Fate and effects of two veterinarian cephalosporins, ceftiofur and cefapirin, in the aquatic environment

Dissertation

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I want dedicate this work to all those who been in my position. Those who have felt lost and hung up on the language and cultural norm of a foreign land. To those who have felt a sense of loneliness at times while still those at home, so many miles away, thought that you were succeeding and achieving. Now today, after countless nights toiling away by my lonesome, I am happy to say I have completed my hardest life's work.

Summary

Human and veterinary cephalosporins can reach the environment due to their widespread consumption, intermittent and diffuse discharge rate. Particular attention is given to this antibiotic group due to their broad spectrum of activity and possible deleterious effects in non-target organisms as well as pressure in antimicrobial resistance acquirement, which may be caused by the parental drugs, metabolites and bioactive byproducts.

Physicochemical properties, consumption, occurrence in aqueous matrices, available ecotoxicity data and technical strategies employed for the degradation of compounds within this group were critically discussed. Classical biological treatment systems cannot provide complete removal of cephalosporins, therefore several technologies have been employed for the removal of these compounds from aquatic matrices. In this regard, photolysis and mass transfer processes were the most investigated ones. Cefalexin, cefradine, cefotaxime and cefazolin possess respectively the highest occurrence score in the aquatic environment. Topics were identified where further investigations are necessary, as: ecotoxicological assessment of parental and transformed compounds, especially using soil organisms, cyanobacteria and biofilms; abiotic degradation rates (hydrolysis, photolysis) and control of natural degradation during abatement technologies; analysis of biologic inactivation and inclusion of metabolites and transformation products in surface water surveillance.

Several biotic and abiotic processes in the environment depend primordially on the xenobiotic pH-dependent speciation. The veterinarian drugs ceftiofur (CEF) and cefapirin (CEPA) are widely used for the treatment of recurrent and economically relevant infections. The acid dissociation constants (pK_a) of CEF and CEPA were studied using two experimental techniques (potentiometry and spectrophotometry) along with computational simulations. *In silico* studies were also used to contrast and discuss the experimental dissociation constants available in the literature of 14 cephalosporins. pK_a values were obtained experimentally for CEF (2.68 ± 0.05, carboxylic acid group deprotonation) and for CEPA (2.74 ± 0.01 for the carboxylic acid deprotonation and 5.13 ± 0.01 for the pyridinium ring deprotonation). The pK_a values available for cephalosporins (n=88) agreed with the *in silico* predicted data (ACD/Percepta RMSE: 0.552 and Marvin RMSE: 0.706).

Therefore, in the biologically and environmentally relevant pH values of 6 - 7.5, CEF and CEPA, as well as many other cephalosporins, are present as anionic species.

CEF and CEPA have been detected in aquatic environments and their fate in surface water as well as during drinking water processing is still unknown. To understand the persistence of cephalosporins in aqueous matrices, hydrolytic and photolytic kinetics were investigated. Both CEF and CEPA showed high instability under alkaline conditions, degrading in few minutes at pH>11 (T = 22 ± 1°C). Cephalosporins speciation did not significantly influence the direct photolysis rates of CEF and CEPA under simulated water disinfection radiation (UV-C, λ = 254 nm). All ionic species of CEF (k^{app} 0.0095 ± 0.0004 mJ cm^{-2}) and CEPA (k^{app} 0.0092 ± 0.001 mJ cm^{-2}) presented fast and similar pseudo-first order degradation rates. In hydrolysis experiments using surface water CEF showed a significant matrix-dependent stability increase with a half-life ($t_{½}$ 14.7 d) tenfold higher than in buffered solutions ($t_{½}$ 1.4 d). Meanwhile, CEPA showed similar hydrolysis in buffered solutions ($t_{½}$ 3 d) and in river water ($t_{½}$ 4.2 d). On the other hand, this antibiotic showed subtle faster photo-degradation rate in this same matrix (k^{app} 0.0128 ± 0.001 mJ cm^{-2}), while CEF photo-degradation showed no matrix effects.

The acute and chronic toxicities of CEF and CEPA towards aquatic organisms were also investigated. CEF and CEPA have significant decay during cladocera (*Daphnia magna)* tests, portraying half-life times ($t_{½}$) of 49 and 53 hours, respectively. During tests with green algae (*Scenedesmus spec*.), CEPA was more instable ($t_{½}$ 88 h) than CEF ($t_{½}$ 267 h). CEF and its hydrolysis products induced deleterious effects in *Daphnia magna* (48h EC_{50} 139, LC_{50} 179 in µM), which was not observed with *Scenedesmus spec.* (72h NOAEC 82.5 ± 2.5 µM). In the case of CEPA, no toxic effects were observed in both tests (48h EC-LC_{50} > 510 and 72h NOAEC 57 ± 6, in µM). The effects of water disinfection radiation (UV-C, λ = 254 nm) on ecotoxicological responses were also studied. Photolysis of CEPA resulted in toxic products, which were effective for cladocera but not for the green algae. On the other hand, the different radiation doses studied did not affect CEF ecotoxicity. This work should provide important data for researchers interested in cephalosporins antibiotics as environmental contaminants, confirming the importance of investigating the fate and effects of cephalosporin antibiotics in the aquatic environment.

Zusammenfassung

Cephalosporine sind wichtige Antibiotika in Human- und Veterinärmedizin. Die Untersuchung des Umweltverhaltens von Cephalosporin-Antibiotika ist von besonderer Bedeutung aufgrund ihres breiten Wirkungsspektrums, was zu möglichen Schäden in „Non-target“ Organismen führen könnte, sowie der Ausbildung von Antiobiotika-Resistenzen durch die Wirkstoffe, deren Metabolite und bioaktiven Nebenprodukte.

Im Rahmen dieser Arbeit wurden in einem kritischen Überblick physikochemische Eigenschaften, Anwendungen, das Auftreten in wässrigen Matrizes, vorhandene Daten über Ökotoxizität und die Eliminierung von Cephalosporinen diskutiert. Cefalexin, Cefradin, Cefotaxim und Cefazolin wurden am häufigsten in der aquatischen Umwelt nachgewiesen. Klassische Verfahren der biologischen Abwasseraufbereitung können keinen vollständigen Abbau der Cephalosporine gewährleisten. Daher wurden vielfach weitere Verfahren untersucht, um diese Substanzen in wässrigen Matrizes zu eliminieren, insbesondere Photolyse und Sorption. Viele Fragestellungen bedürfen weiterer Untersuchungen. Dies umfasst: Umwelttoxikologische Analysen der Substanzen und Transformationsprodukte, einschließlich der Verwendung von Boden-Mikroorganismen, Cyanobakterien und Biofilmen; abiotische Abbauraten (Hydrolyse, Photolyse) biologische Inaktivierung und die Einbeziehung von Metaboliten und Transformationsprodukten in die Überwachung der Oberflächengewässer.

Die pH-abhängige Speziation von Xenobiotika wird durch ihre Säurekonstanten (pK_s) beschrieben und bestimmt in vielen Fällen ihr Verhalten in der Umwelt. Wie auch andere Pharmazeutika weisen Cephalosporine mehrere ionisierbare Zentren auf. Für die beiden veterinären Wirkstoffe Cefapirin (CEPA) und Ceftiofur (CEF) wurden pK_s-Werte mittels zweier experimenteller Methoden ermittelt (Potentiometrie und Spektrophotometrie) und betrugen für CEF 2,68 ± 0,05 (Deprotonierung der Carboxylgruppe), für CEPA 2,74 ± 0,01 (Deprotonierung der Carboxylgruppe) und 5,13 ± 0,01 (Deprotonierung des Pyridinium Ringes). Für diese sowie 14 weitere Cephalosporine wurden experimentell bestimmte Daten (n=88) mit Computersimulationen verglichen. Die experimentellen pK_s-Werte stimmen mit den vorhergesagten *in silico*-Werten (ACD/Percepta RMSE: 0,552 und Marvin RMSE: 0,706) gut überein. CEPA und CEF, sowie viele andere Cephalosporine,

liegen im umweltrelevanten pH Bereich von 6 – 7,5 als anionische Spezies vor, was in der bisherigen Diskussion ihres Umweltverhaltens oft nicht berücksichtigt wurde.

CEPA und CEF wurden in der aquatischen Umwelt nachgewiesen und ihr Verbleib in Oberflächengewässer, sowie in der Trinkwasseraufbereitung ist noch nicht geklärt. Um die Beständigkeit von Cephalosporinen in wässrigen Matrizes zu verstehen, wurde die Kinetik des hydrolytischen und photolytischen Abbaus untersucht. CEF und CEPA wiesen eine hohe Instabilität unter alkalischen Bedingungen auf, mit einem innerhalb von Minuten stattfindenden Abbau, bei einem pH>11 (T = 22 ± 1°C). Die Speziation der Cephalosporine beeinflusste nicht signifikant die Photolyse-Raten von CEPA und CEF bei einer simulierten UV-Desinfektion (UV-C, λ = 254 nm). Sämtliche ionischen Spezies von CEF (k^{app} 0,0095 ± 0,0004 mJ cm^{-2}) und CEPA (k^{app} 0,0092 ± 0,001 mJ cm^{-2}) wiesen hohe und ähnliche Abbauraten pseudo-erster Ordnung auf. Bei Hydrolyse-Experimenten in Oberflächenwasser wies CEF eine signifikante, matrixabhängige Stabilitätserhöhung auf, mit einer Halbwertszeit ($t_{½}$ 14,7 Tage), die zehnfach höher war als in einer gepufferten Reisntwasser-Lösung ($t_{½}$ 1,4 Tage). CEPA wies eine ähnliche Hydrolyse-Rate in gepufferter Lösung ($t_{½}$ 3 Tage) und in Flusswasser ($t_{½}$ 4,2 Tage) auf. Dieses Antibiotikum wies auch einen etwas schnelleren photolytischen Abbau in der gleichen Matrix (k^{app} 0,0128 ± 0,001 mJ cm^{-2}) auf, während der photolytische Abbau von CEF nicht von der Matrix abhing.

Abschließend wurde die akute und chronische Toxizität von CEF und CEPA gegenüber aquatischen Organismen unter Berücksichtigung des zuvor bestimmten abiotischen Abbaus untersucht. CEF und seine Hydrolyse-Produkte waren toxisch für *Daphnia magna* (48 Std. EC_{50} 139, LC_{50} 179 in µM), aber nicht für *Scenedesmus spec.* (72 Std. NOAEC 82.5 ± 2.5 µM). Im Falle von CEPA konnten keine toxischen Effekte während beider Tests (48 Std. EC-LC_{50} > 510 and 72 Std. NOAEC 57 ± 6, in µM) beobachtet werden. Die Photolyse von CEPA unter Bedingungen der UV-Desinfektion (UV-C, λ = 254 nm) resultierte in toxischen Abbauprodukten, welche Auswirkungen auf Cladocera, jedoch nicht auf die Grünalgen hatten. Im Unterschied dazu wurde unabhängig von den Bestrahlungszeiten für CEF keine zusätzliche negative Wirkungen auf die beiden Testorganismen gefunden.

Zusammengefasst liefert diese Arbeit wichtige Daten zum pH-abhängigen Verhalten und Wirkung von Cephalosporin-Antibiotika in aquatischen Systemen.

Content

1. General Introduction

Anthropogenic pollution sources such as urban wastewater and agricultural effluents have been indicated as the main source of human and veterinarian pharmaceuticals in water (Kümmerer, 2009; Khetan and Collins, 2007; Deblonde et al., 2011; Biel-Maeso et al., 2018). Antibiotics are important micropollutants in aquatic matrices due to their large amount of consumption and intermittent discharge rate (Halling-Sørensen et al., 1998; Jansen et al., 2006, Boxall, 2004). The full aspects involving ecological and human exposure effects of antibiotics in the environment are unclear (Slana and Dolenc, 2013). However, some reports evidence the occurrence of multiple antibiotic resistance genes in bacteria from different water matrices (Khetan and Collins, 2007; Chee-Sanford et al., 2011). Furthermore, environmental exposure may contribute with resistance genes transfer to human- and animal-related microbiota (Rizzo, 2011). Toxicity, cytotoxicity and genotoxicity were also identified as adverse effects in non-target organisms exposed to antimicrobials (Opriş et al., 2013). For these reasons a substantial body of research has recently focused on characterizing these compounds' behaviour in the environment (Sacher et al., 2001; Boxall et al., 2011; Opriş et al., 2013), and effective soil and water removal techniques (Rizzo, 2011; De la Cruz et al., 2012, Michael et al., 2013; Serna-Gavis et al., 2017).

Veterinary medicines can reach the environment through improper disposal of pharmaceutical factory effluents or from livestock manures as excreted products. The uncontrolled use of manure or animal sludge as organic fertilizer, common practice in many farms worldwide, may also significantly increase the environmental availability of veterinary antibiotics (Slana and Dolenc, 2013). A large number of medicines are employed as antibiotics to ensure and promote animal health. Most of these compounds are not metabolised or have a low rate of metabolization. From urine and feces the drugs reach directly the soil and, according to chemical properties as ionization state and mobility, they can be sorbed to soil, drained to surface water by run-off or leach to groundwater (Kümmerer, 2009; Blackwell et al., 2009; Boxall et al., 2011; Kim et al., 2011).

Biotic and abiotic strategies can be employed to degrade medicines as antibiotics present in water matrices. Usual biologically based processes do not effectively remove a large

number of drugs (Deblonde et al., 2011; Kim et al., 2011; De La Cruz et al., 2012), mainly because of the recalcitrance of antibiotic containing effluents (Homem and Santos, 2011). In this context, processes such as photolysis and advanced oxidation processes (AOPs) are presented as promising alternatives for water and wastewater pre- or post-treatment, especially for antibiotics degradation (Homem and Santos, 2011; Michael et al., 2013; Serna-Gavis et al., 2017). In the case of photolysis, light radiation is employed to transform xenobiotics via direct photo-transformation or through photosensitizers that promote indirect transformation (Fabbri et al., 2015). The main characteristic of AOPs is the generation of reactive species, in particular OH radicals, to significantly degrade undesirable substances in water. In many cases, photolysis is complemented with AOPs to optimize degradation efficiency (Andreozzi et al., 2004; Kim and Tanaka, 2009; Serna-Gavis et al., 2017).

It is important to point out that contaminant degradation in water and wastewater treatment processes is typically incomplete. The formed transformation products sometimes can be more toxic and persistent than the parental compounds (Wang and Lin, 2012; Li et al., 2013). These transformation products are not easily determined using the usual approaches in water analysis and some of them present high toxicity, as well as carcinogenic and mutagenic properties (Bekbolet et al., 2005; Guay et al., 2005). Therefore, bioassays are strongly recommended to assess the treatment process efficiency as well as the toxicity of possible transformation products formed during and after treatment (Rizzo, 2011; Wang and Lin, 2012; Serna-Gavis et al., 2017).

Nowadays, a large range of veterinary pharmaceuticals are available. In order to evaluate their environmental relevance, data of consumption, presence in the environment and known ecotoxicological effects are necessary (Kemper, 2008; Kümmerer, 2009; Boxall, 2004; Biel-Maeso et al., 2018). The World Health Organization presents a list of important antimicrobials for human medicine, aiming at antimicrobial resistance risk management. In 2011 some antibiotics for veterinary use were included in this list (WHO, 2012). Cephalosporin compounds were highlighted in this report due to their use as last resort to combat gram negative bacteria. For risk assessment and risk management approaches the antibiotics presented in this list were critically categorized. Two veterinarian

cephalosporins should be highlighted: ***Cefapirin*** was classified as highly important and ***Ceftiofur*** was classified as critically important antimicrobial.

Cefapirin is mainly used to treat cow mastitis, an inflammatory disease manifested in dairy cattle mammary gland. It is one of the most important diseases in dairy cow due to its high incidence and impact on farm activities (Rehbein et al., 2013). Mastitis cases are common and they lead to significant financial losses due to the loss in milk quality and production decrease; high use of antibiotics and specialized treatment needs; side effects in animal behaviour and other technical aspects (Sadeghi-Sefidmazgi et al., 2011). Cefapirin can also be used to treat endometritis, another economically significant and common dairy cow disorder that can decrease animal fertility (LeBlanc et al., 2002; Kaufmann et al., 2009). Because of high consumption, traces of cefapirin, its metabolite desacetylcefapirin and other antibiotics are frequently found in bovine milk. To avoid possible adverse effects on final consumers the contaminated milk produced during cattle treatment is discarded. Furthermore, maximum residue limits (MRLs, in $\mu g\ kg^{-1}$) for cephalosporins in milk have been established in several countries (Junza et al., 2011; Hou et al., 2013). In Europe, the second major milk producer in the world, cefapirin is commercialized in several countries with different trade names (EMA, 2008; EMA, 2013; FAO, 2013). This antibiotic is poorly metabolized, being quickly excreted in the active form and as desacetylcefapirin (EMEA, 1996). Recently, some researchers have found low amounts of cefapirin and desacetylcefapirin in surface water and wastewater samples (Cha et al., 2006; Lin et al., 2008). Beyond that, a literature survey carried out by Boxall et al. (2011) showed that this antibiotic represents a potential risk for humans as a drinking water contaminant. Despite these data, environmental and laboratory-based approaches investigating this compound are scarce in literature.

Ceftiofur is active against both Gram-positive and Gram-negative bacteria, *Streptococci*, beta-lactamases-producing bacteria and also anaerobic microorganisms. As other cephalosporins, its antibacterial activity is due to the inhibition of cell wall synthesis (EMEA, 1999). This antibiotic has a large spectrum and is used for bovines, swines, and poultry as well as for equines and canines. For swine, caprine and equine species it is mainly used to treat bacterial pneumonia and other respiratory infections. It is also used to

treat metritis, podotermatitis and urinary infections in canines and bovines. For poultry, it is used to avoid premature death (USP, 2003). Nowadays, more than four products containing ceftiofur are commercially available in Germany. In the European Union this antibiotic is utilized in almost all member countries (EMA, 2008). Similar to cefapirin, ceftiofur residues are also regularly monitored in animal tissues and products, aiming to avoid crossed exposure, i.e., human exposure due to consumption of contaminated products (Keever et al., 1998; Feng et al., 2012). Due to its high consumption some researchers are also interested in this antibiotic as a potential contaminant and it was included in several surface water and wastewater analyses (Gros et al., 2013; Zhou et al., 2012, Zhou et al., 2013). Recently, ceftiofur was detected in Japanese wastewater (Tamura et al., 2017) and in coastal water in Spain (Biel-Maeso et al., 2018).

The behaviour, fate, effects and environmentally relevant concentrations of veterinary medicines in water matrices are still not well understood (Slana and Dolenc, 2013). In order to understand the environmental fate of these two relevant pharmaceuticals the lack of basic environmental data has to be overcome. A baseline document presenting an overview of cephalosporin antibiotics as environmental contaminants is strongly needed. Data is also scarce for a large number of drugs, even for some of the most used products (Grung et al., 2008; Lin et al., 2008). Pivotal physicochemical parameter as the acid dissociation constant (pK_a) values of this group should be available and reliable, which includes correct ionization center identification. Furthermore, fundamental abiotic degradation processes such as hydrolysis mechanisms and rates are scarce for cefapirin and ceftiofur as well as for other cephalosporins. Likewise, the behaviour of such compounds during disinfection strategies, as UV-C photolysis, and the implications concerning ecotoxicological effects are still unknown. These topics and challenges are approached in the presented thesis.

2. Scope and Aim

This work aims at the investigation of the fate and effects of cephalosporin antibiotics in the aquatic environment. To achieve this general aim, a multidisciplinary study is necessary including chemical, biological and environmental knowledge. The piece starts with **Chapter 3**, a critical review presenting an overview and discussion of the most relevant data about cephalosporin antibiotics as aquatic contaminants. This antibiotic group and aspects involving human and animal usage are presented. Moreover, data about consumption, behavior in environmental matrices as well as occurrence and minimization strategies are discussed. The acid dissociation constants of cephalosporins are in detail investigated in **Chapter 4**. The lack of this pivotal physicochemical data and chemical-biological implication are discussed. In this chapter, a close look at the veterinary drugs ceftiofur (CEF) and cefapirin (CEPA) is carried out. As a consequence of Chapter 4, in **Chapter 5** the effects of CEF and CEPA ionization states during photo-transformation are approached. Complementary, the potential of environmental (hydrolysis) and technical abiotic strategies (UV-C photolysis) on antibiotic depletion is investigated. Questions as "To which extent does base-catalyzed hydrolysis act on CEPA and CEF?", "How long may CEPA and CEF last in surface water?" and "Do CEF and CEPA charges affect photo-transformation rates?" are answered. It is important to emphasize: once chemical decay was determined by quantification of parental drugs but not by mineralization indicators (i.e. TOC), the term transformation was used. Finally, **Chapter 6** presents an ecotoxicological study of CEF and CEPA before and after UV-C photo-transformation. Complementing the work carried out in Chapter 5, the aquatic toxicity of untreated and transformed cephalosporins is studied using standard methodologies (i.e. Green Algae Inhibition test and Cladocera Mobility-Viability assays). Are CEPA and CEF toxic to aquatic organism? Does photo-transformation influence toxicity? These and other questions are answered in this chapter. At the end of this work, **General conclusions and Perspectives** are presented to the reader. There, the main lessons learned in this investigation are highlighted as well as new challenges and questions to the scientific community are addressed. The illustration below (Figure 2.1) presents in a summarized way the scope of this investigation. Graphically, it can be observed how the chapters are contributing to the overall topic.

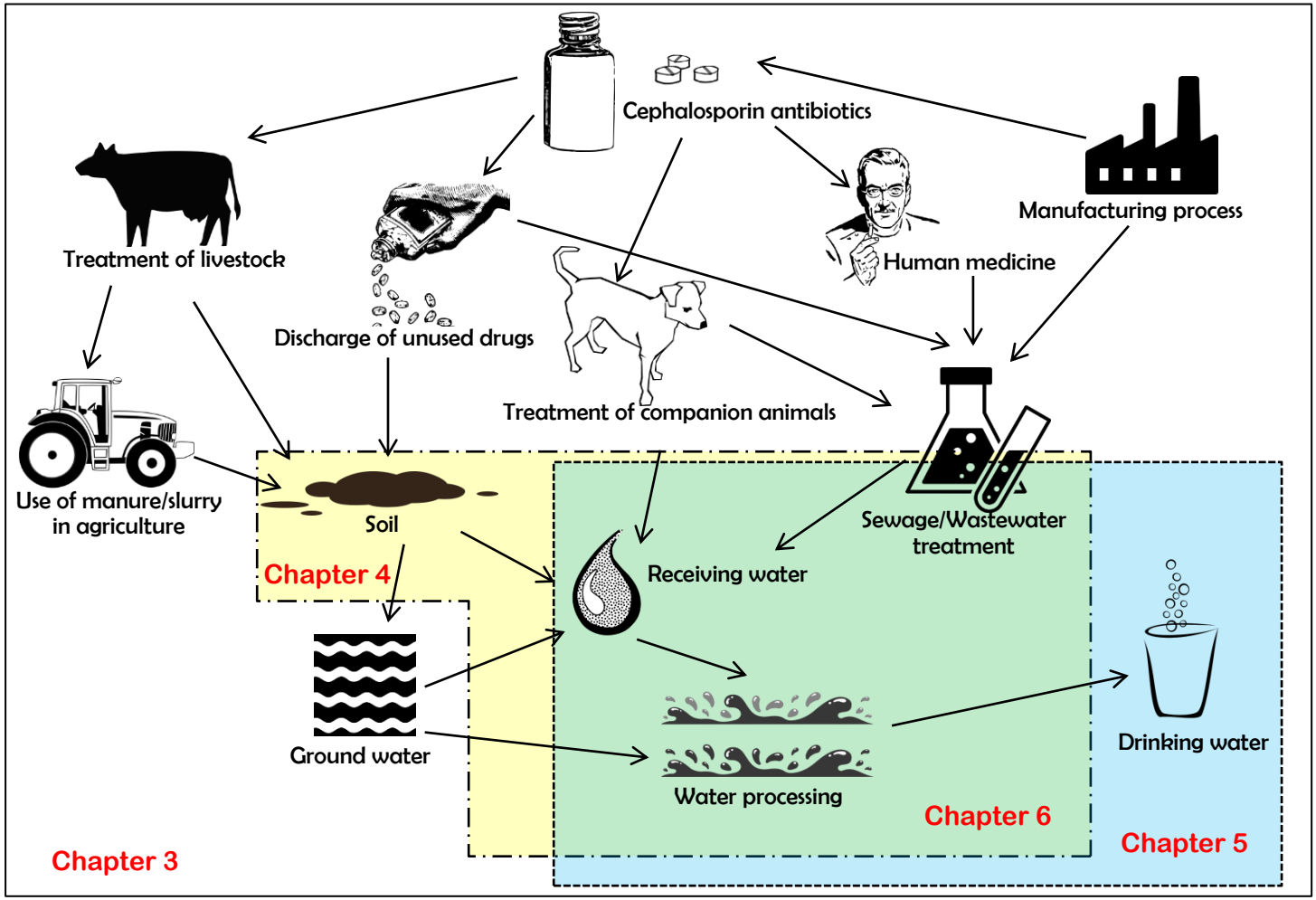

Figure 2.1. Anticipated fate of cephalosporin antibiotics into the environment, adapted from Boxall (2004) and Kümmerer (2009). Geometric shapes indicate the areas of major relevance of each chapter of this thesis. Symbols used are of public domain (Creative Commons, 2017).

2.1. References of Chapters 1 and 2

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3. Cephalosporin antibiotics in the aquatic environment: A critical review of fate, occurrence, ecotoxicity and removal efficiency

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3.1. Abstract

Human and veterinary cephalosporin antibiotics are classified as important water pollutants due to their wide consumption and known inefficiency of classic biologic processes in minimize their input into the aquatic environment. Besides the possibility of affecting non-target organisms, these compounds as well as their metabolites and transformation products can also lead to a pressure on bacterial resistance acquisition. Aiming at the understanding of environmental risks of cephalosporins in aqueous matrices, a literature review was carried out. Relevant data about physicochemical properties, occurrence, ecotoxicity and degradation of cephalosporins were evaluated. Compared to other antibiotics, consumption of cephalosporin s is small. Although their environmental life-time is believed to be short (i.e. days), the available data is insufficient for final conclusions. Several technologies have been used for the treatment of cephalosporins. Long wavelength photolysis (>400nm) is the most investigated strategy for removal of these drugs from aquatic matrices. Mass transfer technologies such as adsorption have also being widely investigated. In most cases, the employed technology led to complete or significant removal (>95%) of parental drugs. Furthermore, the present ecotoxicological data is insufficient for comprehensive ecological risk quotient calculations. Considering the total of 53 cephalosporins, effective values (EC, LC, NOAEC, NOAEL, etc.) are only available for around 30% of parental drugs and are very scarce for cyanobacteria, indicated as the most sensitive organisms to antibiotics. Furthermore, it has been demonstrated that cephalosporins transformation products can be more toxic and more persistent than the parental drugs. Few investigations considering this possibility are available. Consequently, more effort on ecotoxicological data generation and

verification of biologic inactivation of cephalosporins-related products are needed. Likewise, the lack of natural depletion rates and mixture effects on cephalosporins degradation and toxicity has to be overcome.

3.2. Introduction

Cephalosporins belong to a class of semi-synthetic beta-lactam antibiotics widely used in human and veterinary clinic. The World Health Organization (WHO, 2012) classified fluoroquinolones, macrolides and some cephalosporins as critically important and high priority antimicrobials for human medicine. The cephalosporin antibacterial core structure was first isolated in 1945 from the fungus *Cephalosporium acremonium* (Sader and Jones, 1992). As other beta-lactam antibiotics, it inhibits cell wall biosynthesis by connecting to their penicillin binding proteins inside the microbial membrane, resulting in cell lysis and death (Cullmann et al., 1992). Less allergenic and less susceptible to beta-lactamases than penicillins, cephalosporins are accepted as broad-spectrum antibiotics, being used against both Gram-negative and Gram-positive bacteria, effectively interrupting microbial growth (Sader and Jones, 1992; Cullmann et al., 1992; Lin et al., 2000).

