20 to 50% for 3 mg/L.

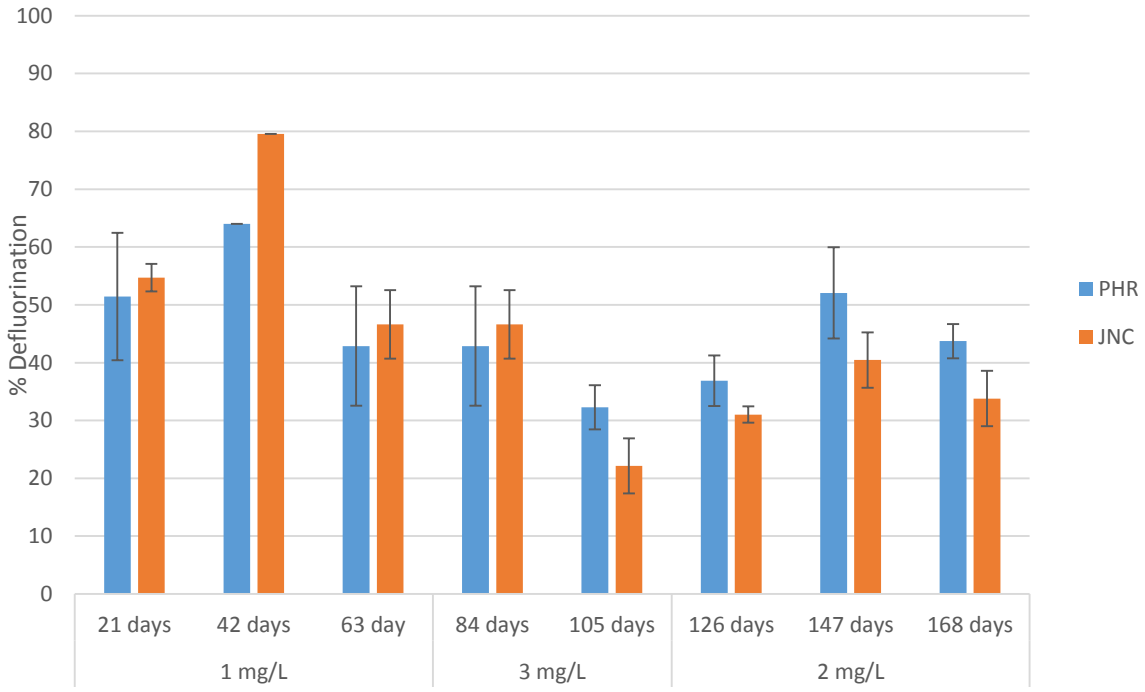

Figure 27: Defluorination percentage (averaged and standard deviation) for the two consortia inoculate with the isolated.

3.4. Discussion

Xenobiotic compounds, resulting from different anthropogenic activities, can enter in the environment from the most diverse ways having potential ecological impact. The ability of different microbial species for the degradation of these compounds can help in the reduction of contamination, decreasing, therefore, their possible impacts.

The degradation on ENR performed by different types of fungi as already been described. In this, several different metabolites, resulting of different alteration processes of ENR have also been described. These differences in alterations are directly related with the type of fungi utilized, with different patterns associated with the type of fungi and their specific characteristics (Karl et al., 2006; Martens et al., 1996; Parshikov et al., 2000; Wetzstein et al., 1997, 2006). Different strategies can be adopt to analyse the degradations of this antibiotic. In the case of the work performed by Martens et al., (1996) the degradation of ENR ^{14}C labelled, by 4 species of white rot fungi and three strains of *Gloeophyllum striatum*, was estimated based on the amount of $^{14}CO_2$ released. In 8 weeks of experiment, degradation reached in some cases over 50%, with brown rot fungi having a better performance than the white ones. Wetzstein et al., 1997, based in the good degradation results showed by *Gloeophyllum striatum,* used this specie to precede to the identification of the metabolites resulting of ENR degradation and to establish degradation routes. Several degradation routes were proposed, one of them involving the loss of F^- (Karl et al., 2006). Considering that, the studied species that have their natural occurrence in agriculture soil could represent a more realistic description of natural degradation. Wetzstein et al., (2006) studies the metabolites resulting of ENR degradation by seven basidiomycetes, indigenous to agricultural soils or animal waste. These metabolites showed to be different from the ones that appeared in the presence of *Gloeophyllum striatum*. The capability of *Mucor ramannianus* to transform ENR as also been study (Parshikov et al., 2000). All of these studies demonstrate that the biotransformation of the same compound performed by different organisms can be a result of different pathways. None of these works utilized a fluoride ion-selective electrode as method. However, this technique have already been used to study the degradation of other fluoroquinolones (Carvalho et al., 2006).

The ability of different type of bacteria for the degradation of a diversity of fluorinated compounds has already been described (Carvalho et al., 2006). However, to our knowledge, there are no data available about ENR degradation by bacteria. For what concerns to this present work, only data of the degradation pathway resulting from the molecular defulorination is available. This is, as showed before, only one of the many

possible biotransformation pathways of ENR. For that, it is not possible to assess if other alteration, that did not result in the liberation of F^-, is occurring. Understanding the alterations of ENR is extremely important. Only in that way it is possible to understand if the resulting compounds also preserved the antimicrobial characteristics of ENR. It can occur that, despite the alterations suffered by ENR the antimicrobial proprieties of the present compounds can continue affecting the microbial communities or that the transformation occurring implies the loss of activity.