Cephalosporin molecules are composed by a core structure, a beta-lactam ring attached to a 7-aminocephalosporanic acid (7-ACA), also known as cephem or dihydrothiazine ring, and two main substituents located at C3- and C7-position (Figure 3.1) (Lin et al., 2000; El-Shaboury et al., 2007). The beta-lactam ring and the acyl side chain at the C7-position are responsible for the antibacterial activity. Meanwhile, the substituents at the C3- and C4-positions mainly rule pharmacokinetics (Cullmann et al., 1992; Sader and Jones, 1992). Normally, cephalosporins are named receiving the prefix cef- or ceph-, being therefore easily recognizable. However, it is not a nomenclature rule. In particular, loracarbef, flomoxef and latamoxef are exceptions. Some cephamycins, an antibiotic group with similar chemical structure, also use the same prefix (WHO, 2017a; WHO, 2017b).

In human medicine, cephalosporins are mainly used as second- or third-line therapy. Specific human genital tract infections as gonorrhea and serious infections as meningitis are primarily combated via cephalosporin usage (Dancer, 2001; BPAC, 2011). However, in veterinary medicine cephalosporins are widely used (EMA, 2016; WHO, 2017b). In fact,

animal respiratory tract infections and intramammary disturbs are commonly combated by using cephalosporins (Gilbertson et al., 1990; Malinowski et al., 2011; Ray et al., 2014). Once inside human or animal body, the excess of antibiotics and/or its metabolites may follow three main routes: i) be distributed into liver, kidney and muscles; ii) be secreted in milk or iii), be excreted in feces and urine (Manzetti and Ghisi, 2014; Ray et al., 2014). Therefore, considering that some active compounds are likely to remain in livestock products, Maximum Residue Limits (MRLs, in $\mu g\ kg^{-1}$) of antibiotics were established to avoid undesired crossed exposure, i.e., human exposure due to consumption of contaminated products (European Commission, 2009; WHO, 2008). On the other hand, the excreted chemicals are released to the environment. In this case, there is no regulation limiting safe or tolerable concentrations for cephalosporins. Furthermore, the estimated life-cycle of antibiotics in the environment is very dynamic and complex (Kemper, 2008; Kümmerer, 2009; Manzetti and Ghisi, 2014).

Nowadays, the main concern about cephalosporins, as for other intensely used antibiotics, is the occurrence and spread of antibiotic-resistant bacteria (Bouki et al., 2013; WHO, 2014; O'Neill, 2016). The first studies in this field focused on the occurrence of resistance acquisition inside hospital environments (Dancer, 2001). Meanwhile, the occurrence of cephalosporins-resistant bacteria has been shown in several environment compartments (Bouki et al., 2013; Veldman et al., 2014). In addition, studies indicate that wastewater treatment plants (WWTPs) actually behave as a stock of resistance genes and bacterial selection hotspot (Bouki et al., 2013; Rizzo et al., 2013). It is a hard task to clearly define the interactions between cephalosporin antibiotic usage and the growth of multiple-resistant organisms. Cullmann et al. (1992) stated three internal processes that can be involved in beta-lactam antibiotic resistance acquirement: i) bacteria may start producing beta-lactamase, the enzyme responsible for the beta-lactam ring cleavage; ii) the target proteins in the microbial membrane are changed, resulting, as an example, in decreased chemical affinity and iii), the access to the target proteins is inhibited, precluding the interaction antibiotic-enzyme. In hospital environments, some studies have shown a positive correlation between cephalosporin usage and increase of resistant organisms (Dancer, 2001). However, only few studies are available to date about agricultural use of

cephalosporins linked to resistant bacteria occurrence (Kemper, 2008; Slana and Dolenc, 2013).

Currently, fifty-three cephalosporin antibiotics (Table 3.1) are catalogued in the Anatomical Therapeutic Chemical (ATC) classification system by the World Health Organization (WHO, 2017a; WHO, 2017b). Of them, five are restricted for veterinary use: cefalonium, cefonicid, ceftiofur, cefovecin and cefquinome. According to their discovery sequence and antibacterial properties cephalosporins are classified as first, second, third and fourth generation (Sader and Jones, 1992; Dancer, 2001; WHO, 2017a; WHO, 2017b). By changing moieties as the substituents at C7- and C3-position, each new generation presented improved characteristics such as better cell permeability, higher stability and broader microbial target spectrum when compared to the previous one (El-Shaboury et al., 2007). Developed during the first years after discovering, first-generation cephalosporins have good activity against Gram-positive bacteria, but they are inefficient against methicillin-resistant-staphylococci (MRSA) and enterococci. Subsequently, second-generation are more stable to hydrolysis by beta-lactamases and have better activity against *Escherichia coli*, Salmonella and other enterobacteriaceae (Saders and Jones, 1992; BPAC, 2011). Compared to the two previous groups, the third one presents the widest activity, being in addition active against Gram-negative organisms and streptococci (BPAC, 2011). The presence of positively charged quaternary nitrogen at the C3-position in the fourth-generation cephalosporins is suggested to be responsible for the observed antimicrobial activity improvement (Saders and Jones, 1992). A representative of each cephalosporin generation is provided in the Supplementary Material.

Considering all aspects presented above, it is assumed that this antibiotic group may be relevant as environmental contaminants. In the last years, several generic reviews about antibiotics in the environment have been published (Kemper, 2008; Kümmerer, 2009; Slana and Dolenc, 2013; Manzetti and Ghisi, 2014; Välitalo et al., 2017). However, there is no similar document focusing specifically on cephalosporins. In the present work, rather than exhaustive data collection, important topics related to the fate, occurrence, ecotoxicology and removal efficiency of this important antibiotic group are presented. Besides the

discussion of the most relevant data, this work highlights the main gaps in current knowledge and points out future challenges.

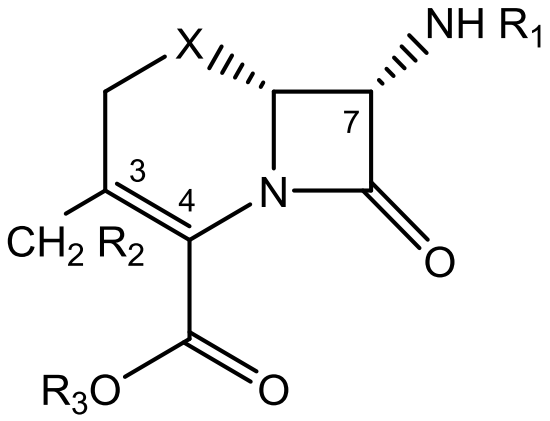


Figure 3.1. Generic structure of cephalosporin antibiotics. X indicates a heteroatom, while R_1, R_2 and R_3 indicate the various substituents. For the 7-amino cephalosporinic acid (7-ACA), X= S, R_1 = H, R_2 = $OCOH_3$ and R_3 = H.

3.3. Entry of cephalosporins into the environment

Similar to other pharmaceuticals, cephalosporins may reach the environment through their medicinal use in humans, livestock and companion animals; inappropriate disposal of unused and/or outdated medicine and through industrial residues (Gilbertson et al., 1990; Kemper, 2008; Kümmerer, 2009; Rizzo et al., 2013; Suárez, 2013). Lately, several publications have presented data about the expected human effects and economic impacts of antimicrobial resistance, in particular due to third- and fourth-generation cephalosporins usage (BPAC, 2011; WHO, 2012; Bouki et al., 2013; Rizzo et al., 2013; Veldman et al., 2014; O'Neill, 2016). This increasing concern is reflected in regulatory changes worldwide, with respect to antibiotics prescription, commercialization and administration (ANVISA, 2011; Almeida et al., 2014; EMA 2016; O'Neill, 2016). The private sector is also aware of this issue. Pharmaceutical industries have implemented Good Manufacturing Practices (GMPs) to minimize the risk of point source emissions (Kümmerer, 2009). In many countries, such as the USA and Denmark, the use of some cephalosporins has been banned or drastically reduced (Schmidt, 2012; Andersen et al., 2015). During the year of 2014, both Norway and Iceland registered no sell of first- and second-generation cephalosporins

for veterinary use. For third- and fourth-generation, less than 1 kg was sold in this period (EMA, 2016). Likewise, densely populated countries such as Brazil implemented regulatory actions to control abuses in antibiotics use and, as a result, minimize the release into the environment (ANVISA, 2011). However, agricultural and hospital contributions are still significant cephalosporin sources (Kemper, 2008; Suárez, 2013; Almeida et al., 2014). The consumption of cephalosporins in hospital care can be very high (Suárez, 2013). Recently, data about the use of human antibiotics in thirteen European countries and territories not belonging to the European Union (EU) was published (Versporten et al., 2014). In this study, Turkey, Montenegro and Tajikistan presented the highest use of cephalosporins, with special preference for second- and third-generation drugs (Versporten et al., 2014). In Portugal, the estimated consumption of cephalosporins for human treatment overcame veterinary use (Almeida et al., 2014). In the years of 2010 and 2011, a consumption of 5.3 and 4.6 tons of human and 0.8 and 0.6 tons of veterinary cephalosporins was projected, respectively (Almeida et al., 2014). The European Medicines Agency (EMA) provides recent data about the sale of veterinary medicinal products (VMPs) in EU countries and Switzerland (EMA, 2016). Considering the 29 countries consulted, third- and fourth-generation cephalosporins accounted for 0.2% of the total of 9,009.5 tons of VMPs sold for food producing animals in 2014 (EMA, 2016). Besides all technical limitations during data collection, the sales figures of VMPs in the years 2011-2014 indicate a steady consumption of third- and fourth-generation cephalosporins (EMA, 2016). When compared to fluoroquinolones and macrolides, other critically important antimicrobial groups presented in the priority list of WHO (2014), the total of cephalosporins sold accounted for just a small proportion. However, only in the EU, approximately 20 tons of veterinary cephalosporins and their metabolites may have reached the environment in 2014 (EMA, 2016).

Cephalosporins can be administrated through oral and parenteral routes. They exhibit fast distribution in biologic systems, presenting half-life times ranging from 0.25 to 9 hours (El-Shaboury et al., 2007; Ray et al., 2014). The majority of antibiotics and their metabolites follow renal excretion, being quickly eliminated (El-Shaboury et al., 2007; Ray et al., 2014). As demonstrated in Table 3.1, most cephalosporins are excreted as unchanged

compounds. The metabolic rate varies from 5 to 65%**.** Therefore, significant amounts of parental drugs may be excreted in feces and urine (Kemper, 2008; Bouki et al., 2013; Manzetti and Ghisi, 2014), as well as in milk (WHO, 2008; European Commission, 2009; Ray et al., 2014). Environmental matrices may receive unchanged cephalosporins, their metabolites generated inside humans and animal bodies, as well as primary transformation products (TPs) (i.e., hydrolytic and photolytic TPs) formed just after excretion (Gilbertson et al., 1990; Kümmerer, 2009).

3.4. Cephalosporin behaviour in soil and water

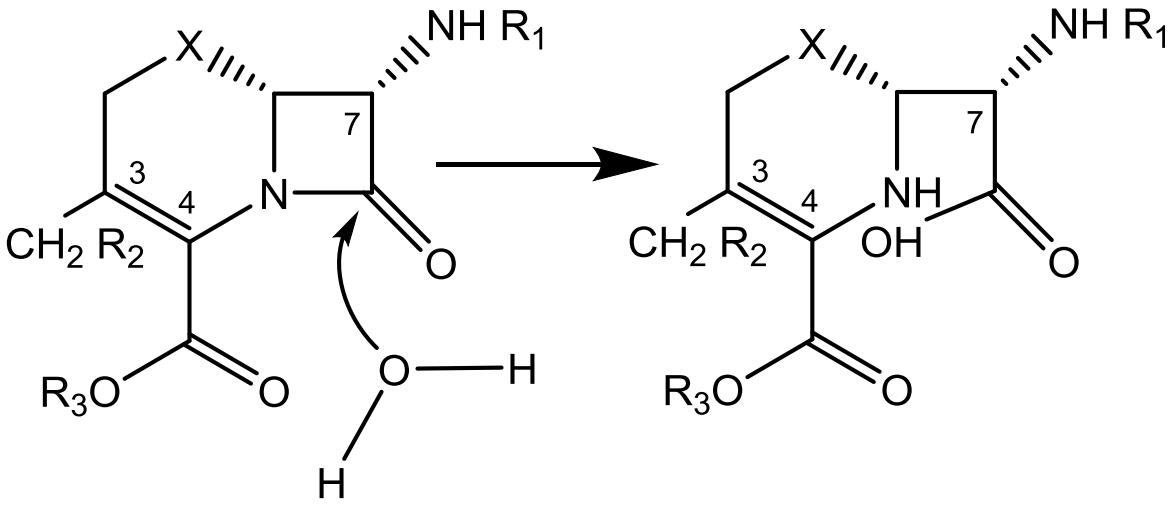
Parental cephalosporins, metabolites and TPs present in urine, feces, manure and in other agricultural-industrial residues may reach soil, ground- and surface waters (Gilbertson et al., 1990; Kemper, 2008; Bouki et al., 2013). Chemical and physicochemical properties of organic contaminants, including antibiotics, play an important role in their fate in these compartments (Schwarzenbach et al., 2003). Cephalosporin R_1, R_2 and R_3 substituents (Figure 3.1) present a wide variety of functional groups (for full structures, see Lin et al., 2000; El-Shaboury et al., 2007; Ribeiro and Schmidt, 2017). These structural changes, even if small, may differentiate their chemical properties and affect environmental behavior (Schwarzenbach et al., 2003; Kümmerer, 2009). For instance, solubility, acid-base characteristics and the octanol-water partition coefficient (K_{ow}) can be strongly affected by substituents. Generally, drugs in this group present low K_{ow} values (Irwin et al., 1988; Mrestani and Neubert, 2000). Therefore, they will be hardly eliminated through biotransformation and soil sorption. Results also indicated that cephalosporins are not readily biodegradable (Alexy et al., 2004). Thus, there is a high potential of these antibiotics to reach ground- and surface water (Slana and Dolenc, 2013). The acid dissociation constant (pK_a) is another important parameter for the environmental fate of organic compounds (Kümmerer, 2009). As well presented by El-Shaboury et al. (2007), Lin et al. (2000) and Ribeiro and Schmidt (2017), cephalosporin structures generally have two or more ionization centers. Hence, those molecules may be positively or negatively charged, as well as be a zwitterion, depending on pH changes. Therefore, a single cephalosporin can behave differently depending on the surrounding pH. Peterson et al.

(2009) and Legnoverde et al. (2014) demonstrated the importance of cephalosporins ionic state during interactions with soil components. At typical environmental pH values, cephalosporins are partially or totally negatively charged (Ribeiro and Schmidt, 2017) and thus may act as ligands for metal cations, such as Cd^{2+}, Cu^{2+}, and Zn^{2+}, forming complexes (El-Maali et al., 2005).

Although the focus of this research is cephalosporins in the aquatic environment, the behavior of such compounds in soil has to be mentioned due to its relevance in the fate of veterinary drugs (Jechalke et al., 2014). However, the literature concerning this issue is very scarce. Gilbertson et al (1990) reported on the degradation of ceftiofur, a broad spectrum veterinary cephalosporin, in feces, urine and different types of soil. The authors reported half-life ($t_{1/2}$) values of days in all tested compartments. Indeed, the resulting TPs showed no antimicrobial activity. Promising alternatives to remove cephalosporins in soil and manure may be the use of biotechnological strategies (i.e. biostimulation, bioaugmention). Specifically, isolated intestinal microflora from bovines has demonstrated great ability to degrade ceftiofur (Rafii et al., 2009; Erickson et al., 2014). However, chemical interactions between cephalosporins and soil constituents, residence time and degradation strategies in soil need to be further investigated.

Hydrolysis may occur in feces, urine and soil. Generally, cephalosporins present fast hydrolysis rates (days < $t_{1/2}$ < weeks) at environmentally relevant condition (i.e. neutral pH and 20°C). (Fubara and Notari, 1998; Berendsen et al., 2009; Jiang et al., 2010; Wang and Lin, 2012). Ideally, when hydrolysis of cephalosporins occurs, the beta-lactam ring undergoes nucleophilic attack, followed by protonation of the N atom and carbon-nitrogen bond cleavage (Scheme 3.1) (Meliá et al., 2015). However, the substituents R_1 and R_2 may be the first moieties to undergo hydrolysis, but this transformation does not change the bioactive part of the beta-lactam structure. Abiotic processes such as hydrolysis and photolysis are strongly influencing cephalosporin degradation in surface water (Jiang et al., 2010). On the other hand, biotransformation predominated in sediment (Jiang et al., 2010). Moreover, several studies demonstrated a pH-dependent stability for cephalosporins, showing the suitability of these drugs to be degraded in alkaline solution (Fubara and Notari, 1998; Gilbertson et al., 1990; Berendsen et al., 2009; Jiang et al., 2010). The

stability of cefapirin, ceftiofur and their most important metabolites was assessed at different pH values and under physiological temperature (Berendsen et al., 2009). All parental compounds showed fast degradation in alkaline environment (pH 12 and 11), while metabolites last longer. Alkaline catalyzed hydrolysis was also reported for cefuroxime (Wang and Notari, 1994), cefuroxime and cefoxitin (Mitchell et al., 2014). However, in environmental systems base-catalyzed reactions contribute to a minor extent in the hydrolysis of cephalosporins (Yamana and Tsuji, 1976; Mitchell et al., 2014). In all cases, hydrolysis may involve two or more reactions and may lead to the formation of bioactive molecules. Therefore, the measurement of antimicrobial activity of hydrolysis products is crucial as well as the study of cephalosporin persistence in surface water under various conditions (i.e. temperature, pH, organic content, ionic strength, etc.).



Scheme 3.1. Possible hydrolysis of a generic cephalosporin structure. This concerted reaction is indicated as the main inactivation route of cephalosporin antibiotics in the aquatic environment.

Table 3.1

Cephalosporin antibiotics: Identification, excretion rates, occurrence in aqueous matrices, ecotoxicological data and new technologies employed for their abatement

Metabolism				**Occurrence**		**Ecotoxicological data**			**Abatement technology used**	
Cephalosporin, Route of administration and Excretion unchanged in urine (%)				Max - Min [ng L^{-1}] and number of positive samples #		Taxon tested and abundance of response values §	Most restrictive response [mg L^{-1}]			
First generation			**Ref.**		**Ref.**			**Ref.**		**Ref.**
Cefalexin	O[b], P[c]	80-90	[1]	0.40 surface water 28889 input WWTP #118	[28] [40] [41] [42] [43] [44] [45] [46] [47] [48] [49] [50] [51] [52] [53] [54] [55] [63]	Macrophyte, fish, proteobacteria §5	*Lemna gibba* 7d EC_{10} >1	[3] [28] [39]	Activated carbon Photolysis (Simulated sunlight) Sulfate radical Electrolysis Nanofiltration Zeolites Chlorination MgO nanoparticles Algae/activated sludge Anodic oxidation, Anodic oxidation/H_2O_2, Electro-Fenton Photocatalysis (Vis/NiO-NCP[d]) Photolysis and Photocatalysis (UV-C and UV-C/$S_2O_8^{2-}$)	[2] [4] [13] [22] [3] [8] [12] [14] [61] [15] [17] [19] [23] [24] [25] [64]

Continuation of Table 3.1

Cefradine	O, P	90 O 60-80 P	[1]	1 Industrial wastewater 20160 influent WWTP #26	[28] [40] [48] [51] [54] [55] [63]	Fish, algae, proteobacteria §4	*Microcystis aeruginosa* 72h EC_{50} 1.38	[3] [28] [30]	Photolysis (Simulated sunlight) Multi-walled carbon nanotubes Algae/Activated sludge	[3] [10] [18] [23]
Cefadroxil	O	90	[1]						Photolysis and Photocatalysis (UV-C and UV-C/$S_2O_8^{2-}$)	[64]
Cefalotin	P	70	[1]			Algae §3	*Pseudokirchneriella subcapitata* 72h EC_{10} 76	[34]	Photolysis and photocatalysis (UV-C, UV-C/H_2O_2, UV-C/$S_2O_8^{2-}$) Sulfate radical	[6] [8]
Cefapirin	P	68-70		5 hospital wastewater 9 surface water #2	[40] [59]	Proteobacteria, fish §2	*Cyprinus carpio*, 96h LC_{50} >60	[3] [28]	Photolysis (Simulated sunlight)	[3]
Cefazolin	P	80	[1]	10 surface water 12850 influent WWTP #11	[28] [40] [53] [63]	Proteobacteria, algae crustacean, Mollusca, fish, Annelida, plantae §11	*Danio rerio* 72h NOAEC 50	[3] [27] [28] [31] [32]	Photolysis (Simulated sunlight) Photolysis (Vis/Ag_3PO_4/BiOBr) Chlorination Sulfate radical Multi-walled carbon nanotube	[3] [16] [7] [8] [18]
Cefazedone	P					Fish §2	*Danio rerio* 72h NOAEC 50	[32]		

Continuation of Table 3.1											
No further data for Cefatrizine (O), Ceftezole (P), Cefalonium[a] (P), Cefacetrile (P, 76), Cefroxadine (O) and Cefaloridine (P)											
Second-generation											
Cefoxitin	P	85	[1]	50 effluent WWTP 13150 influent WWTP #2	[63]						
Cefaclor	O	85	[1]	5.9 coastal water 500 input WWTP #3	[44] [52] [62]					Sulfate radical	[8]
Cefuroxime	O, P	35	[1]	49 influent STP 24380 influent WWTP #4	[63] [65]	Algae, crustacean, annelida, molusca, fish, plantae §8	*Selenastrum capricornutum* 72h EC_{50} >91	[27] [35]		Photolysis (Simulated sunlight)	[10]
No further data for Ceforanide (P), Cefminox (P), Cefbuperazone (P), Flomoxef (P), Loracarbef (O, 90), Cefonicid[a] (P, >95), Cefprozil (O, 60 [1]), Cefotetan (P, 51-81), Cefmetazole (P), Cefotiam (O, P) and Cefamandole (P, 80 [1])											
Third – generation											
Cefixime	O	50								MgO nanoparticles Algae/activated sludge	[19] [23]
Cefdinir	O	15-30		1 coastal water 15.8 coastal water #2	[62]						
Cefotaxime	P	40-60	[1]	0.30 hospital wastewater 18080 influent WWTP #23	[28] [40] [45] [46] [48] [65]	Diptera larvae, proteobacteria, plantae, Annelida, fish, crustacean, Mollusca §10	*Drosophila melanogaster* 48h NOAEC 10	[3] [26] [27]		Photolysis (Simulated sunlight) Sulfate radical Chlorination	[3] [8] [17]

Continuation of Table 3.1										
					[50] [57] [58] [63]				Multi-walled carbon nanotube	[18]
Ceftazidime	P	80-90	[1]			Crustacea, Mollusca, Annelida, fish, plantae §5	*Eisenia foetida* 48h LC_{50} 0.032	[27]	Ozonation/membrane filtration Algae/activated sludge	[20] [23]
Ceftiofur[a]	P			0.9 effluent sept tank 1.7 coastal water #4	[60]				Hydrothermal liquefaction Photocatalysis (Vis/Au-TiO_2) Photocatalysis (Vis/Au-Bi_2CuO_4) Biochars Biodegradation (wetland)	[5] [9] [11] [21] [66]
Ceftriaxone	P	40-65	[1]	2030 effluent WWTP 5150 influent WWTP #2	[63]				Photolysis (Simulated sunlight)	[10]
Cefovecin[a]	P					Crustacea, shrimp, fish §3	*Mysidopsis bahia* 48h LC_{50} 580	[39]		
No further data for Cefcapene (O), Cefpiramide (P), Latamoxef (P), Cefmenoxime (P), Cefditoren (O), Ceftizoxime (P, 100 [1]), Cefsulodin (P), Cefpodoxime (O, 40 [1]), Cefetamet (O), Cefodizime (P, 80), Cefoperazone (P, 20-30) and Ceftibuten (O, 80-90)										
Fourth – generation										
Cefepime	P	85				Crustacea, Annelida, Mollusca, plantae, fish §6	*Lactuca sativa* 120h EC_{50} >100	[27] [33]	Photolysis (Simulated sunlight)	[10]

Continuation of Table 3.1					
Cefquinome[a]		1.8 coastal water #1	[62]		
No further data for Cefpirome (P, 80-90) and Cefozopran (P)					
Others					
Ceftobiprole medocaril	P		Cyanobacteria, cladocera, fish **§**6	ND[e], 72h NOAEC 0.00022	[37]
Ceftaroline fosamil	P		Cyanobacteria, algae, cladocera, fish **§**4	*Anabaena flosaquae*, 72h NOAEC 0.0012	[36]
Ceftolozane	p		Cyanobacteria, cladocera, fish **§**3	*Anabaena flosaquae* 72h NOAEC 0.15	[38]

NOTES

[a] veterinary use only, [b] oral, [c] parenteral , [d] clinoptilolite nanoparticles, [e] not defined, # number of positive samples for this antibiotic, **§** amount of effect number (i.e. EC, LC, NOEC, NOAEL) available for this antibiotic

Table 3.2

References of Table 3.1

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(3) Wang and Lin, 2012
(4) Nazari el at., 2016
(5) Pham et al., 2013
(6) He et al., 2014
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(8) Rickman and Mezyk, 2010
(9) Pugazhenthiran et al., 2014
(10) Jiang et al., 2010
(11) Anandan et al., 2013
(12) Coledam et al., 2017
(13) Liu et al., 2011
(14) Sun et al., 2012
(15) Samarghandi et al., 2015
(16) Xiao et al., 2017
(17) Li and Zhang, 2013
(18) Fakhri et al., 2016
(19) Fakhri and Adami, 2014
(20) Alpatova et al., 2013
(21) Mitchell et al., 2015
(22) Pouretedal and Sadegh, 2014
(23) Guo and Chen, 2015
(24) Estrada et al., 2012
(25) Ajoudanian and Nezamzadeh-Ejhieh, 2015
(26) Rahul et al., 2015
(27) Suárez, 2013
(28) Li and Lin, 2015
(29) Brain et al., 2004
(30) Chen and Guo, 2012
(31) Eguchi et al., 2004
(32) Zhang et al., 2010
(33) Bristol-Myers Squibb, 2016
(34) Magdaleno et al., 2015
(35) GlaxoSmithKline, 2013
(36) EMA, 2012
(37) Vestel et al., 2016
(38) EMA, 2015
(39) Zoetis, 2014
(40) Lin et al., 2008
(41) Lin et al., 2009
(42) Costanzo et al., 2005
(43) Gulkowska et al., 2007
(44) Watkinson et al., 2009
(45) Gulkowska et al., 2008
(46) Leung et al., 2012
(47) Osório et al., 2016
(48) Lin et al., 2010
(49) Minh et al., 2009
(50) Li and Zhang, 2011
(51) Li et al., 2009
(52) Watkinson et al., 2007
(53) Chen et al., 2012
(54) Dutta et al., 2014
(55) Zhang et al., 2013
(56) Sim et al., 2011
(57) Gros et al., 2013
(58) Shraim et al., 2017
(59) Cha et al., 2006
(60) Tamura et al., 2017
(61) Zazouli et al., 2009
(62) Biel-Maeso et al., 2018
(63) Yu et al., 2016
(64) Serna-Gavis et al., 2017
(65) Rossmann et al., 2014
(66) Alexandrino et al., 2017

3.5. Occurrence of cephalosporins

As mentioned above, the consumption of cephalosporins compared to other VMPs can be considered low. Moreover, these compounds are expected to undergo fast hydrolysis in aquatic matrices, being transformed in few days. Nevertheless, cephalosporins have been detected in different aqueous matrices in concentrations ranging from 0.30 ng L^{-1} (cefotaxime) to 0.03 mg L^{-1} (cefalexin) (Table 3.1). Cephalosporins have been detected in surface water (in both fresh and seawater), in raw and treated sewage, in river sediment and in raw and treated wastewaters (see full details in Supplementary Information). A total of 198 positive samples for cephalosporins (#) were reported in the literature (Table 3.1). Figure 3.2 shows the percentage of positive samples for each analyzed matrix and detected cephalosporin. The majority of data (70%) refers to samples directly linked to sewage and wastewaters. In other cases, the occurrence in surface water was reported in areas near to WWTP or sewage treatment plants (STP) discharges (Costanzo et al., 2005; Minh et al., 2009; Zhang et al., 2013; Li and Lin, 2015). Cefalexin was the cephalosporin with most positive samples (#118). In fact, 61% of this total is linked to detections in sewage and wastewater. For cefradine, the second most reported drug (#26), a similar trend was observed, with 84% of occurrences linked to WWTP and STP. In the case of cefotaxime, the third most detected cephalosporin (#23), sewage and wastewater linked occurrences correspond to all reported cases. Therefore, the importance of urban, industrial and hospital related contributions for the release of cephalosporins into the environment is evidenced. However, caution is warranted in the use of positive samples score. This data may be biased by the sampling design, location and reporting methodology used by the authors. As an example, Rossmann et al. (2014) reported on the detection of cefotaxime and cefuroxime in STP but did not provide the frequency of detection of each cephalosporin. In this example and in similar cases, the median values available were assumed as single positive samples, representing therefore an underestimated and limited index. In other cases, the amount of sampling points compromised the overall result. Particularly, Gulkowska et al. (2007) and Minh et al. (2009) carried out a comprehensive investigation of coastal waters of Hong Kong and reported ubiquitous presence of cefalexin. Consequently, seawater presented the second highest incidence of cephalosporins (Figure

3.2). The same is true for the abundance of positive samples related to point sources (#134). Due to practical characteristics as accessibility and high concentration of xenobiotics, WWTPs and STPs are intensively monitored. It reflects directly on the number of positive samples and may bias the occurrence of cephalosporins. On the other hand, investigations of diffuse pollution analyzing for cephalosporins are scarce. In particular, Osório et al. (2016) carried out a comprehensive investigation in four river basins in Spain. Cefalexin was detected in two consecutive years in surface water, in three of the four rivers investigated. The authors also reported this antibiotic in river sediment and correlated the occurrence data of such compounds with human and livestock populations. Likewise, Cha et al. (2006) studied the presence of cefapirin and other beta-lactams in a small mixed-watershed, performing a 12-month monitoring. Despite the large number of campaigns, cefapirin was detected only once (9 ng L^{-1}) in a river strongly influenced by agriculture. In summary, rather little is known about the occurrence of cephalosporins in surface water and sediment, especially for second- and third-generation drugs, compounds significantly consumed (see item 3.3) and highlighted due to their importance for human medicine (WHO, 2012).

According to Junker et al. (2006) and Cha et al. (2006), the main reason for low occurrence registration of cephalosporins in the environment is due to analytical limitations and to the rapid hydrolytic cleavage of the beta-lactam structure. Therefore, besides methods with detection limits in the low ng L^{-1} level, a continuous monitoring including mass-balance directed sampling (Minh et al., 2009; Zhang et al., 2013) is necessary to confirm the introduction and pseudo-persistence of cephalosporins into the aquatic environment. Furthermore, authors have only checked parental cephalosporins. There is no data reporting on detection of cephalosporins' metabolites and TPs. Special attention has to be given to TPs generated by hydrolytic cleavage of the C3- and C7-substituents, which may keep intact their antimicrobial activity (Yamaha and Tsuji, 1976; Kosh and Cazers, 1997). The same holds true for TPs generated after photolysis and disinfection technologies, which may be more toxic to aquatic organisms than the parental drugs (Wang and Lin, 2012; Fabbri et al., 2015).

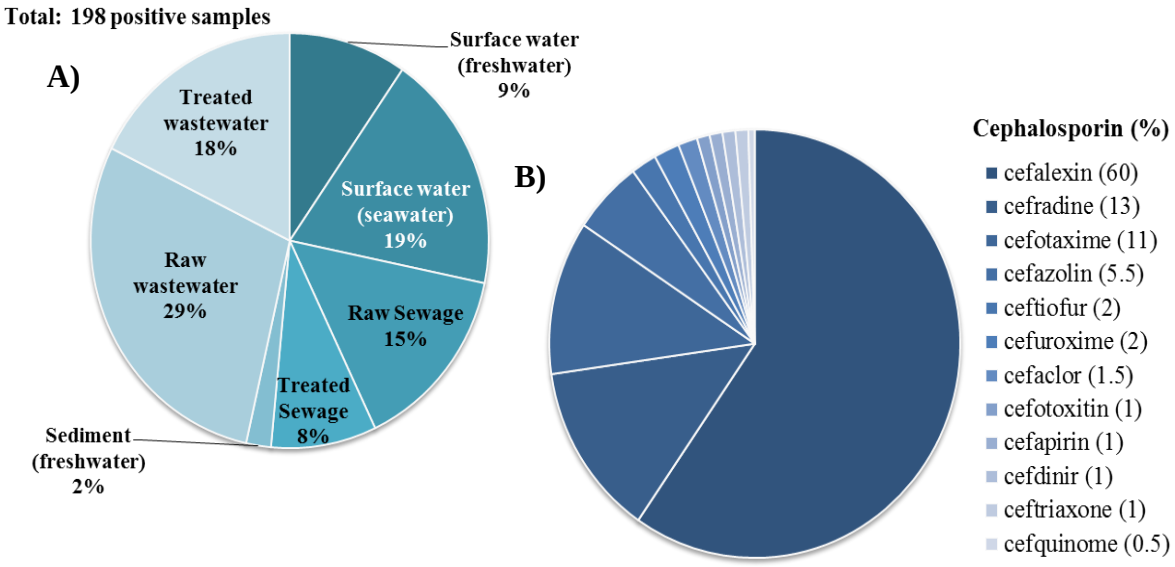

Figure 3.2. Percentual representation of the 198 positive samples for cephalosporin antibiotics in the aquatic environment. **A** ilustrates the contribution of each environmental matrix where positive samples have been reported. Whereas **B** shows the percentual distribuition of positive samples for each detected cephalosporin.

3.6. Effects in non-target organisms

As evidenced by the abundance of response values (i.e. EC, LC, NOEC, NOAEL, etc.) reported in Table 3.1, little is available about the ecotoxicity of cephalosporins. Three trophic level tests were barely performed for drugs within this group. This is mainly due to the lack in the past of regulations concerning the environmental risk assessment (ERA) of antibiotics (European Commission, 2003; EMA, 2012; EMA, 2015). As well, the misleading assumption of low toxicity, similar response and fast hydrolysis for all beta-lactams contributed with the scarcity of data (NRA, 2001; EMA, 2012; EMA, 2015). As an example, contrasting the responses of *Daphnia magna* for cefepime (48h EC_{50} 640) and cefovecin (48h EC_{50} >1000), in mg L^{-1} (Zoetis, 2014; Bristol-Myers Squibb, 2016), one can observe that similar compounds may present different toxicities towards the same species and endpoint. Nevertheless, analyzing the 72 response values available for 14 cephalosporins (Supplementary Information), in general a low toxicity for the tested

organisms is observed. 64% of responses reported are censored values (>) and when deleterious effects were observed, it was in the range of mg L^{-1}, far from the usually detected concentrations in surface water (Table 3.1). This overall low toxicity is plausible since the standardized organisms used for cephalosporins ecotoxicological assessment are evolutionarily more distant to the antibiotics target organisms. Tests with prokaryotes, which are known to be the most sensitive to antibiotics and more ecologically relevant organisms (Välitalo et al., 2017; Le Page et al., 2017), are available for ceftobiprole, ceftoraline and ceftolozane, the most recent cephalosporins. In fact, cyanobacteria results were the most restrictive values for cephalosporins (Table 3.1), with effective concentrations in the same range as detected values in WWTP and STP for several cephalosporins. Recently, Välitalo et al. (2017) demonstrated the relevance of including chronic tests with prokaryotes in the ecotoxicological assessment of antibiotics. Le Page et al. (2017) also stated that classical ecotoxicological tests might underestimate the harm of antibiotics for aquatic micro-organisms. The biological similarities between bacteria and cyanobacteria evidence the risks of deleterious effects due to antibiotic exposure. Furthermore, cyanobacteria play an important ecological role in both aquatic and terrestrial environment, contributing with the cycle of gases and nutrients and biofilms formation (Tan et al., 2016; Välitalo et al., 2017).

Ammonium oxidizing bacteria and green algae also presented sensitivity to antibiotics (Ghosh et al., 2009; Chen and Guo, 2012; Magdaleno et al., 2015; Välitalo et al., 2017). Although the lack of data hampers a thorough comparison, the sensitivity of green algae to cephalosporins seems to be compound and specie specific. The effective values (in mg L^{-1}) reported for cefalotin and *Pseudokirchneriella subcapitata* (72h EC_{50} >600) (Magdaleno et al., 2015) are higher than those observed by Chen and Guo (2012) with cefradine and *Microcystis aeruginosa* (72h EC_{50} 1.38) and *Scenedesmus obliquus* (72h EC_{50} 1.77). No study analyzing the effects of cephalosporins on ammonium oxidizing bacteria is available, which is necessary due to the importance of this group, especially in biological treatment plants (Ghosh et al., 2009). Antibiotics can also lead to bacteria-mediated effects on aquatic organisms. As demonstrated by Gorokhova et al. (2015), trimethoprim exposure resulted in deleterious shift on inner microbiota, affecting *Daphnia magna* digestion and incorporation

efficiencies. This possibility need to be also investigated for cephalosporins both in acute and chronic essays, especially for compounds frequently detected in the aquatic environment (i.e. cefalexin, cefradine, cefotaxime and cefazolin) (Table 3.1).

Soil may be the first environmental destination of veterinary cephalosporins (Gilbertson et al., 1990) but data of ecotoxicological effects on soil-related micro-organisms is scarce. In fact, Soárez (2013) presented in contact tests the acute toxicity of ceftazidime to the earthworm *Eisenia foetida* at low concentration (48h LC_{50} 0.032 mg L^{-1}). A compound specific response was observed since no deleterious effects were observed (48h LC_{50} >100 mg L^{-1}) when *Eisenia foetida* was exposed to filter papers impregnated with cefuroxime, cefepime, cefazolin and cefotaxime (Soárez, 2013). Both ceftazidime and ceftriaxone moderately affected photosynthesis, the fundamental process for plant viability, during chronic experiments with the wheat *Triticum aestivum* (Opriş et al., 2013). Effects were observed through several endpoints (i.e. foliage net assimilation rate, stomatal conductance, carotenoid content, etc.) in concentrations ranging from 0.25 to 1.5 mg L^{-1}. According to the authors, the nitro group substituent present in the cephalosporin structure may inhibit the photosystem II (Opriş et al., 2013). Therefore, possible effects of cephalosporins on soil organisms such as Annelida and plants need to be further explored, especially considering the range of available tests organism (van Gestel, 2016; OECD, 2016) and possible effects on rhizosphere community (Jechalke et al., 2014). Cephalosporin TPs can present higher toxicity to aquatic organisms than the parental drugs (Zhang et al., 2010; Wang and Lin 2012; Li et al., 2013). Cefazolin and cefazedone are examples of cephalosporins with known teratogenic properties demonstrated by zebrafish embryo test (Zhang et al., 2010). When compared with the parental drug, chlorination of cefazolin resulted in higher genotoxicity in *Salmonella typhimurium* tests (Li et al., 2013). In fact, the 2-mercapto-5-methyl-1,3,4-thiadiazole (MMTD) substituent located at C3-position, shared by both cefazolin and cefazedone was suggested as responsible for the teratogenic effects on *Danio rerio* (Zhang et al., 2010). In the case of cefazolin chlorination, the formation of sulfoxide and chlorinated by-products might also contribute with the observed higher deleterious effect. An increasing on *Vibrio fischeri* luminescence inhibition was observed during sunlight simulated photolysis of cefazolin, cefotaxime, cefapirin, cefalexin and cefradine

(Wang and Lin, 2012). The responses were compound specific, where different levels of toxicity were observed within the analyzed drugs. Cefazolin photo-transformation resulted in the highest toxicity increase and its MMTD structure at C3-position was again linked to the toxic response (Wang and Lin, 2012). Therefore, cephalosporin side-chains seem to be relevant as potential precursors of toxic TPs, which unravels further investigation needs.

Considering the known aqueous instability of cephalosporins (Fubara and Notari, 1998; Berendsen et al., 2009; Jiang et al., 2010; Mitchell et al., 2014), the possibility of toxic cephalosporin' TPs (Zhang et al., 2010; Wang and Lin, 2012; Li et al., 2013) and the potential of a higher stability of hydrolysis and photolysis products than parental drugs (Berendsen et al., 2009; Wang and Lin, 2012), the need of assessing the real concentrations of both parental and TPs during ecotoxicological assessment of cephalosporins is evident. Analytical identification and quantification are necessary in order to correctly identify the contribution of each compound in the overall toxicity. However, in that direction, only few studies are available to date (Eguchi et al., 2004; Wang and Lin, 2012; Magdaleno et al., 2015). Another critical point that urges for investigation is potential synergic and antagonistic effects of cephalosporins (Li and Lin, 2015). As recently pointed out by Marx et al. (2015), the lack of such data is alarming and prevents the hazard analysis of mixtures, such as hospital wastewater.

3.7. Technologies used for cephalosporins removal

Classic biological treatment (i.e. WWTP and STP) cannot provide complete removal of cephalosporin antibiotics (Costanzo et al., 2005; Gulkowska et al., 2008; Lin et al., 2009; Minh et al., 2009; Li et al., 2009; Li and Zhang, 2011; Sim et al., 2011; Leung et al., 2012; Rossmann et al., 2014; Yu et al., 2016; Tamura et al., 2017), mainly due to their input concentration, recalcitrance and deleterious impact (as antimicrobials) on the microorganisms responsible for biodegradation. Therefore, alternative technologies have been used for the treatment of such compounds, presenting promising results.

An alternative to improve cephalosporins degradation in biological treatment is the use of pre-treatment as chemical or no bacterial oxidation. A combined system using green algae and active sludge was used for the treatment of cefradine, cefalexin, ceftazidime and

cefixime, resulting in high removal efficiency (>95%) (Guo and Chen, 2015). When compared with the traditional active sludge, the use of *Chlorella pyrenoidosa* as a preliminary step resulted in higher removal efficiency (Guo and Chen, 2015). In fact, the authors suggested that the partially or totally degraded antibiotics presented low impact in the active sludge. Estrada et al. (2012) reported the electro-Fenton (EF) oxidation process as a potential pre-biological treatment of wastewater containing cefalexin. Compared with anodic oxidation, another hydroxyl radical generating techniques, EF enhanced the studied wastewater biodegradability.

Sorption of cephalosporins to new materials was studied intensively (Liu et al., 2011; Ahmed and Theydan, 2012; Pouretedal and Sadegh, 2014; Mitchell et al., 2015; Nazari et al., 2016). To illustrate, activated carbon prepared using walnut shells (Nazari et al., 2016), agricultural waste (Ahmed and Theydan, 2012), lotus stalks (Liu et al., 2011) and wine wood (Pouretedal and Sadegh, 2014) demonstrated great potential for cefalexin adsorption from aqueous solutions, with high removal yields (>80%). In the case of natural zeolites usage, cefalexin removal significantly increased (from 28 to 89%, pH 7) when coated manganese oxide nanoparticles was used (Samarghandi et al., 2015). Similarly, Fakhri and Adami (2014) illustrated the adsorption of cefalexin and cefixime onto magnesium oxide nanoparticles. In this case, basic medium (pH>8) enhanced the removal of cephalosporins from aqueous solution. Carbon nanotubes (CNT) were also employed for cephalosporin abatement (Fakhri et al., 2016). The adsorption of cefotaxime, cefradine and cefazolin onto CNT increased with basic pH (>8). The adsorption medium pH was pivotal for the sorption capacity as well as the contact time, which varied from minutes (Fakhri and Adami, 2014) to hours (Fakhri et al., 2016). As discussed before, cephalosporins are suitable for base-catalyzed hydrolysis (Mitchell et al., 2014) and this process may also be occurring during adsorption experiments under basic pH values. Both Fakhri and Adami (2014) and Fakhri et al. (2016) did not consider this possibility, which may bias the obtained results. Biochars, an adsorbent material produced by pyrolysis of different substrates, presented an overall excellent removal (>99%) of ceftiofur from water (Mitchell et al., 2015). In fact, bioassays using both sensitive and resistant strains of *Escherichia coli* demonstrated the bio-unavailability of the antibiotics and their TPs (Mitchell et al., 2015). During the 24 hours of

experiment neither degradation nor loss of bioactivity due hydrolysis was observed in dark controls without the sorbents. Similar studies are necessary to confirm the efficiency of sequestration strategies. Nevertheless, sorption only sequesters antibiotics from residues or contaminated water. Mass transfer strategies possess as main limitation the need of posterior treatment of the adsorbent-xenobiotic product. On the other hand, such techniques may provide an effective capture and immobilization of contaminants. A posterior treatment as composting or hydrothermal inactivation is needed. In this direction, complete removal of ceftiofur from manure, algae and wastewater biosolids was achieved by hydrothermal liquefaction (Pham et al., 2013). This technique demonstrated high efficiency in the removal (>95%) of both bioactive compounds and antibiotic resistant genes. However, the high use of energy corresponds to high costs, currently limiting the applicability of hydrothermal liquefaction.

Pharmaceutical wastewater rich of cephalosporins and their by-products was treated with electrochemical and sonoelectrochemical catalytic-oxidative processes (Yang et al., 2016). This novel strategy of treatment resulted in efficient removal (94%) of organic content (TOC). However, short-term *Vibrio fischeri* tests provided a clear correlation between the treatment application and the formation of toxic TPs (Yang et al., 2016). Furthermore, the authors did not include discussion on energetic requirements, which is known to be the main limitation of sonochemical treatments. Recently, Coladam et al. (2017) presented the complete elimination of cephalexin and its intermediates during electrolysis with a boron-doped diamond anode electrode. Although the authors reported the formation of recalcitrant acidic species, the application of such technique resulted in biologically inactivated products, which were assessed by *Escherichia coli* inhibition tests. Similarly, electrolysis was used for the degradation of cefepime (Özkan et al., 2002). In this work, the importance of drug ionization state (pK_a) on the reactivity and degradation rates was well discussed, but neither information on toxic effects nor antimicrobial inactivation after treatment was reported. Moreover, the authors did not provide energy consumption information, which is important for both scale up and viability considerations.

As presented in Table 3.1, sunlight simulated photolysis and photocatalysis have been employed for the abatement of several cephalosporins. In fact, photolysis is expected to be

the pivotal process in the aquatic fate of these drugs (Jiang et al., 2010; Wang and Lin, 2012), especially due to their light absorption over a relatively wide wavelength range (Schwarzenbach et al., 2003; Wang and Lin, 2012; Ribeiro and Schmidt, 2017). The photo-transformation rates of cephalosporins under simulated sunlight radiation were compound-specific (Jiang et al., 2010; Wang and Lin, 2012). Compound-specific responses were also observed for water matrix effects, where reasonable similarities were only observed in same generation compounds. Besides, the TPs of cefazolin, cefotaxime, cefapirin, cefalexin and cefradine presented increased acute toxicity to *Vibrio fischeri* after photo-degradation (Wang and Lin, 2012), showing again the importance of assessing ecotoxicological effects of treatment strategies. By itself, photolysis in the visible wavelengths (>400 nm) range provided low degradation efficiency of cephalosporins when compared with the use of short wavelength radiation and catalysts. UV-visible light and photocatalysis were used to degrade cefazolin (Xiao et al., 2017), ceftiofur (Pugazhenthiran et al., 2014; Anandan et al., 2013) and cefalexin (Ajoudanian and Nezamzadeh-Ejhieh, 2015). All authors reported removal efficiency ranging from 70 to 95%, but only Xiao et al. (2017) presented the drug decay during the prior equilibration period necessary for experiments. As demonstrated, cefazolin concentration after 30 min of equilibrium was already reduced by 20 to 80%, which may be related to both drug instability and adsorption onto catalysts. Such information is pivotal for any study involving cephalosporins photocatalysis or any long-term removal strategy.

Advanced oxidative processes (AOPs) are promising for cephalosporins degradation due to the observed fast reaction rates (half-life times ranging from milliseconds to few minutes) and high efficiency in the drugs removal (>95%) (Rickman and Mezyk, 2010; Estrada et al., 2012; He et al., 2014; Serna-Gavis et al., 2017). Cefalotin was mineralized by using UV-C/H_2O_2 and UV-C/$S_2O_8^{2-}$, even when the matrix anionic composition was changed, which can interfere on photons absorption (He et al., 2014). Similarly, Rickman and Mezyk (2010) reported on cefaclor, cefazolin, cefalexin, cefalotin and cefotaxime reaction rates with sulfate radical. The authors indicated the antibiotic core structure as reaction site for $SO_4^{\bullet-}$, which may lead to antimicrobial inactivation of cephalosporins and their TPs. Serna-Gavis et al. (2017) demonstrated that both UV-C and UV-C/sulfate radical had similar

performance in the degradation of cefalexin and cefadroxil. Both cephalosporins underwent direct photolysis in aqueous solution and presented no antimicrobial activity after treatment. The possibility of matrix composition interference on photolysis efficiency has been checked for some cephalosporins (He et al., 2014; Serna-Gavis et al., 2017). However, the possibility of toxic TPs formation needs to be further investigated. The same is true for the potential of chemical speciation to affect photolytic processes, which is unknown for cephalosporins under UV-C radiation. The applicability of chlorination for cephalosporins removal has also been investigated (Li et al., 2013; Li and Zhang, 2013). Li and Zhang (2013) showed fast removal of cefotaxime and cefalexin during municipal wastewater chlorination. The cephalosporins demonstrated high reactivity with all free chlorine doses used, even in the presence of interfering matrix constituents such as ammonia and suspended solids. Likewise, Li et al. (2013) presented the effects of chlorination on cefazolin degradation and genotoxicity. In this case, both pH and disinfectant dose played a role on genotoxicity increase, which was assessed by *Salmonella typhimurium* essays. In all studies, the target antibiotics were totally degraded. However, as properly identified by Li et al. (2013), the formation of chlorinated by-products is an important limitation of this technique and needs to be scrutinized further.

Compound charge is intrinsically related to nanofiltration efficiency and it has been evidenced for multifunctional cephalosporins (Sun et al., 2012). The changes of pH values and the membrane pore size demonstrated to be critical for the removal of cephalexin from aqueous solutions via nanofiltration (Sun et al., 2012). The effects of cefalexin dissociation were also reported for ozone, where the anionic cephalexin (deprotonated carboxylic acid) presented a slight higher depletion rate than the zwitterionic species (Dodd et al., 2006; Ribeiro and Schmidt, 2017). A hybrid system of ozonation and membrane filtration successfully reduced the concentration of ceftazidime spiked in surface water to around 100% (Alpatova et al., 2013). The use of ozone and matrix composition (i.e. alkalinity) played a significant role in the treatment efficiency, possibly due to hydroxyl radicals scavenging. However, no information on pH effects on the process or antimicrobial inactivation was reported.

The works recently carried out by Guo and Chen (2015) and Alexandrino et al. (2017) highlighted the need of measurement of hydrolysis and photolysis degradation during cephalosporins treatment. As reported by these authors, hydrolysis and photolysis contributed with around 17% (Guo and Chen, 2015) and 40% (Alexandrino et al. 2017) of the observed cephalosporin degradation. Guo et al. (2016) reported similar results during the removal of 7-amino cephalosporinic acid (7-ACA) by lipid-rich microalga. Besides the absorption onto microalgae, removal pathways included hydrolysis and photolysis, which contributed with around 30% of the observed compound abatement. As raised above, abiotic degradation of cephalosporins during treatment technologies has been partially neglected, which compromises the achieved removal data in long-term processes, especially when an alkaline environment is used.

3.8. Conclusions and future challenges

Cephalosporin antibiotics have been presented in the literature as a minor harm to the aquatic environment. However, this work raises the need of further investigation to confirm this hypothesis. Beside the importance of cephalosporins in bacterial resistance not approached in this document, information is too limited about cephalosporins release, persistence, transformation and ecotoxicity. Efforts have to be done in the direction of effective control and data disclosure of cephalosporins consumption, providing statistical analysis especially in densely populated (humans and livestock) countries. It is also necessary to determine the fate of cephalosporins in the environment, including chemical speciation (pK_a) effects, persistence in soil and aqueous matrices (degradation rates and $t_{½}$) and the importance of abiotic processes (i.e. photolysis and hydrolysis) in the decay of these compounds both at environmental conditions and during abatement strategies. In engineered technologies, future investigations also need to assess the responses of cephalosporin TPs on sensitive and resistant bacteria, in order to confirm biological inactivation and minimize the risks of bacterial resistance acquirement. Moreover, a direction for microbiologists and ecotoxicologists interested in the study of cephalosporins and their TPs would be the assessment of effects on cyanobacteria, biofilms and rhizosphere community as well as bacteria-mediated effects (i.e. digestive microbiota) on

aquatic (freshwater and marine fauna) and soil microorganisms. These topics are relevant due to both the reported occurrence of cephalosporins in the environment and the ecologic risks that need to be further explored. Given these facts, this review brings up cephalosporins as an aquatic contaminant group partially neglected in the literature, presenting potential and needs for further investigations.

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4. Determination of acid dissociation constants (pK_a) of cephalosporin antibiotics: computational and experimental approaches

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4.1. Abstract

Cefapirin (CEPA) and ceftiofur (CEF) are two examples of widely used veterinarian cephalosporins presenting multiple ionization centers. However, the acid dissociation constants (pK_a) of CEF are missing and experimental data about CEPA are rare. The same is true for many cephalosporins, where available data are either incomplete or even wrong. Environmentally relevant biotic and abiotic processes depend primordially on the antibiotic pH-dependent speciation. Consequently, this physicochemical parameter should be reliable, including the correct ionization center identification. In this direction, two experimental techniques, potentiometry and spectrophotometry, along with two well-known pK_a predictors, Marvin and ACD/Percepta, were used to study the macro dissociation constants of CEPA and CEF. Additionally, the experimental dissociation constants of 14 cephalosporins available in the literature were revised, compiled and compared with data obtained *in silico*. Only one value was determined experimentally for CEF (2.68 ± 0.05), which was associated to the carboxylic acid group deprotonation. For CEPA two values were obtained experimentally: 2.74 ± 0.01 for the carboxylic acid deprotonation and 5.13 ± 0.01 for the pyridinium ring deprotonation. In general, experimentally obtained values agree with the *in silico* predicted data (ACD/Percepta RMSE: 0.552 and Marvin RMSE: 0.706, n=88). For the biological and environmental fate and effect discussion, it is important to recognize that CEPA and CEF, as well as many other cephalosporins, are present as anionic species in the biologic and environmentally relevant pH values of 6 - 7.5.

4.2. Introduction

Cefapirin and ceftiofur are two cephalosporin antibiotics mainly used in the veterinarian clinic and are related to important agricultural activities. Cefapirin is used to treat mastitis, one recurrent intramammary infection that leads to relevant economic impacts in the milk industry (Sadeghi-Sefidmazgi et al., 2011). Likewise, a large range of respiratory, urinal and dermatologic diseases are treated by ceftiofur usage (Salmon et al., 1996). Due to their use as last alternatives to combat infections associated with Gram-negative bacteria and due to their expected pressure on antimicrobial resistance acquirement, both compounds have been highlighted as important antimicrobials by the World Health Organization (WHO, 2012).