3.5. Conclusions

Results obtained for both, initial and isolated consortia from the two selected soil, showed the liberation of F^-, in the different concentration tested. These results are indicators of the capability of the bacteria present in these consortia for the alteration of ENR molecule. This study will proceed with the identification of the different pure strains recovered from the different consortia, in order to understand what type of bacteria can be involved in this process. In addition, HPLC analyses for the quantification of ENR and its metabolites, will allow the assessment of possible biotransformation routes.

CHAPTER 4

General discussion and Conclusions

4. GENERAL DISCUSSION AND CONCLUSIONS

4.1. Discussion

The environmental fate of pharmaceuticals has received increasing attention, especially since the detection of their presence in wastewater treatment plant (WWTP) effluents, surface and groundwater. These compounds can be released into rivers and estuaries directly, by discharge or inadequate treatment of water, or indirectly, through groundwater contamination when contaminated manure is used as agriculture fertiliser. Within the veterinary pharmaceuticals, antibiotics are the group of most concern due to the risk of spread of antibiotic resistance in the environment. In addition to their use to treat diseases, antibiotics are extensively used as enhancer of feed efficiency to, between other things, promote growth. Microorganisms naturally occurring, mainly the ones present in areas that are usually exposed to the presence of several types of pollution, can be adapted to the presence of contaminants. Therefore, some of them can be used in bioremediation, a process that involves the use of microorganisms that catabolize specific molecules, destroy dangerous contaminants or transform them into less harmful forms.

The main objective of this study was to evaluate the potential of autochthonous microorganisms from estuarine environments for bioremediation of pharmaceuticals, namely veterinary antibiotics. This potential was studied in two experiments, one of them using these microorganisms in association with plants, in constructed wetland (CW) microcosms, and the other using them to produce microbial consortia with capacity to degrade antibiotics.

In the first experiment, wastewater from a livestock industry was introduced in CWs microcosms containing plants (*P. australis*) and respective rhizosediment collected at the Lima estuary. The wastewater, doped with two different antibiotics, was added to different microcosms and the response of microorganisms was evaluated, both in terms of community structure and removal performance. Results show that the systems were able to remove more than 90% of the added antibiotics, pointing to the applicability of CWs for the removal of veterinary antibiotics from livestock wastewaters. The potentiality of these systems for the elimination of pharmaceuticals, have already been report for other compounds in different designed CWs (Carvalho et al., 2013a; Hussain et al., 2012; Xian et al., 2010; Zhao et al., 2015).

Results from community structure showed that the microorganism were in an adaptation process, displaying important changes along time. Despite the evolution of

communities over the time, the capability of the systems to treat the wastewater introduced was maintained along the experiment. Furthermore, the impacts of the presence of the antibiotics in the microbial communities, which can results in alteration in ecosystem functionality, were evaluated. Results show that, for all systems, microbial communities were dominated by the phyla *Proteobacteria* (38 to 48%), *Firmicutes* (20 to 27%), *Bacteroidetes* (12 to 15%) and *Actinobacteria* (4 and 9%) but their relative abundance was clearly affected by the presence of the antibiotics. Alteration in communities subject to the presence of different compounds were already seen with a diversity of techniques (Fernandes et al., 2015; Hammesfahr et al., 2008; Zhao et al., 2015). Neverthless, in the present study, data from 454-pyrosequencing analyse provide new knowledge about the bacteria potentially involved in the removal processes.

The potential of the microorganisms, present in plants rhizosediment collected from an estuarine wetland, to biodegrade ENR was studied based on the liberation of the fluorine anion, in the second experiment. To evaluate this, the concentration of the fluorine anion in the culture solution was measured by a fluoride ion-selective electrode. Besides that, the strains present were isolated. For that, part of the initial culture was spread onto plates with different mediums. The result of liberation obtained indicate that these consortia are capable of change ENR molecule, resulting in the liberation of fluorine. However, as described in the case of ENR biodegradation by a diversity of fungi species, a variety of other possible pathways exist (Karl et al., 2006; Martens et al., 1996; Parshikov et al., 2000; Wetzstein et al., 1997, 2006). This fact points to the importance of the identification and quantification of ENR and its metabolites in the cultures, as the non-liberation of fluorine is not a direct indicator of the non-alteration of ENR molecule. The identification of the isolated strains will allow the identification of the bacteria possibly involved in the biodegradation process.

4.2. Conclusion

Both experiments pointed to the capability of autochthones microorganisms present in estuarine plants rhizosediment for the bioremediation of veterinary antibiotics, either in CWs or in enriched consortia. The method applied in the CW experiment makes possible the assessment of changes in microbial community structure at different complexity levels. Although some alteration in community structure could be identified, these systems maintained their depuration capacity along all of the experiment. Besides that, some of the microorganism present were able to interact with a fluorinated antibiotic, leading to the liberation of fluorine from the molecule.

Thus, this work points for the applicability of the use of autochthonous microorganism collected from estuarine environment for bioremediation of pharmaceuticals, namely veterinary antibiotics, providing new knowledge about the bacteria potentially involved in the removal processes.

CHAPTER 5

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