Just as in penicillins, the beta-lactam ring located in the cephalosporin core structure is the main responsible unit for the antimicrobial activity of those drugs. Indeed, it affects the peptidoglycan system leading to bacterial cell wall disruption. Table 4.1 shows the chemical structures of ceftiofur and cefapirin, emphasizing the 7-aminocephalosporanic acid, as known as cephem ring, as well as the substituents R_1 and R_2, which are responsible for both the pharmacokinetic and antibacterial spectrum.

As other antibiotics (Qiang and Adams, 2004), cephalosporins possess a complex chemical structure with diverse functional groups and multiple ionizable moieties. Beyond the analytical point of view (Lin et al., 2000), the degree of ionization also plays an important role in biologic and environmental processes. For example, it affects antimicrobial activity and skin permeation (Hatanaka et al., 1995); the complexation with metals and mineral sorption (Peterson et al. 2009) as well as the photolysis rate (Wang and Lin, 2012). In truth, few authors take into account speciation when reporting and discussing pH dependent processes involving such complex organic compounds. However, Canonica et al. (2008) demonstrated the relevance of the pharmaceutical degree of ionization in the obtained photo-transformation rate constants.

Generally, the degree of ionization can be roughly indicated by the apparent or macroscopic dissociation constants, known as acid dissociation or pK_a values. However, in the case of this antibiotics class, this important physicochemical property is in most cases unreliable or

even unknown. In fact, cefadroxil, cefotaxime, cephalexin, cefepime and cefopirome are known zwitterionic cephalosporins presenting overlapping ionization processes (Streng, 1977; Mariño and Dominguez-Gil, 1981; Fabre et al., 1985; Hatanaka et al., 1995; Evagelou et al., 2003). Therefore, macro- and microscopic dissociation constants are expected for these drugs. Usually, macroscopic dissociation values can be experimentally obtained via potentiometric titration, spectrophotometry and capillary electrophoresis, among other techniques (Streng, 1977; Albert and Serjeant, 1984; Mrestani et al., 1998; Lin et al., 2000; Evagelou et al., 2003; Qiang and Adams, 2004; Andrasi et al., 2007). On the other hand, the complex ionization equilibria may be better explained by using microscopic ionization constants, which describe each specific process composing the observed macroscopic value. However, the former determination requires more refined and multifaceted investigations (Streng, 1977; Mariño and Dominguez-Gil, 1981; Albert and Serjeant; 1984).

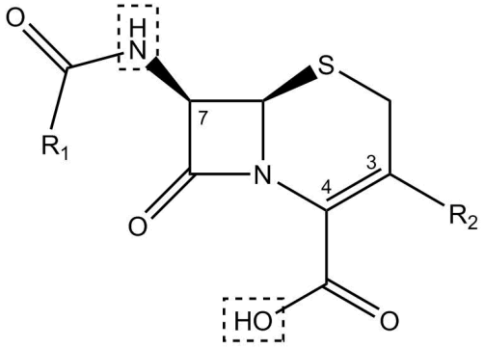
Ceftiofur has been broadly used for decades (Salmon et al., 1996) but, to the best of our knowledge, there is no analytical determination of its macro- neither microscopic dissociation constants. In contrast, two papers reported on the dissociation constants of cefapirin (Streng, 1977, Lin et al., 2000). Furthermore, available data about cephalosporin ionization are scarce, incomplete, have a low agreement or do not connect the obtained pK_a values to the correct ionization centeres. In fact, it is a hard task to compare and to discuss any experimental pK_a values obtained by different researchers once different analytical approaches and parameters can be used (Albert and Serjeant, 1984). In that direction, computational approaches (aka *In silico* prediction) come up as an useful tool for the study of cephalosporin dissociation constants.

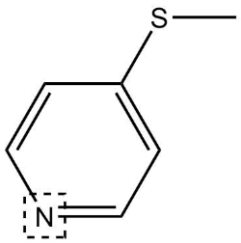
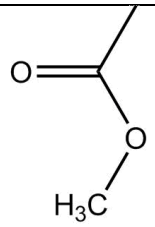
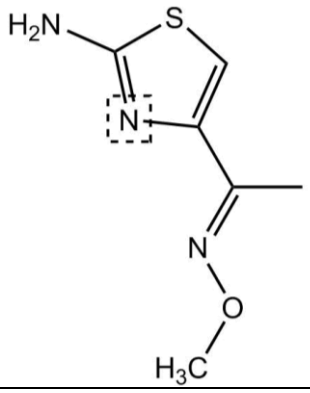
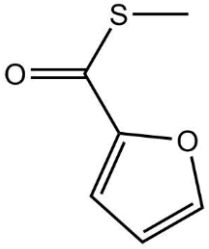
In this paper, we report the acid-base properties of cefapirin and ceftiofur using experimental and computational approaches. Aiming to confirm and fully understand the macroionization processes for those two important veterinarian antibiotics, potentiometry and spectrophotometry titration as well as two broadly and well-recognized pK_a predictors were used. Marvin (ChemAxon, 2016) and ACD/Percepta (Advanced Chemistry Development Inc., 2016) packages were utilized to estimate the macroionization constants of ceftiofur and cefapirin. Using the molecular structures as input, both software packages

estimate dissociation constants using empirical statistic algorithm derived from large compound collections with experimental pK_a data available (Meloun and Bordovská, 2007; Lee and Crippen, 2009; Manchester et al., 2010). Additionally, the available experimental dissociation constants of cephalosporins were revised, compiled and contrasted with the generated *in silico* data.

Table 4.1

Cefapirin and ceftiofur chemical structures and expected ionization centers



Antibiotic Abbreviation	Molecular Formula	**R_1**	**R_2**
Cefapirin CEPA	$C_{17}H_{17}N_3O_6S_2$		
Ceftiofur CEF	$C_{19}H_{17}N_5O_7S_3$		

4.3. Materials and methods

4.3.1. Chemicals

CEPA (CAS#24356-60-3, 98% purity) and CEF (CAS#104010-37-9, 98% purity), both in sodium salt form, were purchased from Sigma Aldrich (Seelze, Germany) and Santa Cruz Biotechnology (California, USA), respectively. Both compounds were diluted in ultrapure water and stock solutions of ≈ $2x10^{-3}$ M of each antibiotic were freshly prepared before each experiment. Hydrochloric acid (Fischer Chemical, Leics, UK), sodium hydroxide (Prolabo, Leuven, Belgium), sodium chloride, phosphoric acid (AppliChem, Darmstadt, Germany), acetic acid (Bernd Kraft, Duisburg, Germany) and potassium hydrogen phthalate (Merck, Darmstadt, Germany) were the main chemicals used. Citric acid monohydrate, boric acid, trisodium citrate, monopotassium phosphate and disodium phosphate dihydrate (Merck, Darmstadt, Germany) were also employed for buffer preparation. Standard pH buffers and potassium chloride solutions from Metrohm (Herisau, Switzerland) were employed for glass electrode conservation and calibration. In addition, all work solutions were prepared using ultrapure water (Elga Purelab flex, resistivity ≈15.3 MΩ-cm). All chemicals were of analytical grade.

4.3.2. Spectrophotometric titration

A Shimadzu-1650PC UV-Vis spectrophotometer running over the wavelength range of 180-500 nm was used for the analytical wavelength determination and for the spectrophotometric titration. At first, both antibiotics were diluted to $7x10^{-5}$ M in buffer solutions at pH 1, 6 and 12.5 and submitted to UV-Vis scan. Afterwards, the generated absorbance spectra were investigated. For both drugs, significant pH-dependent absorbance changes occurred at an absorption wavelength of 291 nm. Consequently, this wavelength was chosen as a suitable analytical wavelength. In other words, it was used to track the spectrophotometric titrations.

Afterwards, a set of buffer solutions ranging from pH 1 to 7 were prepared according to OECD 111 (2004). In addition, solutions with pH ranging from 1.5 to 12 were prepared using Universal buffer (0.1 M H_3PO_4, 0.1 M H_3BO_3 and 0.1 M CH_3COOH), which was

previously used for spectrophotometric titration of cephalosporins (Evagelou et al., 2003). Two mL of fresh cephalosporin stock solution was mixed with 3 mL of each buffer, resulting in a final concentration of $4x10^{-5}$ M and $4.7x10^{-5}$ M of CEF and CEPA, respectively, and 0.01 ionic strength. Next, each solution was inserted in 10 mm path-length quartz cuvettes and UV absorbance spectra recorded immediately. Buffers without antibiotic were used as reference. The whole procedure was carried out in triplicate and at room temperature (23 ± 2°C).

Using a plot of observed absorbance at the analytical wavelength 291 nm versus pH, the pK_a values were calculated. For that, the established second-derivative method was applied. Recently, Lin et al. (2000) successfully used this calculation method to determine the acid dissociation constants of several cephalosporins. The technique is concentration-independent and has shown an appropriate accuracy in our validating method experiments (see description below). Origin Pro9 was used for sigmoidal fitting when monoprotic acid profile was identified. In the other cases, the second-derivative were carried out without fitting. Mean pK_a and standard deviation were calculated according to Albert and Serjeant (1984) recommendations.

4.3.3. Potentiometric titration

Potentiometric measurements were carried out using an automatic Tiamo titrator system (Metrohm) equipped with a 20-mL automatic burette, an automatic stirrer, a glass pH-electrode, and a temperature electrode. Before and after each experiment, the glass electrode was calibrated with standard buffers of pH 4 and 9. This procedure aimed at reducing asymmetric potentials (Qiang and Adams, 2004), checking for alkali errors (Albert and Serjeant, 1984) and calculating the glass electrode accuracy, which was always higher than 97%. Several actions were made to reduce carbon dioxide interference. First, every solution was prepared with CO_2 free ultrapure water and immediately stored in tightly sealed glass vessels. Second, the titrant NaOH was daily standardized by potassium phthalate giving a mean concentration of 0.1005 M. Besides, all experiments were carried out in a 100 mL paraffin-covered beaker under nitrogen atmosphere and the automatic burette content was renewed before each run.

Whenever the system was checked, the plugs for gas and titrant purge, the glass and temperature electrodes and the automatic stirrer were fixed in the 100-mL beaker and the titrand solution was quickly added. This solution was freshly prepared by mixing 5 mL of analyte stock solution, 3 mL of 0.1 M HCl to protonate all ionizable moieties, 5 mL of 0.5 M NaCl to keep the 0.1 ionic strength constant and 17 mL of CO_2 free ultrapure water. To validate this analytical protocol, phosphoric acid was employed. This polyprotic acid with well-known pK_a values (2.2; 7.2 and 12.3) can be used as titration reference (Qiang and Adams, 2004). To that end, a stock of 0.1 M H_3PO_4 was prepared and titrated before any antibiotic analysis, according to the method described above. Using the second-derivative, the following pK_a values were obtained: 2.2 ± 0.01; 7.0 ± 0.04 and 12.0 ± 0.01 (n=3). Considering that certain error is expected when pH readings are higher than 11 (Albert and Serjeant, 1984), the present method showed an acceptable ± 0.2 pK_a value accuracy. Finally, each cephalosporin at final concentration of $5.8x10^{-5}$ M was titrated at least in triplicate and under controlled room temperature (23 ± 2°C). pK_a values were calculated using the $d^2(pH)/dV^2$ of the plot pH versus volume of titrant (in mL).

4.3.4. Computational prediction

Marvin version 16.2.29 (ChemAxon, 2016) and ACD/Percepta version 2015 (Advanced Chemistry Development Inc., 2016) were used for pK_a estimation of CEF and CEPA. Additionally, these programs were used to predict the macro dissociation constants of fourteen cephalosporins, which possess experimentally determined pK_a values. From the available literature, we selected only data generated under both controlled temperature and ionic strength (I), factors that have significant influence in this parameter (Albert and Serjeant, 1984). Marvin and ACD/Percepta were used due to their significant accuracy and fast performance compared with other estimation packages (Meloun and Bordovská, 2007; Manchester et al., 2010). Based on empirically determined partial charges, Marvin provides micro and macro ionization constants as well as the ionic species distribution diagram (Szegezdi and Csizmadia, 2004). ACD/Percepta pK_a predictor works by using a pre-defined set of ionization centers with associated core pK_a microconstants and a database of various interaction constants and interaction calculation methods (Advanced Chemistry

Development Inc., 2016). In our study, the chemical structure of each antibiotic was analyzed using standard temperature (25°C) and zero ionic strength. Marvin was run in macro mode and ACD/Percepta was run in the GALAS algorithm mode, which provides more details about macro-ionization when compared with the Classic mode (Advanced Chemistry Development Inc., 2016). The ionic species distribution in solution of each drug, the obtained pK_a values and their corresponding ionization centers were tabulated and compared with the available literature values. To assess the performance of the *in silico* models, the root-mean-squared error (RMSE) of each single predicted pK_a value versus each corresponding experimental data was calculated.

4.4. Results and discussion

4.4.1. Spectrophotometric titration

As the first step, we had obtained the analytical wavelength 291 nm for CEPA and CEF by diluting the drugs in hydrochloric acid, sodium hydroxide and ultrapure water. CEPA shows a notable spectral variation upon pH changes (see Figure 4.1a). At alkaline and neutral pH the maximum absorbance lies around 259 nm. However, there is a bathochromic shift to 291 nm at acidic pH. In addition, this antibiotic presented higher absorbance intensity at pH 1 when compared to other pH values. For CEF, the absorbance changes were more subtle (Figure 4.1b). Although intensity changes were observed in the peaks at 291 nm and 225 nm, they were smaller than 0.4 absorbance units. Once the pH decreases, the intensity at 291 nm increases. On the other hand, the peak at 225 nm remains stable at pH 12.5 and 6, however it had a subtle decrease at pH 1.

Supplementary studies were done to check if the observed spectral changes were due to ionization instead of degradation. Similarly to the available literature (Berendsen et al., 2009), CEPA and CEF were stable in the pH range 1 - 9, but showed significant degradation at highly alkaline pH (CEF 50% and CEPA 20% degradation after 30 minutes at pH 12.5, data not shown). Considering this behavior, all experiments above pH 9 were carried out promptly.

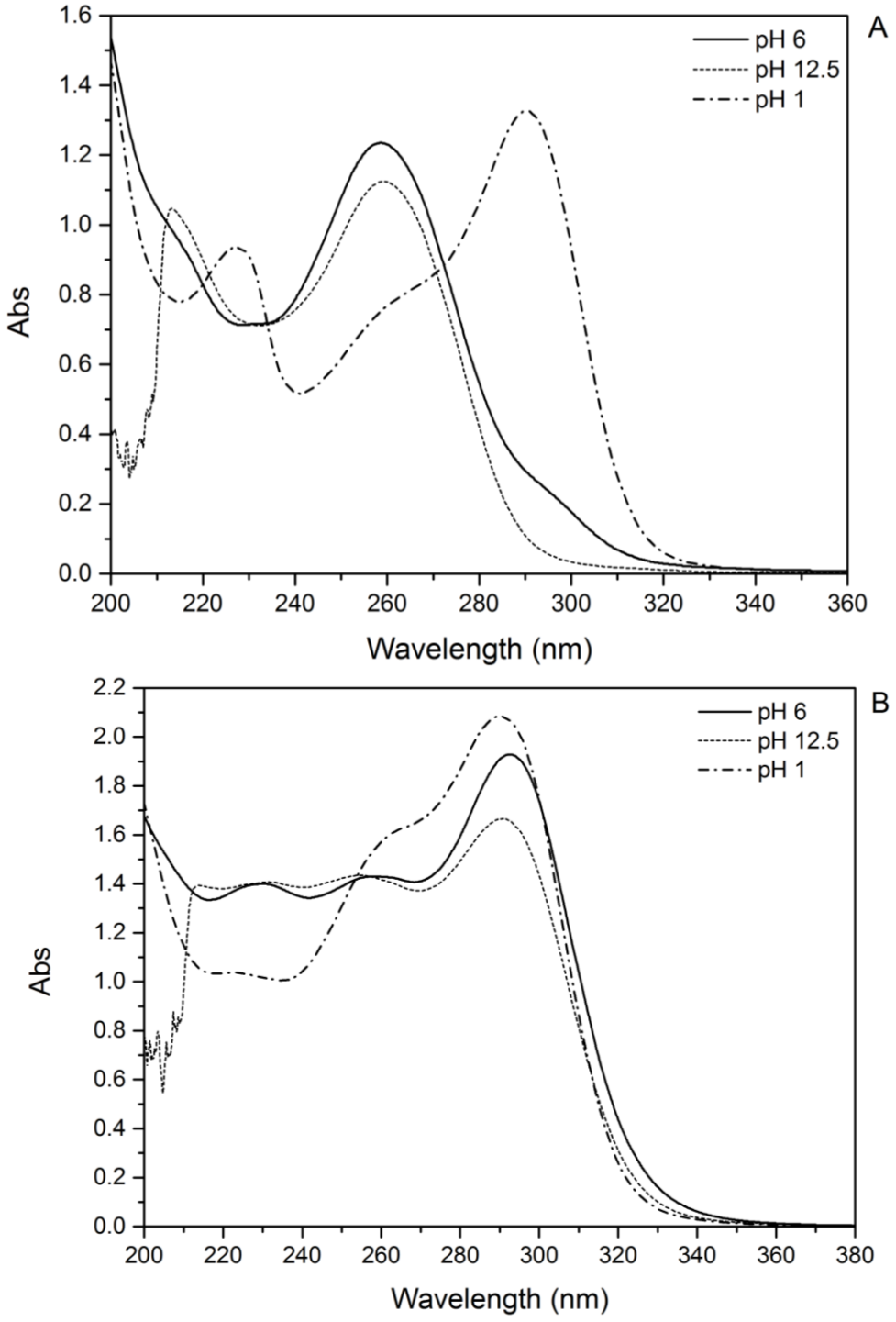


Figure 4.1. UV absorption spectra of $7x10^{-5}$ M CEPA (A) and CEF (B) at different pH values.

According to the plot absorbance at 291 nm versus pH it was possible to determine one pK_a value for CEPA (5.13 ± 0.01), which can be associated to the pyridinium group (pK_{a2}) located at the R_1 substituent of this drug. Between the pH range 1.5 – 7 the observed titration curve exhibited a monoprotic acid profile (Figure 4.2). Although two equivalent points may be expected due to the carboxylic and pyridine group ionizations, it is known that the protonated and deprotonated species of carboxylic acid have very similar absorbance spectra (Albert and Serjeant, 1984). So, spectrophotometric titration by itself is not recommended to study dissociation of this moiety. As in our spectrophotometric experiments, Streng (1977) used the maximum absorptions at 258 nm and 290 nm to follow the pyridine ring protonation, whereas potentiometry was employed for the carboxylic pK_a determination. As a result, he reported two p*K*a values for CEPA: 1.83 (carboxylic) and 5.48 (pyridine). Lin et al. (2000) obtained only one pK_a value for CEPA, which was associated to the pyridine functional group. In this study, the drug presented a sigmoidal electrophoretic mobility in citrate buffer in the pH range 4 – 6.4, resulting to pK_{a2} values 4.72 and 4.65 according to the calculation method used. Although the authors did not discuss it in the manuscript, we believe that the pyridine ring protonation at acid pH as well as its dissociation masked the carboxylic acid ionization, making it undetectable. In Gennaro's (1990) reference book, two pK_a values for CEPA were reported: 2.15 and 5.44. This second value is believed to be the pK_{a2}, related to the pyridine functional group. Considering the expected error of our results (± 0.2 pK_a units) and the analytical differences between the author procedures, the obtained values agreed satisfyingly.

It is known that many cephalosporins are zwitterions (Streng, 1977; Fabre et al., 1985; Evagelou et al., 2003). As Figure 4.2a shows, the large shift from long to medium wavelength spectra presented by CEPA under alkaline conditions confirm the zwitterionic behavior of this compound (Albert and Serjeant, 1984).

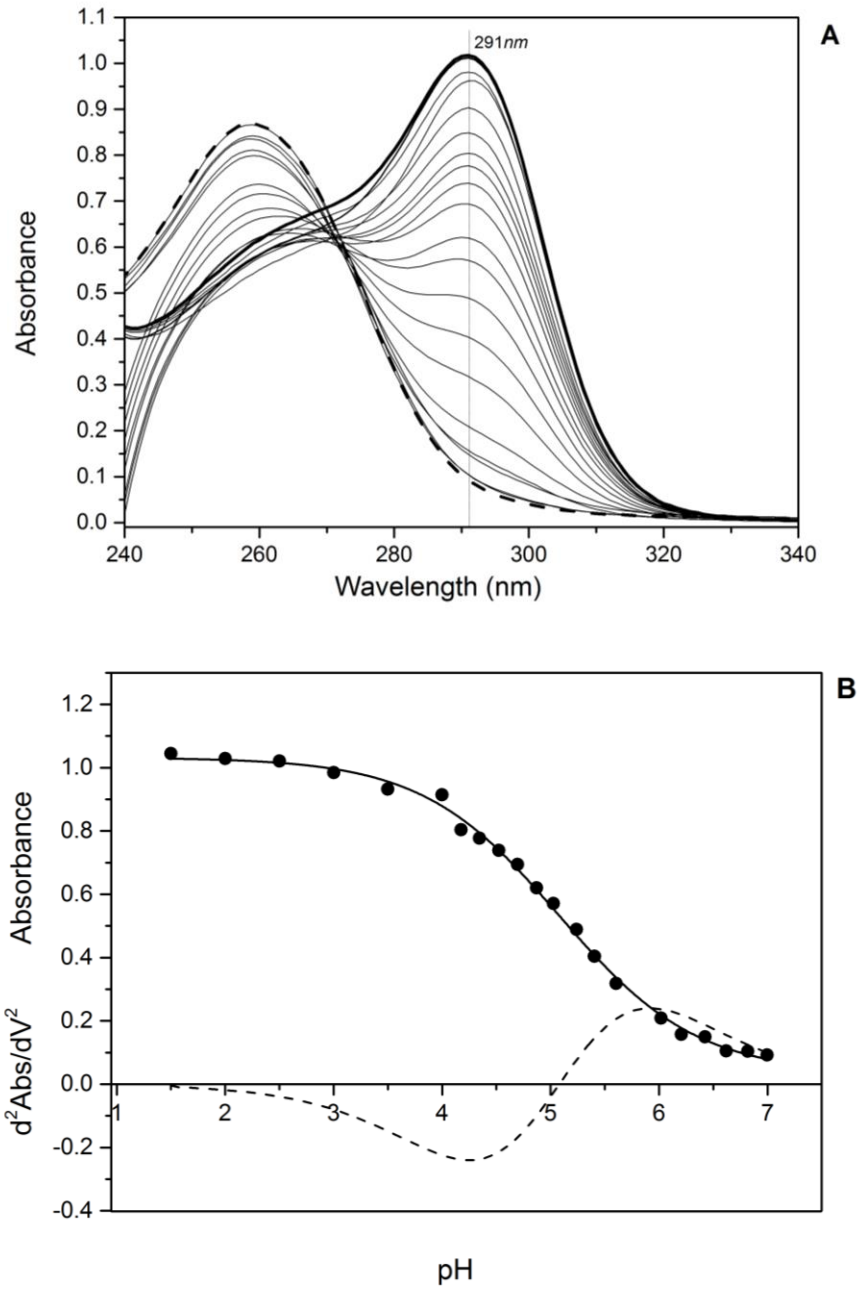


Figure 4.2. Spectrophotometric titration of CEPA. A) UV absorption spectra of 4.7×10^{-5} M CEPA in the pH range 1.5 (thick black curve) - 7 (dashed curve). The vertical line indicates the analytical wavelength (291 nm) chosen for pK_a calculations. B) Absorbance of CEPA at 291 nm versus pH and second-derivative curve (dashed line). The inflection point indicates the obtained mean pK_{a2} at 5.13 ± 0.01.

Two different buffer systems in the pH range 1 – 12 were used for the CEF spectrophotometric titration. However, no pK_a value could be obtained using this technique. As indicated during the analytical wavelength determination (Figure 4.1b), absorbance variations were very small. Therefore, even using two wavelengths (225 and 291 nm) and sigmoidal curve fitting no acid profile was observed. Moreover, the chemical structure of CEF is very similar to cefepime and cefpirome, presenting three expected ionization centers: one carboxylic group at C-4; an amide group at C-7 and an aminothiazole ring as R_1 substituent (Table 4.1). As main difference, cefepime and cefpirome possess quaternary nitrogen at C-3, which is constantly charged. Although there is no literature reference for CEF dissociation behavior, the chemical similarity of these three cephalosporins suggests similar pK_a values. In fact, Evagelou et al. (2003) determined the dissociation constants of cefepime and cefpirome. Using UV spectrophotometry, they derived the following pK_a values: 3.00 and 10.60 for cefepime and 3.04 for cefpirome. The values near to 3.00 were associated to the aminothiazole ionization (pK_{a2}), whereas the second was linked to the amide group (pK_{a3}). As in our results with CEPA, these authors could not detect the carboxylic ionization via UV.

4.4.2. Potentiometric titration

Plotting d^2pH/dV^2 against pH, dissociation constants for CEPA and CEF were determined. Potentiometric titration experiments resulted in one equivalent point for each cephalosporin. Hence, the mean pK_a values 2.74 ± 0.01 and 2.68 ± 0.05 were determined for CEPA and CEF (n=3), respectively. In fact, CEFA and CEF showed very similar titration curves. As exemplified in Figure 4.3, they presented a monoprotic acid profile under our experimental conditions.

Although the expected polyprotic behavior of CEF and CEPA may lead to incorrect attribution of pK_a to its respective ionization center, we suggest that the obtained values are representing the carboxylic acid functional group dissociation (pK_{a1}), which also agrees with the available literature. Typically, values reported for pK_{a1} of cephalosporin antibiotics are in the range 1.12 – 3.15 (Table 4.2). For CEPA, the reported pK_{a1} values are 1.85 (Streng, 1977) and 2.15 (Gennaro, 1990). The differences may be explained by different

temperature, ionic strength and analytical methods used. The accuracy of the method employed in this work was checked by phosphoric acid titrations. As mentioned before, a ± 0.2 pK_a value deviation may be expected in our results.

To the best of our knowledge, no experimentally obtained dissociation constants have been reported previously for CEF. For cefepime and cefpirome, two drugs with similar structure, pK_{a1} values of 1.12 and 1.62 were determined (Evagelou et al., 2003). These values are lower than our results. However, deviations may be expected since pK_a values can be affected by other substituents as well as by other analytical parameters like ionic strength and temperature (Albert and Serjeant, 1984). Ionic interactions between the carboxylic acid and the constantly charged quaternary nitrogen of cefepime and cefpirome (see chemical structures at Table S4.2, Supplementary Information) may justify this observed variation.

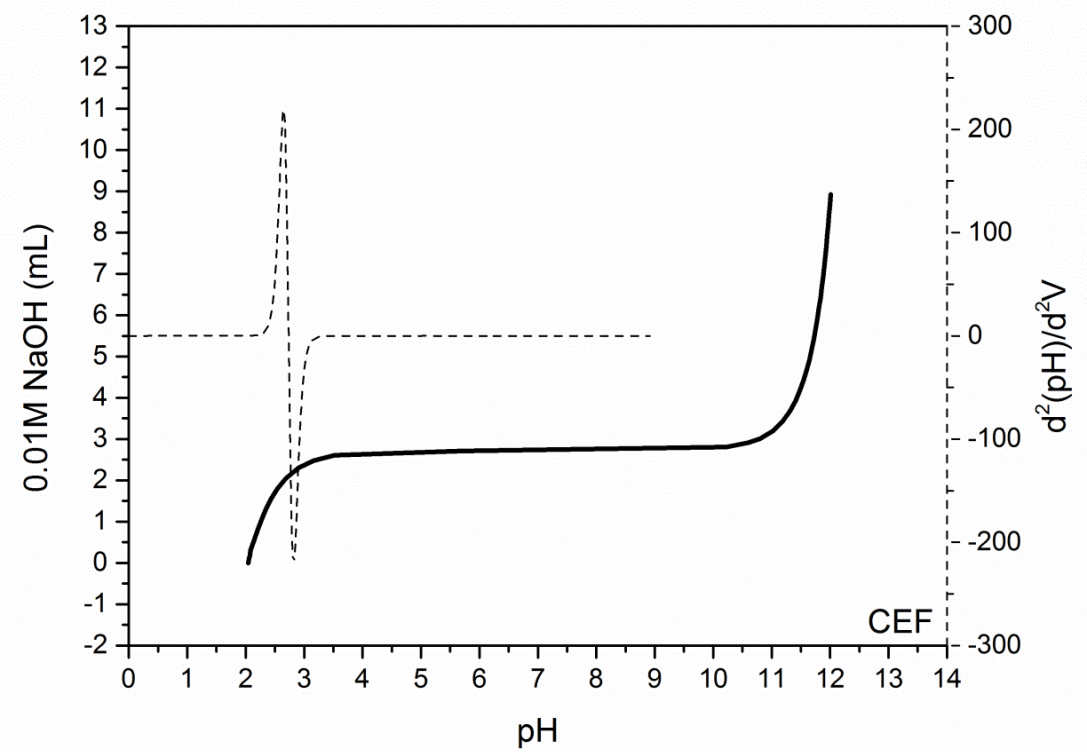


Figure 4.3. Potentiometric titration of 5.8×10^{-5} M CEF (solid curve) and second-derivative plot (dashed curve). The inflection point indicates the obtained mean pK_{a1} 2.68 ± 0.05.

4.4.3. Computational prediction

We have shown before the pK_{a1} and pK_{a2} of CEPA as well the pK_{a1} of CEF. Similarly, the macro dissociation constants of these two drugs were calculated according to the chemical structure analyzed by two reliable pK_a predictors (Table 4.2). For CEPA, ACD/Percepta generated the following pK_a values: 2.30 to the C-4 carboxylic acid, 5.30 to the R_1 pyridine ring and 10.60 to the amide bound to C-7. In the case of CEF, 2.40 were associated to the C-4 carboxylic, 3.60 to the aminothiazole in the R_1 and 10.70 to the C-7 bound amide. This predictor was run using the GALAS model, which uses an internal training set of >20,000 individual pK_a values and gives more detailed information about micro- and macrospeciation (Manchester et al., 2010; Advanced Chemistry Development Inc., 2016).

Marvin running in the macro mode associates 3.54 (carboxylic acid deprotonation), 5.00 (pyridinium deprotonation) and 11.54 (amide deprotonation) for CEPA. Likewise, for CEF, 2.83 and 10.75 were predicted to the carboxylic acid and to the amide, respectively, and 4.19 to the imine in the R_1. Marvin pK_a predictions are based on the calculated partial charge of the atoms located in the analyzed structure, using a Hammett-Taft approach (Manchester et al., 2010; ChemAxon, 2016). In fact, this program showed a good agreement (RMSE: 0.72) in the pK_a prediction of 1670 organic compounds and pharmaceutical molecules (Szegezdi and Csizmadia, 2004).

Besides macro dissociation constants, the ionic species distribution chart of CEPA and CEF were also predicted using the software ACD/Percepta (Figure 4.4). The most significant species (>10% in solution) were obtained for cefapirin and ceftiofur. For CEPA, once the solution pH increases the following ionization sequence was obtained: $CEPA^+$ (pyridine ring protonated) > $CEPA^{+/-}$ (pyridine ring protonated and carboxylic acid deprotonated) > $CEPA^-$ (carboxylic acid deprotonated) > $CEPA^{2-}$ (carboxylic acid and amide deprotonated). Similarly, for CEF the following sequence was suggested: CEF^+ (protonated aminothiazole) > $CEF^{+/-}$ (protonated aminothiazole and deprotonated carboxylic acid) > CEF^- (carboxylic acid deprotonated) > CEF^{2-} (carboxylic acid and amide deprotonated). Schemes 4.1 and 4.2 summarize the main ionic speciation of CEPA and CEF, respectively. According to the

distribution charts, both drugs behave mostly as an anion in environmental and biological relevant pH (6 – 7.5).

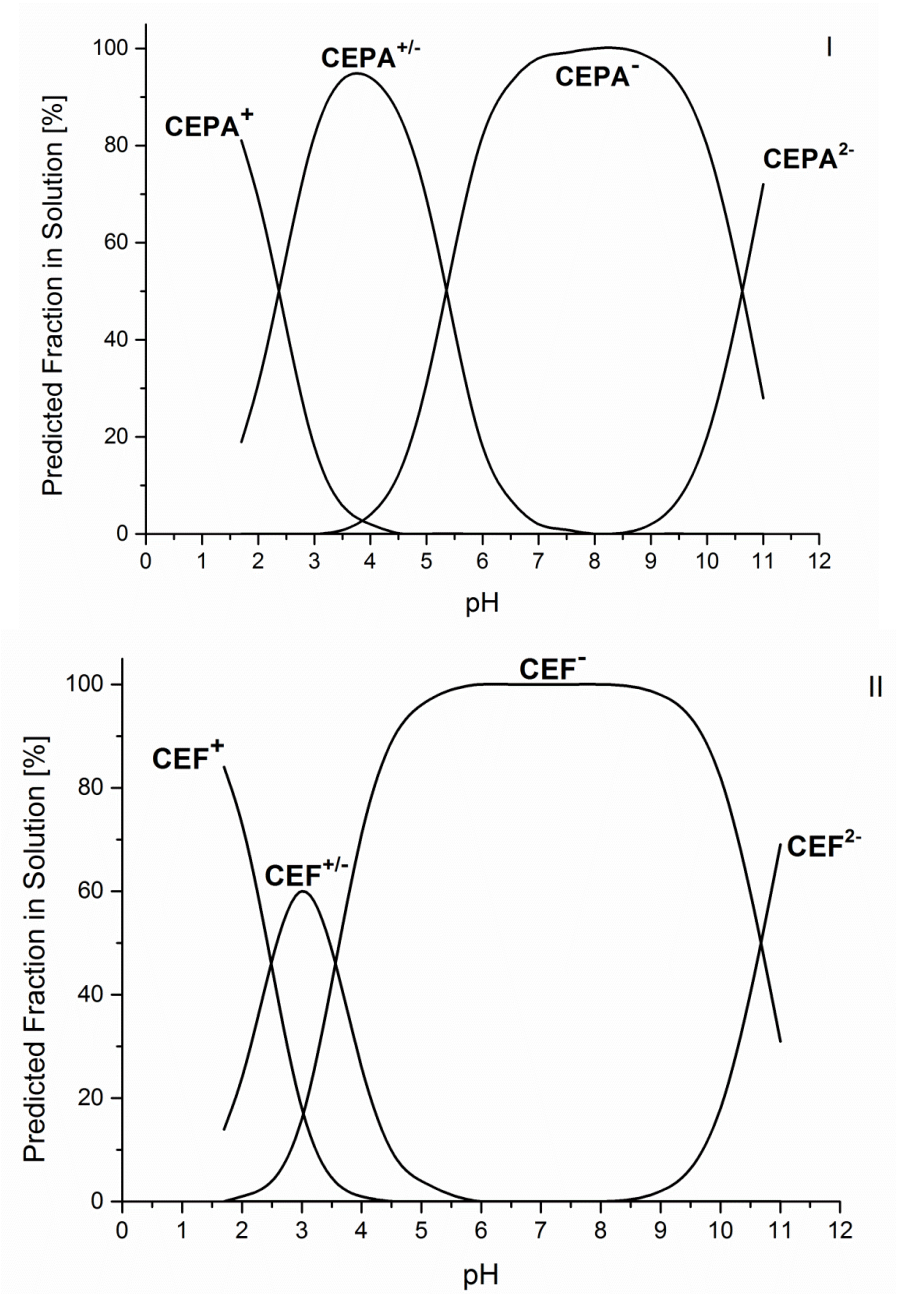
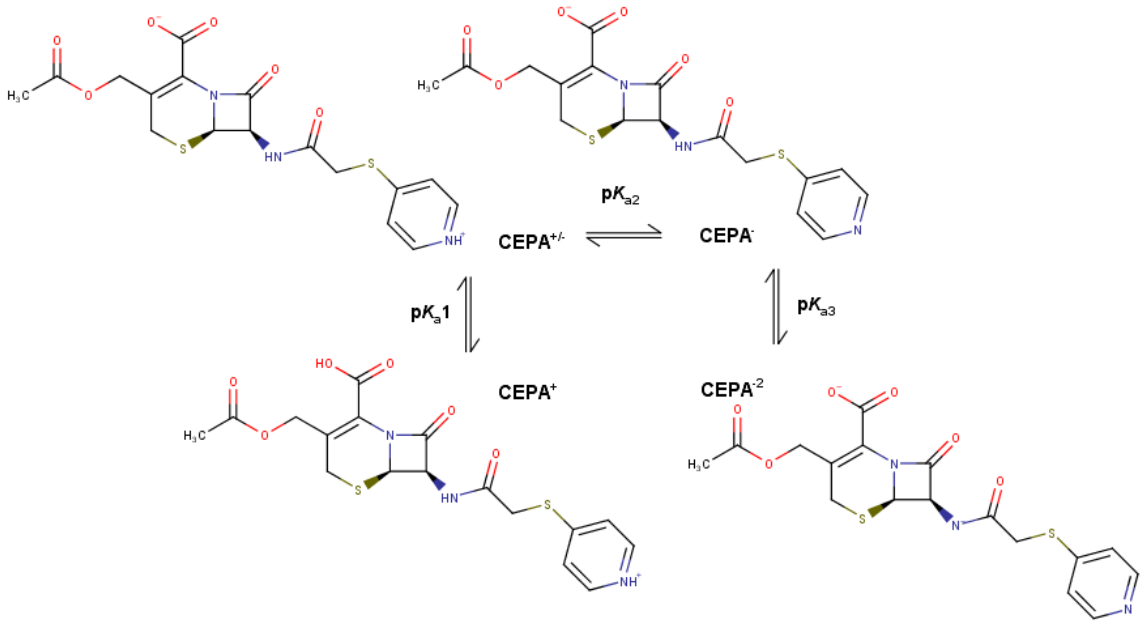
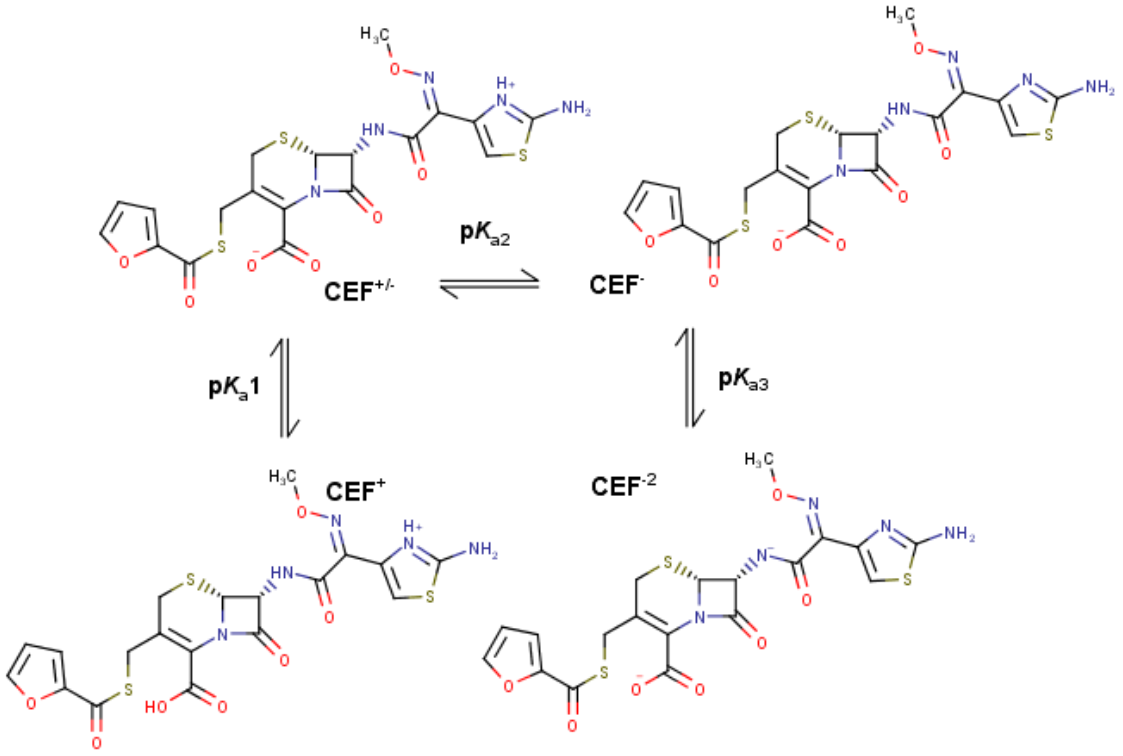


Figure 4.4. ACD/Percepta predicted speciation diagram of CEPA (I) and CEF (II) in solution as a function of pH.

We also performed the ionic species distribution analysis using Marvin and the obtained data were very similar to the ones obtained with ACD/Percepta (See Figure S4.4, Supplementary data). However, Marvin does indicate the dissociation of the cephen ring in high alkaline pH. Nevertheless, in the case of CEF, this program contradicts ACD/Percepta predictions, the latter predicts the protonation of the R_1 aminothiazole instead of the imine as the ionization center responsible for the CEF^+ and $CEF^{+/-}$ species formation. This finding will be further discussed below.



Scheme 4.1. Main ionic species of CEPA predicted by ACD/Percepta and Marvin



Scheme 4.2. Main ionic species of CEF predicted by ACD/Percepta

Additionally, experimentally determined pK_a values of several cephalosporins were compared with the predicted ones (Table 4.2). Marvin and ACD/Percepta predictors provided a complete overview of the dissociation of sixteen compounds (fourteen from literature plus our results with CEPA and CEF). Both software packages provide a good agreement of experimental and predicted values (Figure 4.5, Marvin RMSE: 0.706; ACD/Percepta RMSE: 0.552, N=88) and prove their applicability in the investigation of cephalosporin dissociations. Manchester et al., (2010) used both software packages to investigate 211 multiprotic druglike compounds and, as in our results, they observed a similar performance of ACD/Percepta (RMSE: 0.80) and Marvin (RMSE: 0.90). Nevertheless, the pK_a of some ionization centers were here for the first time reported. As examples, values for the deprotonation of the hydroxyphenyl group of cefadroxil, for the deprotonation of the amide of cephalexin and cefaclor as well as for the carboxyl acid and amide moieties deprotonation of cefradine were here identified (Table 4.2).

However, as mentioned above, conflicting data were observed for some compounds. For ceftriaxone, ceftazidime, cefepime, cefpirome, ceftamet, and cefotaxime the literature reported pK_a values disagree with the ones predicted by Marvin. In all previous studies, authors associated the obtained apparent pK_{a2} to the R_1 aminothiazole ionization center (Fabre et al., 1985; Mrestani et al., 1998; Evagelou et al., 2003; Aleksić et al., 2005, Andrasi et al., 2007). However, this software indicated another functional group present in these compounds, the R_1 imine, as the pK_{a2} ionization center.

As previously discussed, most cephalosporins are zwitterionic drugs with overlapping ionization (Streng, 1977). Consequently, macro and microionization processes are expected. The experimentally determined pK_a value, also known as apparent macroconstant, describes only part of a complex equilibrium. Moreover, macroconstants are composed of microconstants and this multifaceted relation was very well discussed previously (Albert and Serjeant, 1984). To check Marvin's algorithm was using the wrong ionization center for micro- and macro dissociation values predictions, this program was run in micro mode using the cefotaxime structure as an example of this controversial group. Following, the resulting ionization values for the R_1 imine (4.12) and the aminothiazole group (3.28) were close. Analyzing cefotaxime again in macro mode, the same aminothiazole presented a negative acid dissociation constant -0.31 while the imine 4.21. In fact, this result indicates that the overlapping ionization of these two groups is likely to create some problems in this program.

In this direction, it is important to point out that Fabre et al. (1985) and Aleksić et al. (2005) deeply investigated the overlapping constants of cefotaxime employing experimental work. Potentiometric titrations of this drug resulted in 3.40 as pK_{a2} (Fabre et al., 1985). Using the ultraviolet absorption at 235 nm, wavelength associated to the aminothiazole group, the same authors additionally obtained 3.33 as pK_{a2} value. Likewise, 3.15 was associated to the aminothiazole center by Aleksić et al. (2005) after potentiometric experiments. In general, all these experimental data had a good agreement and the R_1 imine group as one possible ionization center was never mentioned.

Table 4.2

Macroscopic acid dissociation constants (pK_a) of cephalosporin antibiotics: experimental and predicted data

Antibiotic Method	**pK_{a1}**	**pK_{a2}**	**pK_{a3}**	**pK_{a4}**	**Source**
Ceftriaxone	Carboxyl	Aminothiazole	Hydroxytriazinone	Amide	
Pot	2.37	3.03	4.21	10.74	[1]
Marvin	3.14	3.28*	5.58	10.96	
ACD/Percepta	2.50	3.50	4.20	10.70	
Cefadroxil	Carboxyl	Ammonium	Hydroxyphenyl	Amide	
Pot	2.48	7.37	9.64		[2]
CZE	2.52	7.65			[2]
Pot	2.65	7.59			[3]
CZE	2.86	7.14			[3]
Pot	1.40	7.50	10.00		[4]
Pot	1.38	7.55	10.10		[4]
Marvin	3.45	7.22	9.48	11.97	
ACD/Percepta	2.80	8.60	10.00	11.40	
Ceftazidime	Carboxyl R_1	Carboxyl	Aminothiazole	Amide	
Pot		2.91	3.81		[3]
CZE		2.19	3.98		[3]
Marvin	3.38	2.77	3.28*	10.88	
ACD/Percepta	2.20	3.10	3.70	10.40	
Cefepime	Carboxyl	Aminothiazole	Amide		
Pot	1.12	3.07	10.80		[5]
Spec		3.00	10.60		[5]
Pot		3.03			[3]
CZE		3.36			[3]
Marvin	3.25	3.28*	11.15		
ACD/Percepta	0.90	3.30	10.20		
Cefpirome	Carboxyl	Aminothiazole	Amide		
Pot	1.62	3.11	10.80		[5]
Spec		3.04			[5]
Pot		3.04			[3]
CZE		3.10			[3]
Marvin	3.09	3.28*	10.80		
ACD/Percepta	2.90	3.60	10.20		
Cefetamet	Carboxyl	Aminothiazole	Amide		
Pot	2.93	3.07	10.65		[1]
Marvin	3.22	3.28*	11.07		
ACD/Percepta	2.50	3.60	10.70		
Cefotaxime	Carboxyl	Aminothiazole	Amide		
Pot	2.21	3.15	10.87		[1]
Pot	2.30	3.37			[2]
CZE		3.20			[2]
Pot	2.90				[3]
CZE	2.09				[3]
Pot	2.10	3.40	10.90		[6]
Spec		3.33			[6]
Marvin	3.22	3.28*	11.07		
ACD/Percepta	2.10	3.60	10.60		

Continuation of Table 4.2

Cefalexin	Carboxyl	Ammonium	Amide		
Pot	2.53	7.13			[2]
CZE	2.93	7.18			[2]
Pot	2.34	7.08			[3]
CZE	3.11	6.79			[3]
Pot	2.71	6.62			[7]
CZE		6.96			[8]
Pot	2.53	7.14			[9]
Marvin	3.45	7.23	11.91		
ACD/Percepta	2.80	7.40	10.00		
Cefaclor	Carboxyl	Ammonium	Amide		
Pot		7.07			[2]
CZE	1.74	7.19			[2]
Pot		7.19			[3]
CZE	2.69	7.38			[3]
CZE		6.92			[8]
Marvin	3.03	7.23	11.65		
ACD/Percepta	1.60	7.30	9.80		
Cefradine	Carboxyl	Ammonium	Amide		
CZE		7.27			[8]
Marvin	3.47	7.59	11.99		
ACD/Percepta	2.80	8.40	10.60		
Cefoperazon	Carboxyl	Hydroxyphenyl	Amide R_1	Amide	
Pot		9.15			[2]
CZE	3.13	8.99			[2]
Marvin	3.38	9.47	12.33	11.34	
ACD/Percepta	2.60	9.40	11.50	13.30	
Cefoxitin	Carboxyl	Amide			
Pot	2.75				[2]
CZE	3.15				[2]
Marvin	3.59	10.96			
ACD/Percepta	2.80	11.2			
Cefamandole	Carboxyl	Amide			
Pot	2.60				[3]
CZE	2.46				[3]
Marvin	3.32	11.25			
ACD/Percepta	2.60	10.8			
Cefuroxime	Carboxyl	Amide			
Pot	2.17				[3]
CZE	2.04				[3]
Marvin	3.15	10.97			
ACD/Percepta	2.90	10.6			
Cephapirin	Carboxyl	Pyridine	Amide		
CZE		4.72			[8]
CZE		4.65			[8]
Pot	1.85				[10]
Spec		5.44			[10]
Spec		**5.13**			This work
Pot	**2.74**				This work
Marvin	3.54	5.00	11.54		
ACD/Percepta	2.30	5.30	10.60		

Continuation of Table 4.2

Ceftiofur	Carboxyl	Aminothiazole	Amide	
PT	**2.68**			This work
Marvin	2.83	3.28*	10.75	
ACD/Percepta	2.40	3.60	10.70	

Notes:

Respective ionization center deprotonation: Carboxyl acidic $COOH \rightarrow COO^-$; Aminothiazole $NH^+ \rightarrow N$; Hydroxyphenyl $OH \rightarrow O^-$; Hydroxytriazinone $NH \rightarrow N^-$; Ammonium $NH_3^+ \rightarrow NH_2$; Pyridine $NH^+ \rightarrow N$; Amide $NH \rightarrow N^-$.
Pot – Potentiometry
CZE – Capillary zone electrophoresis
Spec – Spectrophotometry
Marvin – ChemAxom's pK_a predictor
ACD/Percepta – ACD/Labs' pK_a predictor
I – Ionic strength
* For this predictor, this value was presented as microconstant

(1) Aleksić et al., 2005; I = 0.1, 25 ± 0.1°C
(2) Andrasi et al., 2007; I = 0.2, 25 ± 0.1°C
(3) Mrestani et al., 1998; I = 0.16, 25.1°C
(4) Mariño and Dominguez-Gil, 1981; I= 0.05, 20 ± 2°C
(5) Evagelou et al., 2003; I= 0.15, 25 ± 8°C
(6) Fabre et al., 1985; I = 0.5, 20 ± 1°C
(7) Hatanaka et al., 1995, I= 0.5, 37°C
(8) Lin et al., 2000; I= 0.1, 25°C
(9) Völgyi et al., 2007; I = 0.15, 25 ± 0.5°C
(10) Streng, 1977; I = 0.1, 25 ± 2°C
Predictions: I=0, 25°C
This work: I=0.01 UV and 0.1 P, 23 ± 2°C

In fact, ACD/Percepta result also corroborates the experimental data and did not indicate the R_1 imine of cefotaxime as the pK_{a2} ionization center. According to ACD/Percepta calculations, the aminothiazole group is the one responsible for this pK_a value. Therefore, our results indicate an error when Marvin predicts overlapping values of compounds possessing imine and aminothiazole as R_1 ionizable groups. Considering that CEF possesses these controversial moieties, the data obtained for this drug using Marvin predictions were revised. We have replaced the macro dissociation value predicted for the imine (4.19) for the micro dissociation value predicted for the aminothiazole group (3.28) as final pK_{a2} of CEF. The same was done for the similar drugs as indicated in Table 4.2.

In fact, the inaccuracy demonstrated here by Marvin may be corrected by using the training logarithm presented in this software (ChemAxon, 2016). This program presents the option of creating a correction gallery using a roll of experimental data and known ionization centers. Unfortunately, for build a robust model, a bigger set of experimental data about cephalosporin pK_a would be necessary.

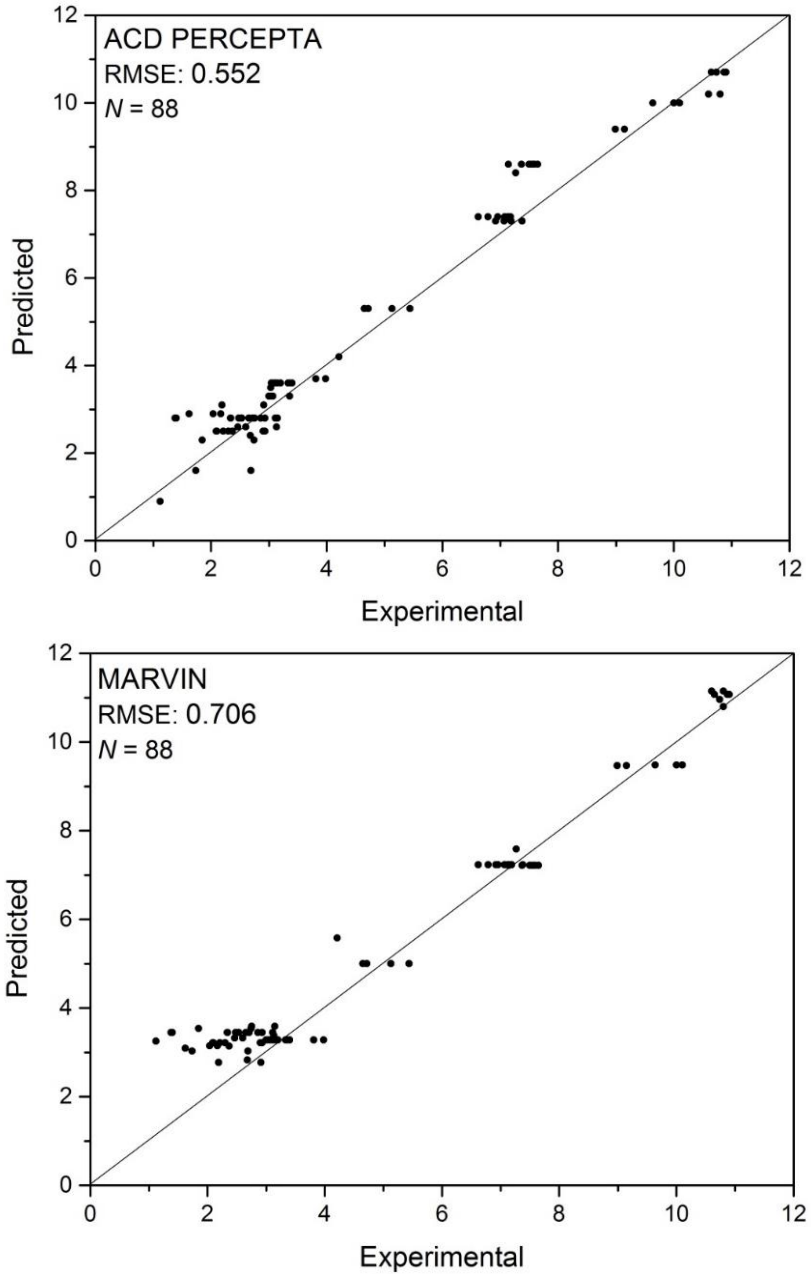


Figure 4.5. Predicted versus experimental dissociation constants of cephalosporin antibiotics. Each single experimentally determined pK_a was compared with its respective predicted value. The similarity between both set of values was assessed via the root-mean-squared error (RMSE), for each predictor.

4.5. Conclusions

The use of experimental along with computational approaches has shown to be useful for the reliable determination of dissociation constants of two relevant cephalosporin antibiotics. We reported the first experimental pK_a value for ceftiofur as well as confirmed the previously published values for cefapirin. Our data help to discuss results of environmental studies like the ones reported by Wang and Lin (2012). Although these authors mixed up the pK_a values with their respective ionization centers, it was observed that the protonation of the pyridinium ring and the deprotonation of the carboxylic acid of CEPA clearly affected the obtained pseudo-first-order photolysis rate constants. In the same direction, the pseudo-first-order photolysis rate constant of CEF presented by Kim et al. (2009) can now be assigned to the transformation of the anionic form of this drug.

In addition, we demonstrated *In silico* prediction as an useful tool for the determination of cephalosporin dissociation constants. Nevertheless, we indicate that caution must be taken when Marvin predictor is used to evaluate drugs having imine and aminothiazole groups as substituents. Finally, all available experimental pK_a values of cephalosporin antibiotics were for the first time revisited**.**

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5. Base-catalyzed hydrolysis and speciation-dependent photolysis of two cephalosporin antibiotics, Ceftiofur and Cefapirin

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5.1. Abstract

Lately, special attention has been given to veterinary cephalosporin antibiotics due to their broad activity spectrum and significant consumption. Indeed, the determination of hydrolytic and photolytic kinetics provides a better comprehension of the undesired persistence of cephalosporins in aqueous matrices. In this work, the two widely used veterinary antibiotics ceftiofur (CEF) and cefapirin (CEPA) showed high instability under alkaline conditions, degrading in few minutes at pH >11. In buffered solutions at neutral pH and natural temperature (T = 22 ± 1°C), both drugs presented moderate stability (t½ = 1.4 h – 3.7 d, respectively). Our study also demonstrated that CEPA and CEF speciation did not significantly influence the direct photolysis rates. Using a simulated water disinfection set-up (λ = 254 nm), all ionic species of CEF and CEPA presented fast and similar pseudo-first order degradation rates, k^{app} 0.0095 ± 0.0004 and 0.0092 ± 0.001 mJ cm^{-2}, respectively. Furthermore, using surface water in hydrolysis experiments, CEF demonstrated significant matrix-dependent stability with a half-life (t½ = 14.7 d) tenfold higher than in buffered solutions. In contrast, CEPA presented a very similar hydrolysis rate in river water (t½ = 4.2 d) and a subtle faster photo-degradation rate in this same matrix (k^{app} 0.0128 ± 0.001 mJ cm^{-2}), highlighting the importance of disinfection radiation for cephalosporin depletion in aqueous environments

5.2. Introduction

Cephalosporin antibiotics, as other pharmaceuticals, have been reported to be present in the range of ng L^{-1} to μg L^{-1} in aqueous matrices (Cha et al., 2006; Lin et al., 2008). Besides the potential effects in non-target organisms (Wang and Lin, 2012; Alexandrino et al., 2017), the observed increase of bacterial resistance in the last years has been linked to the presence of antibiotics in the environment (Boxall, 2004; Kümmerer, 2009), especially the ones employed in veterinary medicine (Zhang et al., 2017). In this context, cephalosporin antibiotics are of environmental concern due to their high consumption (Sadeghi-Sefidmazgi et al., 2011), significant activity against Gram-negative bacteria (EMA 1999), and unclear fate in complex matrixes (Gilbertson et al., 1990; Peterson et al., 2009; Jiang et al., 2010). Indeed, investigations of abiotic transformation processes (i.e., hydrolysis, photolysis, and partitioning/sorption), as well as biotic data (i.e., aerobic and anaerobic degradation rates) are incomplete or non-existent for several pharmaceuticals. However, previous studies suggest that abiotic transformations, as hydrolysis and photolysis, may be more important than biodegradation for the environmental depletion of cephalosporins (Gilbertson et al., 1990, Jiang et al., 2010; Li et al., 2011; Wang and Lin, 2012; Mitchell et al., 2014, Alexandrino et al., 2017). As for other penicillin derivatives, relative fast hydrolysis rates of cephalosporins are expected due to side-chain degradation and beta-lactam ring cleavage (Yamana and Tsuji, 1976). However, hydrolytic half-life times of days have been reported for this antibiotic group at pH 7 and 20 - 25°C (Gilbertson et al., 1990, Jiang et al., 2010; Mitchell et al., 2014). Consequently, the presence of such compounds in aquatic systems cannot be neglected.

The semi-synthetic antibiotics ceftiofur (CEF) and cefapirin (CEPA) have significant usage in veterinary clinic due to their broad activity spectrum against both Gram-positive and Gram-negative bacteria and high efficiency in the treatment of respiratory, urinal, dermatologic and infectious diseases (Gilbertson et al., 1990; EMEA, 1999; Berendsen et al., 2009; Zhang et al., 2017). CEF has been widely used to treat respiratory and dermatologic diseases in dairy and beef cattle, swine, equines and chicks (Sunkara et al., 1999; Zhang et al., 2017). In vivo studies demonstrated that intramuscularly applied CEF is metabolized to the equally biologically active desfuroyl ceftiofur (DFC), desfuroyl ceftiofur

cysteine disulphide and furoic acid (EMEA, 1999). Li et al. (2011) suggested DFC as the major hydrolysis product of CEF, presenting similar bioactivity and effectiveness than its precursor. On the other hand, ceftiofur aldehyde is the main CEF biodegradation transformation product, being formed by the beta-lactam ring cleavage, which induces reduced or neutralized antimicrobial activity (Li et al., 2011). Considered as one of the most important dairy cattle medicines, CEPA is mainly used for the treatment of mastitis, one recurrent and economically relevant intramammary infection (Sadeghi-Sefidmazgi et al., 2011). Desacetyl cefapirin (DAC) is the main CEPA metabolite, sharing similar structure, stability and antimicrobial activity with its parental drug (Berendsen et al., 2009). Recently, the occurrence of CEPA and other cephalosporins in aquatic environments has been reported (Cha et al., 2006; Lin et al., 2008). The average detected concentrations of CEPA were 5 and 9 ng L^{-1}, respectively, obtained in hospital wastewater of Taiwan (Lin et al., 2008) and in an agriculturally influenced river in USA (Cha et al., 2006). However, little information is available about the environmental fate of those important antibiotics, such as hydrolysis rates and photolysis.

In fact, temperature, pH and matrix composition are important parameters for hydrolysis of organic compounds (Mabey and Mill, 1978; Schwarzenbach et al., 2003; OECD, 2004). Likewise, chemical speciation can play a significant role in the photo-transformation of active products (Canonica et al., 2008; Baeza and Knappe, 2011), specifically in multifunctional antibiotics (Avisar et al., 2010). Hydrolysis kinetic data are hardly available for CEPA, where only body temperature was considered (i.e., 37 °C) (Berendsen et al., 2009). Meanwhile, other authors already studied pH and temperature effects on the degradation of CEF (Gilbertson et al., 1990; Koshy and Cazers, 1997; Sunkara et al., 1999). However, the reported data gives conflicting evidence. The only consensus is the complexity of CEF hydrolysis. Koshy and Cazers (1997) stated that DFC, the major CEF hydrolysis product, is later converted to dimers of desfuroylceftiofur, thiolactone, among other more complex structures. Recently, Ribeiro and Schmidt (2017) reported on the pH dependent ionization of CEPA and CEF, including their expected speciation yields in aqueous solution. At pH values typical for water treatment (6 to 8), it was observed that both compounds are mainly present as anions (i.e., CEPA- and CEF-) or as zwitterions (i.e.,

CEPA+/- and CEF+/-). Particularly, Wang and Lin (2012) carried out the only available study approaching pH effects on cephalosporin photo-transformation. Using simulated sunlight-induced photolysis (290 nm - 700 nm), these authors studied CEPA and other cephalosporins depletion and indicated high influence of speciation in the observed degradation rates. Approaching disinfection radiation, few data are available for cephalosporins. Specifically, Kim and Tanaka (2009) and Kim et al. (2009) investigated the effects of UV-C and UV/H_2O_2 in the transformation of CEF. However, these authors did not consider the speciation propriety of this drug.

In summary, considering that speciation of a pollutant and matrix composition can influence UV-C photolysis and hydrolysis rates, the available data for CEPA and CEF is inconclusive for predicting its environmental behavior. The present study aims to close this gap by investigating the influence of pH and environmental temperature on hydrolysis kinetics of CEF and CEPA, as well as to provide more information regarding the impact of cephalosporin speciation on photo-transformation kinetics in synthetic- and real water matrices.

5.3. Materials and methods

5.3.1. Chemicals

Ceftiofur sodium (CEF, purity >98%, CAS# 104010-37-9) and Cefapirin sodium (CEPA, purity 99%, CAS# 24356-60-3) were purchased from Santa Cruz Biotechnology (California, USA) and Sigma Aldrich (Seelze, Germany), respectively. Generally, each powdered cephalosporin was diluted in ultrapure water resulting in stock solutions of 2 mM. All solutions were prepared freshly before use and stored under refrigeration (4°C) for at maximum 2 days. The following chemicals were used for buffer solutions preparation: potassium chloride (Sigma Aldrich, Seelze, Germany), hydrochloric acid (Fischer Chemical, Leics, UK), boric acid, monopotassium phosphate, dipotassium phosphate (Merck, Darmstadt, Germany), sodium hydroxide (Prolabo, Leuven, Belgium) and acetic acid (Bernd Kraft, Duisburg, Germany). Acetonitrile, methanol and sulfuric acid (Fischer Chemical, Leics, UK), as well as sodium carbonate and sodium bicarbonate (Merck,

Darmstadt, Germany) were necessary for chemical quantification. In addition, uridine (URI) and atrazine (ATZ) purchased from Sigma Aldrich (Seelze, Germany) were used for actinometry and modeling purposes. Solutions were prepared with ultrapure water (UW) (Elga Purelab Flex, resistivity 15.5 ≈ MΩ-cm). All reagents were of analytical grade.

5.3.2. Surface water sampling

Ruhr River surface water (10-30 cm depth) was collected in Kupferdreh (RK), N 51°23'32"; E 7°4'31", district of Essen, Germany, in March 2017. The grabbed samples (6 L) were filtered with 0.45 µm mixed cellulose ester filters (Whatman, Germany) and stored at 4°C and dark until use. Right after water sampling, the following relevant parameters were assessed: total carbon (TC), non purgeable organic carbon (NPOC), inorganic carbon (IC), dissolved organic carbon (DOC) as well as total nitrogen, pH, alkalinity and the ions Cl^-, SO_4^{2-}, NO_3^- and NO_2^-.

5.3.3. Hydrolysis

The pH-dependent stability of CEPA and CEF in aqueous solutions and environmentally relevant temperature was investigated. Testing each cephalosporin separately, experiments were performed in triplicate, in darkness, under room temperature (22 ± 1°C) and without ionic strength adjustment. First, ten buffer solutions (pH 1 to 12.5, Table S5.4, Supplementary Information) were prepared according to OECD (2004) recommendations. Next, buffer solutions of each investigated pH value and UW (4 mL each) were inserted into inert brown-glass vials (15 mL). Following, 2 mL of fresh antibiotic stock solution was added to the test vial for starting the reaction. The resulting test conditions were 70 µM antibiotic in 0.04 M buffer, seventy minutes hydrolysis time and sampling every fourteen minutes. For sampling, 0.5 mL was removed and immediately mixed with 4.5 mL phosphate buffer pH 7, for stabilizing cephalosporins according to Berendsen et al., (2009). Finally, 50 µL of each quenched sample was analyzed by high performance liquid chromatography (HPLC) to monitor the abatement of the compounds under study (see further details below, section 5.3.5). To verify reproducibility and deviations, all reactions and measurements were carried out in triplicate. Complementary, hydrolysis experiments

were carried out with RK. For that, 50 mL of each matrix was spiked with antibiotics, resulting in 70 μM starting concentration. Later, the test solution was split in brown-glass vials (1 mL), which were immediately analyzed for the antibiotic concentration by HPLC-DAD (section 2.5). Using this design, CEPA and CEF were determined every 22 minutes for 6 hours.

5.3.4. Photolysis

The UV-C photolytic transformation of environmentally relevant ionic species of CEF and CEPA was studied. Aiming at a better comprehension of the degradation processes involved in the aquatic fate of these chemicals, two approaches were used. First, for kinetic studies, experiments were carried out in UW under different pH values. Afterwards, experiments were performed using the target cephalosporins spiked in surface water.

5.3.4.1. Kinetics studies

Photolysis was performed using a merry-go-round reactor (H&Th Schneider Glasapparatebau, Germany), which was previously described and applied for similar purposes (Wegelin et al., 1994; Canonica et al., 2008; Lutze et al., 2015). As inner radiation source, a monochromatic low pressure mercury lamp (15 W, GPH303T5L/4, Hereaus, Germany) with main emission at 254 nm was used. Additionally, a fluid-circulator (Ministat 125, Huber, Germany) filled with UW was used to keep the reactor internal temperature always constant at 25 ± 0.2°C. Finally, quartz tubes (20 mm internal diameter) were placed in the circular water bath for sample exposure. Before and after each experiment, the system absolute fluence rate was chemically measured by URI actinometry (von Sonntag and Schuchmann, 1992; Lutze et al., 2015). Accordingly, the obtained average fluence rate (E^0) was 62 ± 3 μeinstein m^{-2} s^{-1}, equivalent to 3 mJ cm^{-2} s^{-1}.

Considering that speciation of pharmaceuticals can influence their photo-transformation (Canonica et al., 2008; Avisar et al., 2010; Baeza and Knappe, 2011), the cationic, zwitterionic, and anionic species of CEPA and CEF (Figure 5.1) were investigated. Therefore, several buffer solutions (pH 2 to 9) were prepared using adjusted phosphate and sulfuric acid buffers. Afterwards, test solutions were prepared in a 100-mL volumetric flask

by adding antibiotic stock solution, 5 mL buffer and making up with UW. To ensure no interference in the light transmittance through the test tubes, all chemicals were studied at low concentration (i.e., CEPA 4 μM; CEF 5 μM; buffer 5 mM). Consequently, the initial test solutions presented maximum absorbance below 0.01 cm^{-1} at wavelengths ≥ 200 nm. Following, 50 mL of each test solution were inserted in quartz tubes previously conditioned to 25 ± 0.2°C. Next, 5 mL was sampled after 0, 18, 36, 54, 72 and 90 seconds of exposure. Finally, cephalosporin depletion was determined for every performed condition by HPLC-DAD (section 5.3.5). All experiments and measurements were performed at least in triplicate.

5.3.4.2. Photolysis in surface water

To study the behavior of CEPA and CEF during UV-C disinfection of water, a real matrix was tested. Radiation experiments were performed as described above (section 5.3.4.1). Since the real water matrix RK presented significant light absorption (i.e., 0.045 cm^{-1}), it was necessary to introduce a correction factor for internal fluence rate calculation. Therefore, the Morowitz factor (Katsoyiannis et al., 2011) was used to correct the applied fluence rate determined by URI actinometry. For comparison, the model contaminant ATZ was used for internal fluence determination according to Canonica et al. (2008) and Lutze et al. (2015). In fact, both internal and corrected fluence rates showed good agreement (Fig. S5.3, Supplementary Information).

5.3.5. Analytical methods

Bicarbonate concentration was determined as alkalinity by 0.01 M sulfuric acid titration using an automatic titrator (Tiamo, Metrohm). The concentration of total carbon (TC), non purgeable organic carbon (NPOC), inorganic carbon (IC) and dissolved organic carbon (DOC) as well as total nitrogen of the river water was determined by a TOC-Analyzer (5000A, Shimadzu). Absorbance measurements were carried out using a UV-Vis spectrophotometer (1650PC, Shimadzu) running over the wavelength range of 180-500 nm. pH measurements were done using a daily calibrated pH meter (6.0258.600, Metrohm). Cl^-, SO_4^{2-}, NO_3^- and NO_2^- were determined using ion chromatography (Basic 883, Metrohm)

equipped with ion suppression and conductivity detection. Finally, CEPA, CEF and ATZ quantification was performed using a Shimadzu high performance liquid chromatography (HPLC) system (controller, SCV-10-Avp; degasser unit, DGU-20 5R; liquid chromatograph, LC-10Atvp; auto injector, SIL-10Advp; diode array detector SPD-M10avp and column oven, CTO-10Asvp), equipped with a diode-array detector (DAD). Chromatographic conditions are fully described in Table S5.1, Supplementary Information.

5.4. Results and discussion

5.4.1. Hydrolysis in pure water

Generally, hydrolysis can be categorized as acid-catalyzed (k_A), neutral or pH-independent (k_N) and base-catalyzed (k_B) reaction (Mabey and Mill, 1978; Schwarzenbach et al., 2003). The observed pseudo-first-order hydrolysis rate constant (k_{obs}) can be defined as the sum of all reactions following Eq. (5.1)**:**

$$k_{obs} = k_A[H^+] + k_N + k_B[OH^-] \quad (5.1)$$

In our experiments, CEPA presented similar hydrolysis rates under acidic and neutral environment (Figure 5.2a). From pH 1.3 to 7.1, the mean k_{obs} values were similar (Table 5.1), resulting in half-life of days. In contrast, base-catalyzed reactions appeared to be important in the transformation of this antibiotic. As Figure 5.2a demonstrates, above pH 9 a linear increase of log(k_{obs}) values with pH was observed. In fact, CEPA presented high instability at pH 12, showing a half-life of approximately 10 minutes. Furthermore, the mean k_{obs} obtained at pH 7.1 was in the same range of the data obtained at acid environment but hundredfold smaller than the ones observed at strongly alkaline conditions (Table 5.1). This result indicates that CEPA is rather stable at typical pH values (6 - 8) of natural water matrices and that acid-catalyzed reactions are not significant at pH $\geq$ 2. Assuming k_N equals the k_{obs} at pH 7.1 (Schwarzenbach et al. 2003), the pH independent rate constant for CEPA was derived from Eq. (5.1) (k_N = 0.03 x 10^{-4} s^{-1}, 22 ± 1°C).

Consequently, considering that only neutral and base-catalyzed hydrolysis take place at pH>9, the k_B value for CEPA was also obtained rearranging Eq. (5.1) and using the previously calculated k_N. Therefore, the base-catalyzed rate constant (k_B) calculated for

CEPA at 22 ± 1°C was 0.14 M^{-1} s^{-1}. Later, using the obtained k_N and k_B values and rearranging Eq. (5.1), the acid-catalyzed rate constant (k_A) calculated for CEPA was 0.08 x 10^{-4} M^{-1} s^{-1}. With k_N, k_A and k_B at hand the pH values where two hydrolysis reactions are equally important, *I*, (Mabey and Mill, 1978; Mitchell et al., 2014) can be calculated. For this purpose, Eqs. (5.2 – 5.4) were used:

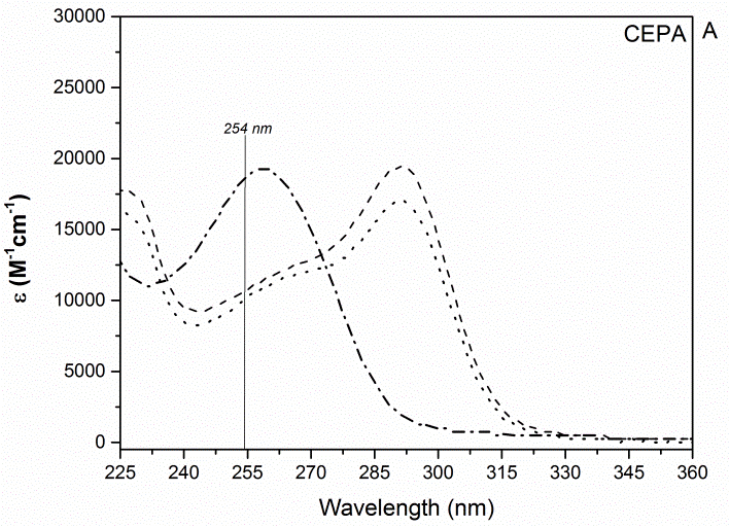
$$I_{AN} = log\left(\frac{k_A}{k_N}\right) \quad (5.2)$$

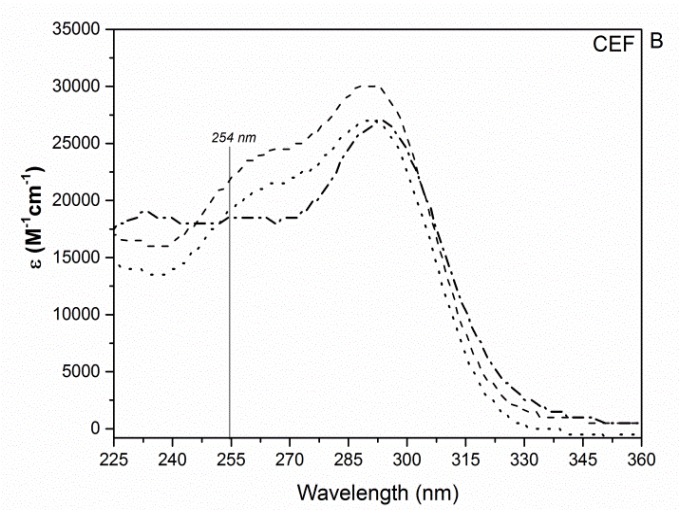
$$I_{AB} = 0.5log\left(\frac{k_A}{k_B K_w}\right) \quad (5.3)$$

$$I_{NB} = log\left(\frac{k_N}{k_B K_w}\right) \quad (5.4)$$

Eqs 5.2 – 5.4 gave following results: I_{AN} = pH 0.5, I_{AB} = pH 4.9 and I_{NB} = pH 9.4. Therefore, at typical pH of real water, neutral and base-catalyzed reactions are the most important for the hydrolysis of this antibiotic. CEF and CEPA display a similar hydrolysis profile. As indicated in Figure 5.2b, base-catalyzed reactions are the most significant ones in the aqueous transformation of CEF. However, CEF showed a faster transformation rate in acid catalyzed hydrolysis than CEPA. From pH 1.5 to pH 4.2, the obtained half-life times were in the range of hours. In fact, even under neutral conditions CEF presented significant hydrolysis rate (pH 7.2, k_{obs} = 0.06 x 10^{-4} s^{-1}, $t_{½}$ 1.4 days). Nevertheless, CEF was subtly more stable than CEPA in a highly alkaline environment (Table 5.1).

The hydrolytic kinetic parameters for CEF at 22 ± 1°C were calculated. In analogy to calculations for CEPA, k_N (0.06 x 10^{-4} s^{-1}), k_B (3.80 x 10^{-2} M^{-1} s^{-1}) and k_A (3.26 x 10^{-4} M^{-1} s^{-1}) values were obtained. Furthermore, values for *I* were determined (Table 5.1).





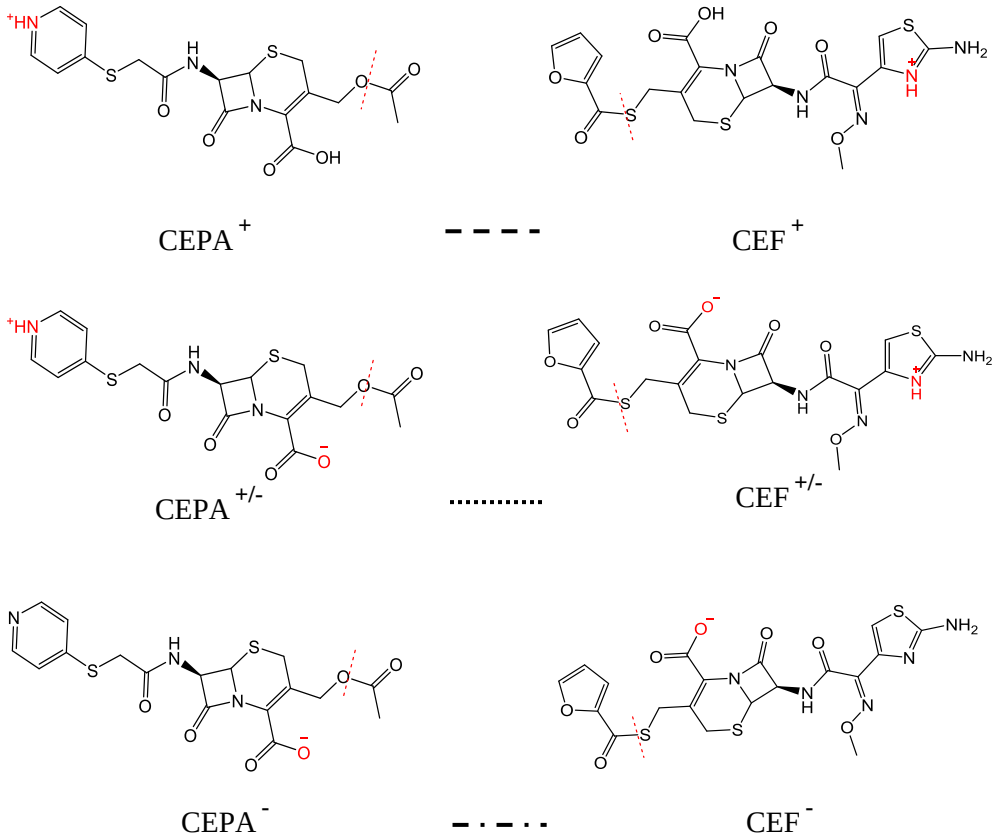


Figure 5.1. Average spectra and ionic species of CEPA and CEF. In **A**, CEPA (4 µM) at pH 2 (dashed curve, protonated pyridine ring), pH 3.7 (dotted curve, protonated pyridine ring and deprotonated carboxylic acid) and pH 9 (dash-dotted curve, deprotonated carboxylic acid). In **B**, CEF (2 µM) at pH 2 (dashed curve, protonated aminothiazole), pH 3.2 (dotted curve, protonated aminothiazole ring and deprotonated carboxylic acid) and pH 9 (dash-dotted curve, deprotonated carboxylic acid). The red dashed lines indicate main hydrolytic pathways, while red highlighted are the charged moieties.

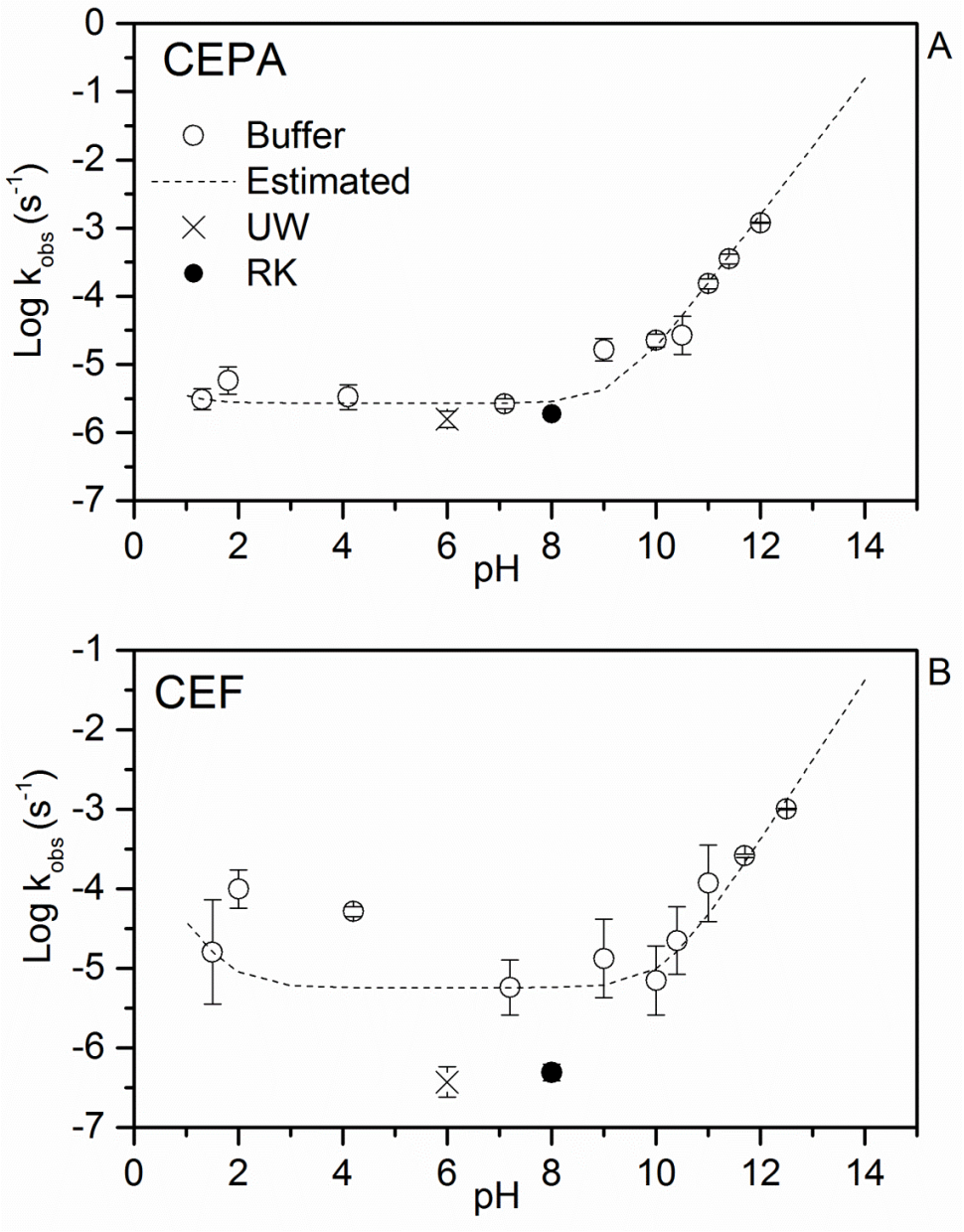


Figure 5.2. Matrix and pH-dependent hydrolysis of CEPA (**A**) and CEF (**B**) demonstrated by plotting Log k_{obs} (s^{-1}). Each antibiotic (70 µM as starting concentration) underwent hydrolysis under controlled room temperature (22 ± 1°C). Opened circles represent data in buffered solution (40 mM buffer). Ultrapure (UW, closed circle) and Ruhr River (RK, cross) waters were used as matrix references. All experiments were done at least in triplicate.

Table 5.1

Cephalosporin hydrolysis as a function of pH. Mean pseudo-first-order hydrolysis rate constants (k_{obs}) (n=3), half-life times ($t_{1/2}$) and derived kinetic parameters

CEPA				**CEF**			
pH	k_{obs} (x 10^{-4} s^{-1})	$t_{1/2}$		pH	k_{obs} (x 10^{-4} s^{-1})	$t_{1/2}$	
12	11.93	9.7min	k_N (10^{-4} s^{-1})	12.5	10.04	11.5min	k_N (10^{-4} s^{-1})
11.4	3.55	32.9min	0.03	11.7	2.61	44.3min	0.06
11	1.52	1.3h	k_B (M^{-1} s^{-1})	11	1.17	1.6h	k_B (M^{-1} s^{-1})
10.5	0.27	7.2h	0.14	10.4	0.22	8.7h	3.80 x 10^{-2}
10	0.22	8.6h	k_A (M^{-1} s^{-1})	10	0.07	1.1d	k_A (M^{-1} s^{-1})
9	0.16	11.7h	0.08 x 10^{-4}	9	0.13	14.5h	3.26 x 10^{-4}
7.1	0.03	3d		7.2	0.06	1.4d	
4.1	0.03	2.4d	I_{NB} = 9.4	4.2	0.51	3.7h	I_{NB} = 10.3
1.8	0.06	1.4d	I_{AB} = 4.9	2	0.99	1.9h	I_{AB} = 6.0
1.3	0.03	2.6d	I_{AN} = 0.5	1.5	0.16	12h	I_{AN} = 1.8
UW	0.02	4.9d		UW	0.003	30d	
RK	0.02	4.2d		RK	0.01	14.7d	

Notes:
The ionic strength was not maintained. T = 22 ± 1°C. UW, ultrapure water (pH 6 after antibiotic spiking). RK, surface water from Ruhr River (pH 8 after antibiotic spiking). Buffer concentration of 40 mM. k_{obs} deviations can be observed in Fig. 5.2

The base catalyzed hydrolysis of several cephalosporins has been reported, but in few cases complete hydrolytic kinetic parameters or intersection points have been established. These data are important for the understanding or even prediction of cephalosporins behavior during analytical procedures, in biological systems and in other environmentally relevant matrices. Analyzing both cephalosporins hydrolysis profiles, no visible shoulder caused by dissociation was observed. Consequently, hydrolysis kinetics were not influenced by the pK_a values of CEF and CEPA, indicating that all analyzed species behave similar regarding hydrolysis (Yamana and Tsuji, 1976). Therefore, Eq. (5.1) was used along with the obtained k_N, k_B and k_A values for CEPA and CEF (Table 5.1) to estimate and fit the hydrolysis degradation rate for both compounds at any pH value and 22 ± 1°C. The resulting modeled curves are plotted in Figure 5.2 along with measured values. The measured k_{obs} for CEF presented large scattering, especially under basic and acidic conditions. However, the fitted curve indicates acid-catalyzed hydrolysis of CEF occurring

at pH ≤ 3, which confirms the results obtained by Berendsen et al. (2009). For CEPA, the fitted curve agrees well with the measured values. To the best of our knowledge, this is the first time that hydrolytic kinetics parameters are provided for CEPA.

Although the authors did not calculate $t_{1/2}$ or hydrolysis rate constants, Berendsen et al. (2009) reported similar alkaline catalyzed degradation for CEF. The degrees of degradation of 100% (30 minutes at pH 12) and 80% (3 hours at pH 11) observed by these authors follow the same tendency as presented in our study. Berendsen et al. (2009) also investigated pH effects on the degradation of CEPA and its major metabolite, desacetyl cefapirin (DAC). It was demonstrated that CEPA and DAC were instable at pH above 7.5. In fact, results of this previous investigation agree well with our findings, indicating that CEPA degrades faster than CEF under alkaline conditions. However, differences were noticed in the available data concerning the neutral and acid catalyzed hydrolysis of CEF. For example, Sunkara et al. (1999) reported a rate constant of $k_{obs} = 0.03 \times 10^{-4}\ s^{-1}$ at pH 5 (citric acid-NaH_2PO_4 buffer) and $k_{obs} = 0.05 \times 10^{-4}\ s^{-1}$ at pH 7.4 (phosphate buffer) for CEF. As shown in Table 5.1, our results at pH 7.2 agree with the data of Sunkara et al. (1999), but reveal a faster reaction at acidic condition. Gilbertson et al. (1990) also reported on CEF hydrolysis and observed a decrease in reaction rates at acidic pH. CEF half-life times of 100, 8, and 4.2 days were obtained at 22°C and pH values of 5, 7 and 9, respectively (Gilbertson et al. 1990).

Koshy and Cazers (1997) studied CEF hydrolysis and product formation. Upon cephalosporin hydrolysis in a high ionic strength environment, desfuroyl ceftiofur (DFC) was formed in analogy to hydrolysis of thioesters. This transformation does not change the bioactive part of CEF (beta-lactam structure). Hence, the biological activity may be preserved after CEF hydrolysis (Koshy and Cazers, 1997), which may also apply to CEPA. In analogy to carbon esters hydrolysis, likely results in formation of hydroxo CEPA and acetic acid upon hydrolysis of the ester-group (Yamana and Tsuji 1976).

Previous studies investigated the hydrolysis of other cephalosporins in aqueous solution. Wang and Notari (1994) demonstrated that both [H+] and [OH-] are significant for

cefuroxime hydrolysis. Indeed, the hydrolysis of cefuroxime's alkyl carbamate group as well as of its beta-lactam ring were the major degradation pathways.

Yamana and Tsuji (1976) studied the pH-dependent hydrolysis kinetics of such 3-acetoxymethylcephalosporins, indicating that, in both neutral and alkaline pH, the ester moiety is hydrolyzed resulting in high yields of desacetyl intermediates. Under acidic conditions the desacetyl product is also formed, but quickly undergoes lactonization. Hydrolysis rates of several cephalosporins determined by Yamana and Tsuji (1976) were very similar to CEF, also revealing significant acid catalyzed hydrolysis at pH ≤ 3 (Figure 5.2b). Besides sharing the same R_2 substituent, cefaloglycin was more susceptible to base and acid-catalyzed hydrolysis than CEPA. In fact, cefaloglycin showed significant stability only in the pH range 2 to 4 (Yamana and Tsuji., 1976), whereas CEPA presented similar k_{obs} values at pH ≤ 9 (Figure 5.2a). However, it is important to consider that under environmental conditions several other parameters beside pH may influence hydrolysis.

5.4.2. Matrix effects on CEPA and CEF hydrolysis

The hydrolytic degradation of CEPA and CEF was studied using UW and RK. The latter was sterilized by filtration and chemically characterized (Table S5.3). For both matrices, no pH adjustments were carried out. As Table 5.1 illustrates, CEPA presented similar degradation in both matrices. For this drug, the observed k_{obs} values for UW and RK are in the same range of the ones obtained using buffered solutions and agree with the estimated curve (Figure 5.2a). Consequently, matrix composition did not affect CEPA hydrolysis, at least under our experimental conditions (dark, 22 ± 1°C, 70 μM starting concentration). However, the results obtained for CEF are controversial. Degradation in unbuffered UW and RK was much slower than the ones exhibited and predicted for similar pH values (Figure 5.2b). For instance, half-life times of 30 and 14.7 days were observed in UW (pH 6 after antibiotic spiking) and RK (pH 8 after antibiotic spiking), respectively. In contrast, in phosphate buffer pH 7.2, CEF presented $t_{1/2}$ of 1.4 days. Li et al. (2011) reported a k_{obs} of 0.05×10^{-4} s^{-1} for CEF hydrolysis in neutral solutions at 25°C. This value is very similar to our results using pH 7.2 phosphate buffer (i.e., $k_{obs} = 0.057 \times 10^{-4}$ s^{-1}) but ten times higher

than our results using UW water (k_{obs} = 0.005 x 10^{-4} s^{-1})(Table 5.1). Sunkara et al (1999) reported 0.007 x 10^{-4} s^{-1} as CEF k_{obs} in distilled water (25°C), a value in the same range as our results with similar matrix. Furthermore, using phosphate buffer at pH 7.4 and citric acid-NaH_2PO_4 buffer at pH 5, Sunkara et al. (1999) obtained k_{obs} = 0.046 x 10^{-4} s^{-1} and k_{obs} = 0.025 x 10^{-4} s^{-1}, respectively. These values are very similar to our results with buffers (Table 5.1) and confirmed that CEF hydrolysis can be influenced by buffer composition. Consequently, the strong influence of buffers on CEF hydrolysis may explain the discrepancy between data of experiments in synthetic solutions and in UW and RK, which were not buffered with phosphate.

Phosphate, borate and acetate buffers did not catalyze cefuroxime, cefalotin and cefoxitin hydrolysis (Wang and Notari, 1994; Mitchell et al., 2014). In the study of CEPA and CEF stability during typical sample preparation, Berendsen et al. (2009) indicated the applicability of phosphate buffers (pH 7) to quench further degradation. Likewise, the standard guideline for studying the pH-dependent hydrolysis (OECD 2004) also includes phosphate in the list of suggested buffers. The observed scattering of CEF degradation kinetics may also result from fluctuations in the baseline of the chromatographic determination of CEF, which appeared during the analysis of the samples. However, standard solutions were not affected (Figure S5.2, Supplementary Information).

5.4.3. Photolysis as a function of speciation

Considering that photolytic reactions may depend on speciation (Canonica et al., 2008; Avisar et al., 2010; Baeza and Knappe, 2011), the cationic, zwitterionic and anionic species of CEPA and CEF underwent direct photolysis under controlled pH, temperature and fluence rate. The apparent molar absorption coefficient (ε^{app}) at 254 nm for each ionic species of CEF and CEPA was determined (Table 5.2**)**, which is important for characterizing photochemical reactions. For that, the predicted species-distribution in solution as a function of pH (Ribeiro and Schmidt, 2017) was used and UV-vis spectra of solutions containing high yields of each species were performed (Figure 5.1). If small concentrations of different ionic species may be present simultaneously in solution, the

term apparent (app) was adopted (Canonica et al., 2008). Each ionic species was photolyzed using at least two pH values. Using the average fluence rate multiplied by the irradiation time, (tE^0), the observed concentration variation, $[C]_t$ and $[C]_0$, and rearranging Eq. (5.5), the apparent pseudo-first-order rate constant (k^{app}) for each ionic species was determined.

$$ln\left(\frac{[C]_t}{[C]_0}\right) = -k^{app} \times (tE^0) \quad (5.5)$$

As shown in Figure 5.3, CEPA cationic and zwitterionic species presented fast and similar transformation rates, with $t_{1/2}$ ranging from 25 to 29 seconds (Table 5.2). In contrast, CEPA anionic species showed somewhat faster reaction, having half-life times of 19 and 21 seconds. A small variation (± 0.001) in the calculated average of k^{app} was observed (Table 5.2), demonstrating that CEPA's electronic density alteration hardly affects photo-transformation. Moreover, all investigated CEF species presented very similar transformation rates (Figure 5.4). This antibiotic revealed a comparable photolysis rate as CEPA, with half-life times ranging from 23 to 26 seconds (Table 5.2).

Table 5.2

Cephalosporin photo-transformation as a function of speciation. UV-C (254 nm) photo-kinetic parameters

CEPA							**CEF**						
pH	Major specie [a]	ε^{app} (m^2 mol^{-1})[b]	k^{app} (mJ cm^{-2})	r^2	$t_{1/2}$ (s)	ϕ^{app} (mol $einstein^{-1}$)	pH	Major specie[a]	ε^{app} (m^2 mol^{-1})[b]	k^{app} (mJ cm^{-2})	r^2	$t_{1/2}$ (s)	ϕ^{app} (mol $einstein^{-1}$)
2	+	1050	0.0090	0.999	25.7	0.178	2	+	2400	0.0097	0.966	23.9	0.078
2.3	+	1000	0.0083	0.993	27.8	0.176	2.2	+	2200	0.0097	0.965	23.9	0.085
3.7	+/-	1000	0.0079	0.998	29.2	0.162	3.2	+/-	2200	0.0088	0.981	26.7	0.076
4.3	+/-	1530	0.0079	0.998	29.2	0.109	3.5	+/-	1950	0.0087	0.984	26.7	0.086
7	-	1900	0.0116	0.997	19.9	0.128	3.7	-	1900	0.0098	0.992	23.9	0.098
9	-	1850	0.0110	0.999	21.0	0.123	7.2	-	1850	0.0098	0.996	23.9	0.101
							9	-	2150	0.0101	0.997	23.1	0.090
			0.0092 ± 0.001[c]			0.146 ± 0.02[c]				0.0095 ± 0.0004[c]			0.087 ± 0.007[c]

Notes:

[a] Predominant ionic specie in solution according to pK_a values (Ribeiro and Schmidt, 2017)
[b] Calculated at 254 nm
[c] Average and Standard Deviation

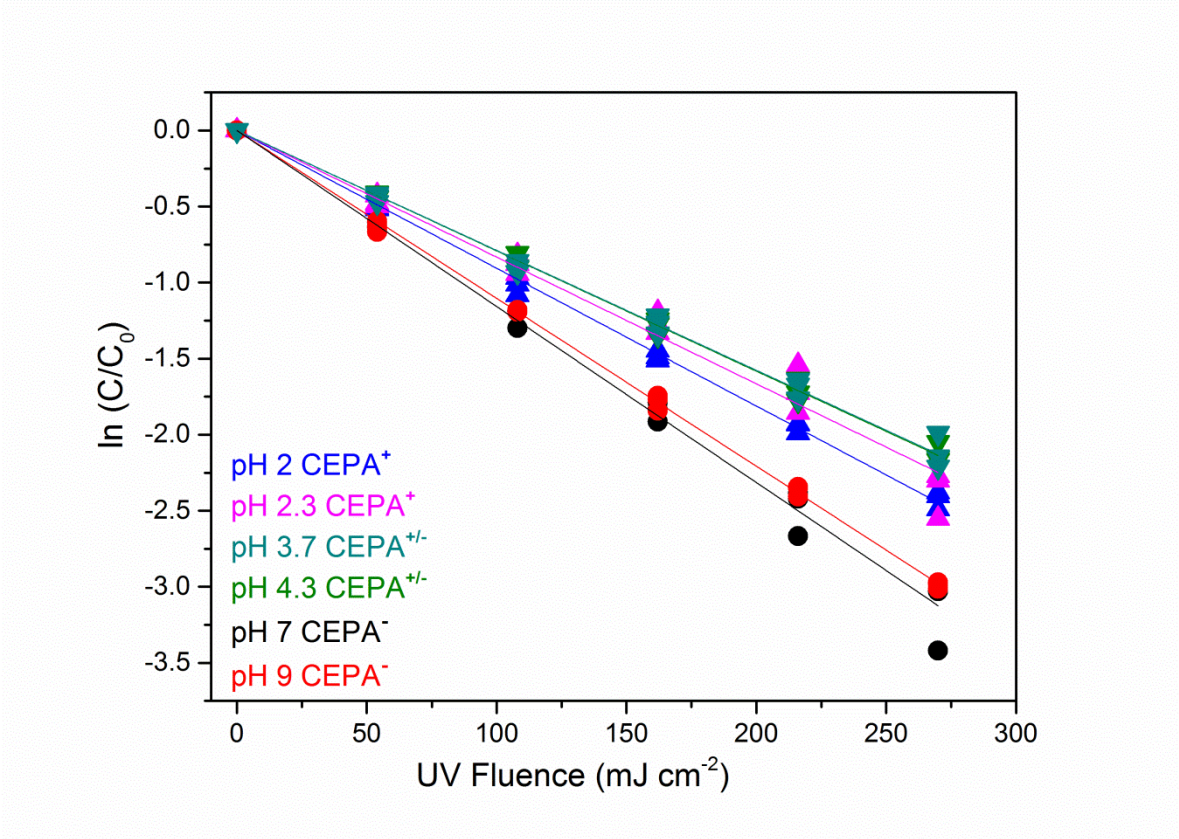


Figure 5.3. Photolysis of CEPA ionic species as a function of (UV) fluence. Buffered solutions (5 mM) containing CEPA (4 µM) underwent photolysis. All experiments were done in triplicate, at constant temperature (25 ± 0.2°C) and pH ranging from 2 – 9 (see caption). Linear regression forced through (x, y) = 0.

The apparent quantum yield (ϕ^{app}) for each analyzed species at 254 nm was calculated by rearranging Eq. (5.6), which describes direct photolytic degradation (Canonica et al., 2008). For that, the obtained apparent molar absorption coefficient (ε^{app}), the average photon fluence rate of the employed photo-reactor (E^0, 62 ± 3 µeinstein m^{-2} s^{-1}) and the time-based apparent pseudo-first-order rate constant (tk^{app}, in s^{-1}) (Table S5.5, Supplementary Information), were used.

$$tk^{app} = 2.303E^0\varepsilon^{app}\Phi^{app} \quad (5.6)$$

As demonstrated in Table 5.2, the quantum yields for CEPA species presented small variation, with the mean at ϕ^{app} 0.146 ± 0.02 mol einstein^{-1}. For CEF, the mean apparent

quantum yield was 0.087 ± 0.007 mol einstein^{-1}. As for CEPA, all studied species of CEF showed similar ϕ^{app} values. Our results differ from those reported by Wang and Lin (2012). Using a sunlight simulator (290 nm < λ < 700 nm), the authors demonstrated significant pH dependence for CEPA photo-transformation. Our results may be explained by the different wavelength used for both quantum yield determination and for the cephalosporins photo-transformation. Furthermore, differences in photo-transformation parameters may be also explained by variations of starting concentration, matrix characteristics and experimental setup (Lin and Reinhard, 2005).

Using a low-pressure lamp of 8 W emitting at 254 nm and pH 7, Kim and Tanaka (2009) and Kim et al. (2009) also reported on the direct photo-transformation of CEF. First, Kim et al. (2009) reported a ε^{app} 254 nm of 1466 m^2 mol^{-1} for CEF. The present study reveals a similar ε^{app} (1850 m^2 mol^{-1}). To allow a rate comparison, the available tk^{app} values were normalized ($k^{app}(E^0) = tk^{app}/E^0$). Consequently, it was observed that in our experiments less energy was required for CEF depletion (i.e., $k^{app}(E^0)$ = 0.93 x 10^{-3} J^{-1}m^2) compared to results of Kim and Tanaka (2009) ($k^{app}(E^0)$ =1.72 x 10^{-3} J^{-1}m^2). However, with both degradation rates > 30% of CEF will be degraded upon 400 J m^{-2}, UV-exposure recommended for water disinfection (DVGW, 1997).

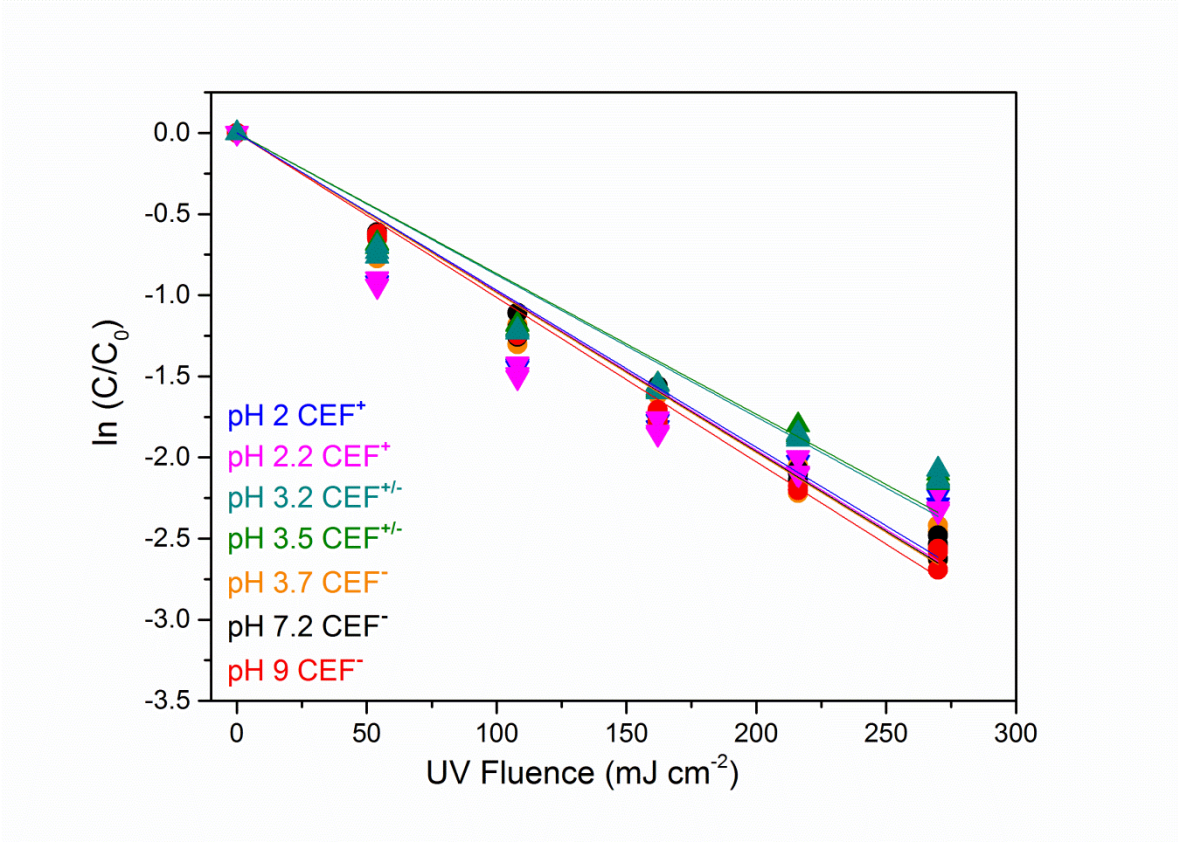


Figure 5.4. Photolysis of CEF ionic species as a function of (UV) fluence. Buffered solutions (5 mM) containing CEF (2 µM) underwent photolysis. All experiments were done in triplicate, at constant temperature (25 ± 0.2°C) and pH ranging from 2 - 9 (see caption). Linear regression forced through (x, y) = 0.

Interferences by buffer which can catalyze hydrolysis can be ruled out since the rates observed in our photolytic study were thousandfold higher than the ones observed for hydrolysis. k^{app} and ε^{app} can but must not correlate, due to the fact that the quantum yield can be affected by speciation of the compound under study (Canonica et al., 2008). In fact, analyzing diclofenac, sulfamethoxazole and other pharmaceuticals photo-transformation, Baeza and Knappe (2011) did not observe clear correlations between ε^{app} and photolytic transformation rates. Even including quantum yields for comparing the pH-dependence of photo-kinetics parameters, these authors could not observe the patterns suggested by Canonica et al. (2008). Indeed, the generated data referring to CEPA and CEF photolysis, compiled in Table 5.2, were also investigated according to the methodology employed by

Canonica et al. (2008), Baeza, and Knappe (2011). For CEF, all derived parameters present similar low deviation and no clear correlation between them was observed. In contrast, for CEPA a clear correlation was observed, with concomitant increase of ε^{app} and k^{app} (Figure S5.5. Supplementary Information). Our results with cephalosporins corroborate the results of Baeza and Knappe (2011), where the authors illustrate that despite structural similarities, sulfonamide antibiotics showed different correlations of photo-kinetic parameters.

5.4.4. Photolysis in surface water

In natural waters, the presence of photosensitizers and radical scavengers may lead to different photo-transformation processes than in UW. Previous studies indicated significant enhancement of pharmaceutical degradation in surface waters due to indirect photolysis (Lin and Reinhard, 2005; Katsoyiannis et al., 2011; Wang and Lin, 2012). In the present work, the photolytic kinetics of CEF and CEPA spiked in RK were assessed. As illustrated in Figure 5.5, photolysis of CEF and CEPA is not strongly affected by constituents of the water matrix. In case of CEF, hardly any effect of the water matrix can be observed, while photolysis of CEPA is slightly enhanced by the presence of the water matrix, which indicates that photosensitizers where involved in degradation of CEPA in RK. Yet, the observed matrix effect on CEPA photo-oxidation compared to CEF cannot be explained. However, different behaviors in photo-oxidation of compounds with similar structures has already been reported (Baeza and Knappe, 2011; Wang and Lin, 2012). Using sunlight simulator Wang and Lin (2012) reported a different behavior, slower photolysis rate of CEPA among several cephalosporins in surface water than in pure water. Alkalinity and nitrite are indicated as major photosensitizers in surface water (Schwarzenbach et al., 2003). As discussed by Katsoyiannis et al. (2011) and Wang and Lin (2012), dissolved organic matter may play a role as both, photosensitizer and radical scavenger. Accordingly, the investigated surface water was chemically characterized (Table S5.3). Although RK and the water matrix investigated by Wang and Lin (2012) had different qualities, the difference observed in CEPA photolysis may be also explained by the difference of radiation sources used. The UV-C radiation from the LP-Hg arc may more effectively excite aqueous species than the sunlight simulator, which emits only a small fraction of UV-C radiation.

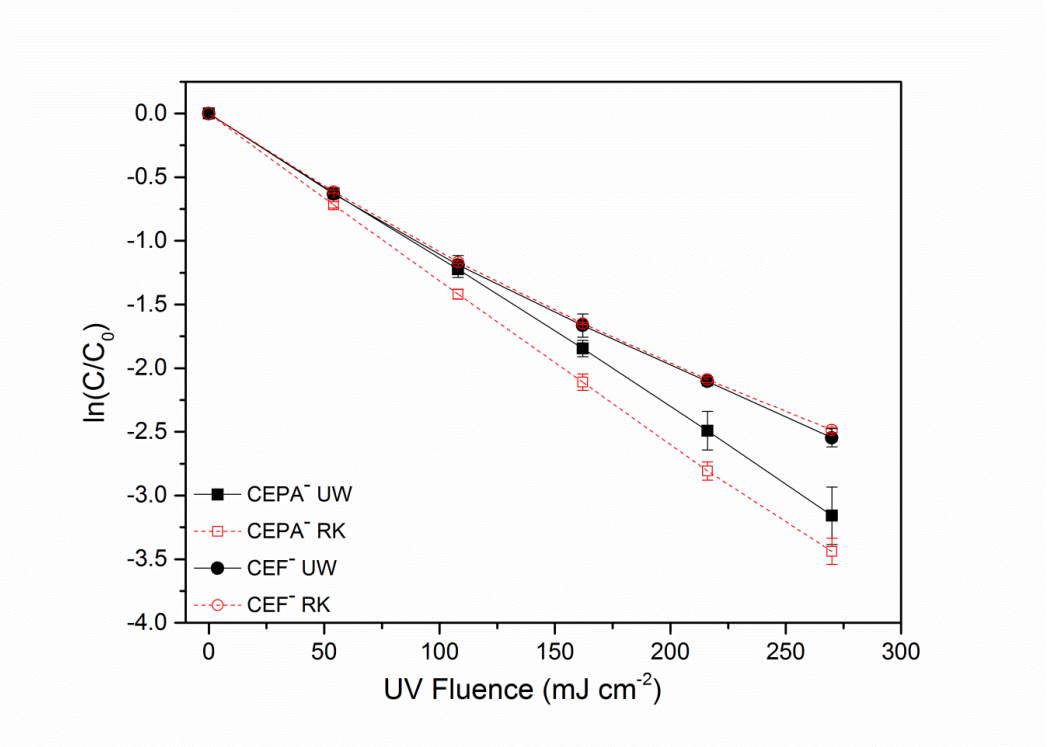


Figure 5.5. Photo-transformation of $CEPA^-$ and CEF^- in ultrapure (UW, pH 7.2, straight line) and in Ruhr River (RK, pH 8, dashed line) waters, as a function of (UV) fluence. T = 25 ± 0.2°C

To transfer the observed transformation kinetics of CEPA (k^{app} = 0.0128 mJ cm^{-2}) and CEF (k^{app} = 0.0093 mJ cm^{-2}) in RK to a real scenario of water disinfection, the necessary radiation dose for removal of 90% of each drug was calculated, as proposed by Avisar et al. (2010). Consequently, 1800 J m^{-2} were necessary for 90% transformation of CEPA in RK. For CEF, 2500 J m^{-2} was the observed value. Accordingly, typical UV doses employed in water disinfection (i.e., 400 J m^{-2}) (DVGW, 1997) seem to be insufficient for significant photo-transformation of the target cephalosporins.

5.5. Conclusions

Kinetic rates for the hydrolysis and photo-transformation of CEF and CEPA in artificial and natural waters were presented. The conclusions of this work can be summarized as follows:

- pH-dependent hydrolytic kinetic parameters (22 ± 1°C), including intersection points (*I*) for CEPA and CEF have shown that these compounds are relatively stable under neutral and acid environment, presenting half-life times ranging from 1.4 to 3 days (CEPA) and 1.9 hours to 3.7 days (CEF). Whereas, for both drugs base-catalyzed reactions (pH > 9) led to fast degradation (day > $t_{½} \geq$ min).
- CEF was more recalcitrant in surface water ($t_{½}$ = 14.7 days) than in buffered solutions.
- In contrast to sunlight induced photolysis, photo-degradation kinetics under UV-C radiation (λ = 254 nm) was not affected by speciation of CEPA. The same applied for CEF ionic species.
- During UV-disinfection applying 400 J m^{-2}, CEF and CEPA will be degraded in RK by 32 and 40 %, respectively.

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6. Ecotoxicity of the two veterinarian antibiotics ceftiofur and cefapirin before and after photo-transformation

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6.1. Abstract

The release of antibiotics into the environment may lead to deleterious effects in non-target organisms as well as pressure in antimicrobial resistance acquirement. Ceftiofur (CEF) and cefapirin (CEPA) are veterinary cephalosporins used for recurrent and economically relevant infections. Both antibiotics have been detected in aquatic environments and their fate during drinking water processing is still unknown. This work investigated the acute and chronic toxicities of CEF and CEPA towards aquatic organisms including stability tests. Complementary, the effects of water disinfection radiation (UV-C, 254 nm) on ecotoxicological responses were studied. CEPA and CEF have significant decay during *Daphnia magna* tests, portraying half-life times ($t_{½}$) of 49 and 53 hours, respectively. During tests with green algae (*Scenedesmus spec*.), CEPA was more instable ($t_{½}$ 88 h) than CEF ($t_{½}$ 267 h). CEF and its hydrolysis products induced deleterious effects in *Daphnia magna* (48h EC_{50} 139, LC_{50} 179 in μM), which was not observed with *Scenedesmus spec.* (72h NOAEC 82.5 ± 2.5 μM). In the case of CEPA, no toxic effects were observed in both tests (48h EC-LC_{50} > 510 and 72h NOAEC 57 ± 6, in μM). Photolysis of CEPA resulted in toxic products, which were effective for cladocera but not for the green algae. On the other hand, the different radiation doses studied did not affect CEF ecotoxicity. This investigation illustrates the importance of cephalosporin hydrolysis during standard toxicity tests. Furthermore, the potential formation of specie-specific toxic compounds during water processing is demonstrated, highlighting the need of further assessing toxicity of both cephalosporins and their transformation products.

6.2. Introduction

As for other pharmaceuticals, the behavior of cephalosporin antibiotics in the aquatic environment is very dynamic and complex (Yamaha and Tsuji, 1976; Gilbertson et al, 1990; Kümmerer, 2009; Wang and Lin, 2012; Ribeiro et al., 2017). Environmental concern about cephalosporins involve possible undesirable effects on non-target organisms (Wang and Lin, 2012; Chen and Guo, 2012; Magdaleno et al., 2015; Li and Lin, 2015), as well as the occurrence, spread, and increasing of antibiotic-resistant bacteria (EMA, 2012; WHO, 2014; EMA, 2015). Representatives of these antibiotics have been detected in aqueous matrices at concentrations raging from ng L^{-1} to μg L^{-1} (Cha et al., 2006; Lin et al., 2008; Tamura et al., 2017; Biel-Maeso et al., 2018). Recently, traces of ceftiofur were detected in two locations in Japan (Tamura et al., 2017) and in coastal water of Spain (Biel-Maeso et al., 2018). The measured concentrations by Tamura et al. (2017) were 1.6 (WWTP effluent in Kyoto) and 0.9 (sept tank effluent in Tokushima), both in ng L^{-1}. Likewise, ceftiofur was detected in rivers discharge area (Sancti-Petri Channel, Cadiz Bay), with concentrations ranging from not detected to 1.7 ng L^{-1} (Biel-Maeso et al., 2018). Average detected concentrations of cefapirin were 5 and 9 ng L^{-1}, obtained respectively in hospital wastewater of Taiwan (Lin et al., 2008) and in an agriculturally influenced river in USA (Cha et al., 2006). However, when compared to other antibiotics, the frequency of reported cephalosporins' occurrence in the environment can be considered low (Cha et al., 2006; Junker et al, 2006; Tamura et al., 2017). According to Junker et al. (2006), this is mainly due to analytical limitations and to transformations of the parental structure. However, considering the wide use and importance of this antibiotic group, the knowledge of cephalosporins' environmental fate is insufficient (Eguchi et al., 2004; Ribeiro et al., 2017).

In human medicine, cephalosporins are mainly used as second- or third-line therapy (BPAC, 2011). However, in veterinary treatments cephalosporins are intensively used (Gilbertson et al, 1990; Salmon et al., 1996; Eguchi et al., 2004; WHO, 2014). Ceftiofur (CEF) and cefapirin (CEPA) are used especially for the treatment of common and recurrent animal diseases (Gilbertson et al, 1990; Salmon et al., 1996; Sadeghi-Sefidmazgi et al., 2011). The intramammary infection mastitis, which leads to relevant economic impacts in

the milk industry, is usually treated by cefapirin (Sadeghi-Sefidmazgi et al., 2011). Likewise, ceftiofur is used for treatment of a large range of respiratory, urinal and dermatologic diseases (Salmon et al., 1996). These drugs possess as core structure the 7-aminocephalosporanic acid, known as cephem ring, as well as substituents at C-3 and C7-position (Table 6.1), which are responsible for both the pharmacokinetic and antibacterial effects (Salmon et al., 1996). This antibiotic group has a broad activity spectrum presenting high efficiency towards both Gram-positive and Gram-negative bacteria by interrupting bacterial cell walls (EMA, 2012; EMA, 2015).

Several data suggested that abiotic transformations are more significant than biodegradation for the environmental degradation of cephalosporins in surface water and wastewater (Gilbertson et al., 1990, Jiang et al., 2010; Li et al., 2011). Hydrolysis is a relevant mitigation process due to the expected fast degradation of cephalosporins' side-chains and the beta-lactam ring cleavage (Yamana and Tsuji, 1976; Mitchell et al., 2014; Ribeiro et al., 2017). Wang and Lin (2012) studied photolysis of cephalosporins under simulated sunlight radiation and reported a significant increase of acute toxicity towards the luminescent bacteria *Vibrio fischeri.* Similarly, cefradine and its photo-degradation products presented adverse effects to chlorophyceae and cyanophyceae (Chen and Guo, 2012). Besides the importance and increasing use of ultraviolet radiation (UV-C) for water disinfection and wastewater purification, few data are available with regard to cephalosporins' fate during drink water process (Kim and Tanaka, 2009). In fact, even basic data reporting on ecotoxicity of cephalosporins and their transformation products is lacking (Zhang et al., 2010; Liu et al., 2011; Wang and Lin, 2012; Chen and Guo, 2012; Rahul et al., 2015; Magdaleno et al., 2015). This situation requires further investigation, once the presence of such compounds in aquatic systems cannot be neglected.

This work aims at the investigation of acute and chronic toxicities of CEPA and CEF. Complementary, the effects of different UV-C doses on CEPA and CEF ecotoxicity were assessed using two well established toxicity essays. Acute tests were carried out using the cladocera *Daphnia magna* (OECD, 2004; Zimmermann et al., 2017), while chronic tests were performed with the green algae *Scenedesmus spec.* (Eisentraeger et al., 2003; OECD, 2011; ISO, 2012). Knowing that cephalosporins' hydrolysis may interfere in exposure

concentrations and results interpretation, the whole investigation followed the recommendations for test validations presented in the respective OECD standard guidelines (OECD, 2000; OECD, 2004; OECD, 2011).

6.3. Materials and methods

6.3.1. Chemicals

The investigated cephalosporins CEPA (CAS# 24356-60-3) and CEF (CAS# 104010-37-9), both in the sodium salt forms and purity >98%, were purchased from Sigma Aldrich (Seelze, Germany) and Santa Cruz Biotechnology (California, USA), respectively. Table 6.1 provides further chemical information of CEPA and CEF. Monopotassium phosphate, dipotassium phosphate (Merck, Darmstadt, Germany), sodium hydroxide (Prolabo, Leuven, Belgium) and hydrochloric acid (Fischer Chemical, Leics, UK) were used for buffer preparation and pH correction. Acetonitrile (Fischer Chemical, Leics, UK), uridine, potassium permanganate and potassium dichromate (Sigma Aldrich, Seelze, Germany) were also used. All reagents were of analytical grade. If not specified, solutions were prepared with ultrapure water (UW) (Elga Purelab Flex, resistivity 15.5 ≈ MΩ-cm).

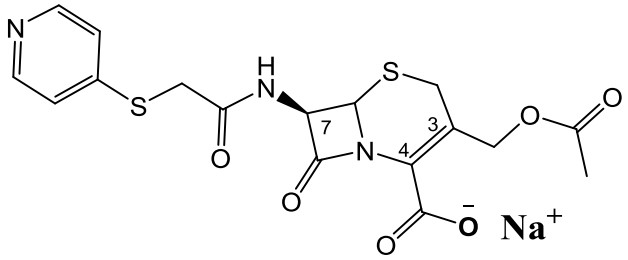
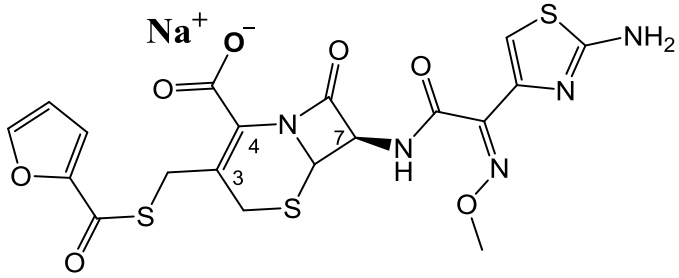
6.3.2. Photolytic transformation

To study the effects of water disinfection on cephalosporins, the anionic species of CEPA and CEF underwent UV-C photo-transformation according to Ribeiro et al., 2017. In brief, test solutions were prepared mixing antibiotic stock solution, phosphate buffer (pH 7) and UW. The resulting concentrations were 4 μM CEPA, 2 μM CEF and 5 mM buffer. Analyzing single cephalosporins, radiations were performed using a merry-go-round reactor (H&Th Schneider Glasapparatebau, Germany) coupled to a fluid-circulator (Ministat 125, Huber, Germany) for inside temperature control (25 ± 0.2°C). A 254 nm monochromatic low pressure mercury lamp (15 W, GPH303T5L/4, Hereaus, Germany) (185 nm band suppressed) was used as radiation source. The applied energy, measured as average fluence rate (E^0), was chemically determined by uridine actinometry, resulting in 62 ± 3 μeinstein m^{-2} s^{-1}, equivalent to 3 mJ cm^{-2} s^{-1}.

The pseudo-first-order degradation rates of CEPA and CEF (Table 6.1), previously determined by Ribeiro et al. (2017), were used for prediction of different depletion degrees (Supplementary Information). Accordingly, sampling was carried out after four radiation doses (0, 75, 150 and 300 mJ cm^{-2}), equivalent respectively to the following modeled antibiotic degradation: 0, 58, 82 and 97% for CEPA and 0, 52, 77 and 95% for CEF. Complementary, the treated samples were analyzed via HPLC-DAD for the target cephalosporins to confirm the resulting concentration.

Table 6.1

Description of investigated cephalosporins

Name Abbreviation	Chemical properties [a]	
Cefapirin CEPA		$C_{17}H_{16}N_3NaO_6S_2$ MW = 445.44 g mol^{-1} pK_{a1} = 2.74 (COOH → COO^-) [b] pK_{a2}= 5.13 (NH^+ → N) [b] k^{app} = 0.0116 mJ cm^{-2} [c]
Ceftiofur CEF		$C_{19}H_{16}N_5NaO_7S_3$ MW = 545.54 g mol^{-1} pK_{a1} = 2.68 (COOH → COO^-) [b] pK_{a2} = 3.53 (NH^+ → N) k^{app} = 0.0098 mJ cm^{-2} [c]

Notes:

[a] ChemAxom (2017), [b] Ribeiro and Schmidt (2017),
[c] pseudo-first order degradation rate (k^{app}), determined during photolytic transformation at pH ≈ 7 and 254 nm (Ribeiro et al., 2017)

6.3.3. Stability study

Considering the known aqueous instability of cephalosporins (Mitchell et al., 2014; Ribeiro et al. 2017) and the OECD guideline 23 determination (OECD, 2000), the stability of

CEPA and CEF under the conditions of ecotoxicological tests was assessed. Test media (descriptions below, item 6.3.4 and at Supplementary Information) were spiked with antibiotics in 50-mL volumetric flasks, resulting in 70 μM starting concentration. Next, solutions were split in 1.5-mL brown-glass vials, kept in darkness and controlled temperature (20 ± 1°C). Following, antibiotic concentration was determined every 3 hours for 2 days via HPLC-DAD. The resulting pseudo-first-order hydrolysis rate constants (k_{obs}) and respective half-life times ($t_{½}$) of both compounds were determined according to Ribeiro et al. 2017.

6.3.4. Toxicity testing

Ecotoxicological characterization of untreated and photodegraded drugs was performed. Therefore, the *Daphnia magna* acute toxicity test was accomplished using the DaphToxKit F^{TM} (MicroBioTests, Belgium) following the OECD guideline 202 (OECD, 2004). Animal maintenance, chemical stock solutions and sample dilutions were carried out using standard freshwater (SFW), which was prepared in accordance with ISO 6341 (ISO, 2010) by using stock vials available in the DaphToxKit F^{TM} (MicroBioTests, Belgium). SFW is constituted by calcium chloride dehydrate (294 mg L^{-1}), magnesium sulphate heptahydrate (123.25 mg L^{-1}), sodium hydrogen carbonate (67.75 mg L^{-1}) and potassium chloride (5.75 mg L^{-1}) dissolved in UW. To assess untreated cephalosporins, the powdered drugs were diluted in SFW using the following range: 0.01 to 1000 μM (CEPA) and 8 to 2000 μM (CEF). Treated solutions were serially diluted (5 to 85% of treated solutions) right after photolytic experiments. In both cases, the solvent SFW was aerated before use, which resulted in dissolved oxygen > 4 mg L^{-1} during the whole exposure period (48 h). Using 50-mL glass beakers, each experiment was performed with 4 replicates, each corresponding to 5 healthy neonates (< 24 h old) exposed to 10 mL of each concentration. Negative controls using only SFW were included. Subsequently, the test tray was kept in darkness at constant temperature (20 ± 1°C). Finally, cladocera immobilization and mortality were recorded after 24 and 48 h via visual observation under a stereomicroscope (Model SZX9, Olympus, Germany). As defined by OECD Guideline 202 (OECD, 2004), organisms unable to swim or to move antennae after gentle stimulation were considered motionless (see also

Zimmermann et al., 2017). Meanwhile, the lack of heart beat indicated animal death. A positive control with potassium dichromate was carried out to assess methodological reliability. The software Origin Pro9 was used for plotting dose-response curves and the respective lethal and effective concentrations ($LC_{20/50/80}$ and $EC_{20/50/80}$ values). Furthermore, data not fitted by probit analysis were reported qualitatively only.

Complementary, chronic toxicity tests were performed. The freshwater green algae *Scenedesmus spec.* was used for growth inhibition tests following the respective guidelines (OECD, 2011; ISO, 2012). Algae were maintained and harvested according to ISO 8692 (ISO, 2012), using a modified cultivation medium (AM) (Supplementary Information). The tests were performed in sterile 24-well polystyrene microplates (Cat. #10062-896, VWR, USA), with 2 mL sample in each well. For biomass determination, the chlorophyll content was measured via fluorescence using a multimode reader Infinite M200 (Tecan, Switzerland) and a conversion factor (Supplementary Information). Untreated antibiotics and treated samples were assessed using a similar procedure as used for acute toxicity. For both drugs, the tested concentration range was 9.8×10^{-4} to 98 μM, whereas treated samples were serially diluted (2.65 to 85% of treated solutions). In all cases, AM was used as solvent and the initial biomass ($\approx 10^6$ cells mL^{-1}) was included for experimental concentration calculations. Potassium permanganate and AM were used as positive and negative controls, respectively. Six replicates per concentration were inserted into the microplates using a pre-defined experimental design (Supplementary Information). Following, the initial biomass concentration in each well was confirmed via fluorescence measurements. Next, test plates were sealed with parafilm plus their original cover and incubated in a Celltron shaker (Infors AG, Switzerland). The incubation conditions were as follows: 100 rpm, 23 ±1°C and light intensity of 120 μeinstein m^{-2} s^{-1}, measured respectively by a thermometer HI 98128 (Hanna, USA) and a quantum-radiometer-photometer LI-185B (Li-Cor, USA) coupled to a LI-190SB quantum sensor. Biomass growth was measured daily using uncovered microplates and automatic 30s pre-shaking. Full parameters used for fluorescence measurements are provided in Supplementary Information. Growth rates and the percent inhibition were calculated according to OECD 201 (OECD, 2011). Response curves were plotted using the logarithm of tested

concentration and treated as described for acute toxicity. Before and after both ecotoxicological procedures, the tested samples were quantified via HPLC-DAD for the target cephalosporins.

6.3.5. Chemical quantification

A high performance liquid chromatography (HPLC) system (Shimadzu) equipped with a Prontosil 120 C-18 column (250 x 4 mm, 5 μm particle diameter, Bischoff) and a diode-array detector (DAD) was used for antibiotics quantification. Acetonitrile (A) and UW at pH 2 (adjusted with hydrochloric acid) (B) were used as mobile phase. The A:B ratios used were 10:90 and 25:75 isocratic for CEPA and CEF, respectively. Using a flow rate of 0.5 mL min^{-1} and injection volume of 100 μL, the obtained limits of quantification and detection, in nM, were respectively 439 and 145, for CEPA, and 913 and 345, for CEF.

6.4. Results and discussion

6.4.1. Photo-transformation experiments and antibiotic stability under testing conditions

The depletion of CEPA and CEF measured during disinfection-like photolysis was in the range of the values predicted from experimentally determined pseudo-first order rate constants (see Table 6.1). As demonstrated in Figure 6.1, both drugs showed a significant decay that increased with radiation dose. Therefore, the resulting samples presented different cephalosporin concentrations (Figure 6.1).

Hydrolysis experiments indicated significant instability of CEPA and CEF in SFW. According to the obtained pseudo-first-order hydrolysis rate constants and half-life times (Table 6.2), antibiotic concentrations will decrease by more than 20% at the end of toxicity tests. During tests in AM, hydrolysis showed low significance, at least for CEF. The expected decay due to hydrolysis after 72 hours of testing in AM is 17% for CEF and 44% for CEPA. Consequently, the aqueous instability of CEPA and CEF may affect the interpretation of ecotoxicological experiments. Since daphnia experiments were performed in darkness, photolysis was ruled out. Although the physical barriers of algae tests in

microplates may difficult photolysis, this process may occur due to constant lighting usage. In both tests, biodegradation can also contribute to drug decay. Therefore, cephalosporins were quantified after all experiments (see results below).

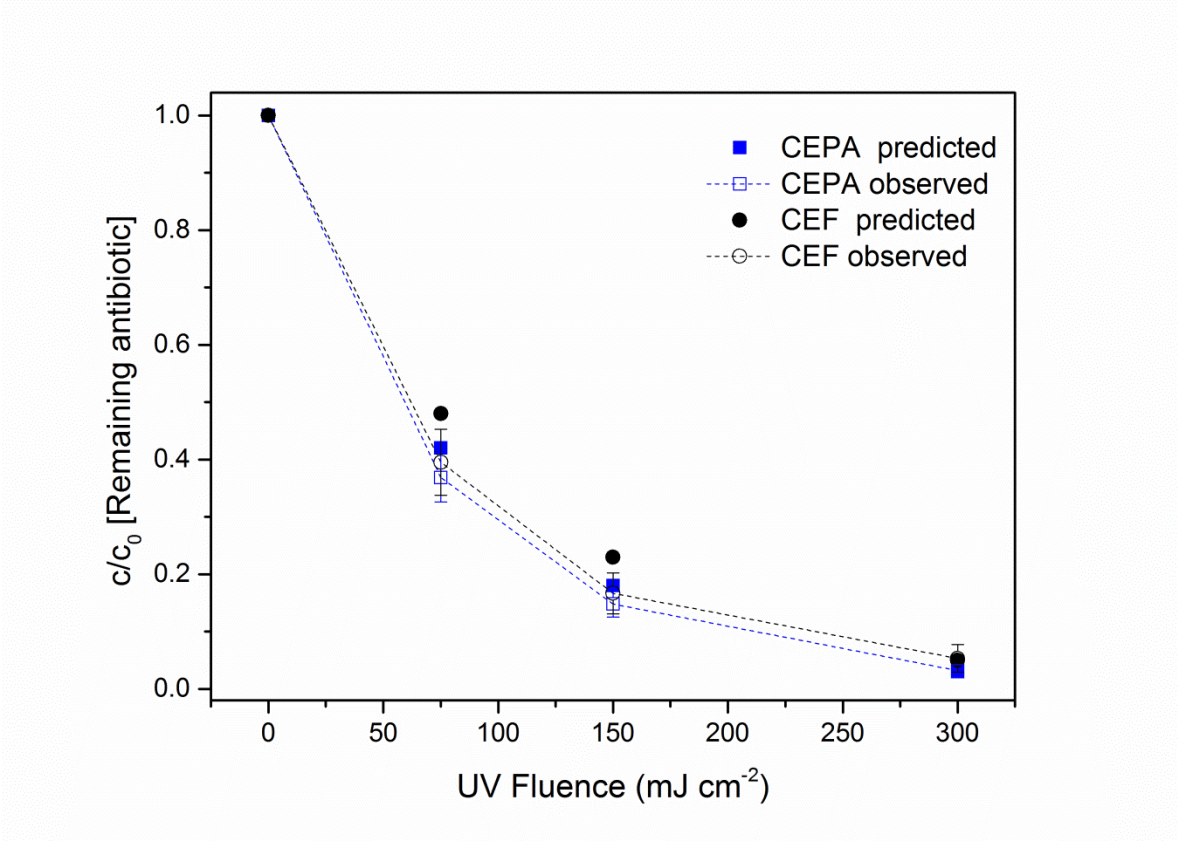


Figure 6.1. Remaining CEPA and CEF, in %, as a function of applied UV Fluence, in mJ cm^{-2}. Squares and circles represent respectively predicted (closed) and observed (open) degradation of CEPA and CEF.

A previous study indicated fast degradation rates of CEPA and CEF under disinfection UV-C photolysis (main emission 254 nm) (Ribeiro et al., 2017). As observed in Figure 6.1, the use of the known pseudo-first-order degradation rates of CEPA and CEF (Ribeiro et al., 2017) allowed a precise prediction of photolytic transformation of these two cephalosporins. As a result, samples containing different concentration of cephalosporin

and direct UV-C photo-degradation products were obtained. Therefore, it was possible to perform ecotoxicological study of both raw and photodegraded cephalosporins, simulating water disinfection.

Table 6.2

Cephalosporin stability in ecotoxicological media

	Hydrolytic parameters			
Drug	Solvent	k_{obs} in $hours^{-1}$	r^2	$t_{½}$ in hours
CEPA	SFW	0.014 ± 0.001[a]	0.95	49
	AM	0.008 ± 0.0001	0.99	88
CEF	SFW	0.013 ± 0.005	0.98	53
	AM	0.003 ± 0.0002	0.97	267

Notes:
[a] average and standard deviation; $t_{½}$ half-life, k_{obs} pseudo-first-order hydrolysis rate constant;
SFW standard freshwater; AM modified algal media

Hydrolysis half-life times obtained in our preliminary stability studies (Table 6.2) demonstrated that both CEPA and CEF are difficult substances for aquatic toxicity testing. Once both compounds degraded more than 20% in cladocera test system, organisms were exposed to a mixture of parental cephalosporins and hydrolysis by-products. Therefore, exposure concentrations were analyzed according to OECD 23 (2000) determination. The same was true for green algae tests with AM, where CEPA demonstrated high instability (Table 6.2).

Daphnia tests were carried out in darkness, indicating that drugs decay during test would be only related to hydrolysis and biotransformation. Differently, algal growth experiments were carried out under constant lighting. In this case, besides hydrolysis and assimilation, photolysis may also contribute to overall degradation. However, two important barriers minimize photolysis during algal tests. First, the fluorescent light source employed for incubation emits high wavelength radiation, which is less effective for cephalosporins

degradation (Wang and Lin, 2012). Second, parafilm and polystyrene covers used during microplates incubation act as radiation filters (Eisentraeger et al., 2003).

Photolysis and hydrolysis are important processes for aquatic contaminants abatement (Schwarzenbach et al., 2003). In fact, data concerning these processes are necessary for the understanding and validation of ecotoxicological results (OECD, 2004; OECD, 2011). Although OECD standard guidelines (OECD, 2004; OECD, 2011) recommend a preliminary stability study for all substances submitted to ecotoxicological characterization, the lack of such information is recurrent in many reports concerning cephalosporin toxicity (Zhang et al., 2010; Chen and Guo, 2012; Wang and Lin, 2012; Suárez, 2013; Magdaleno et al., 2015). For this antibiotic group the especial attention should be taken due to the expected quick degradation of beta-lactams in aqueous matrices (Yamaha and Tsuji, 1976, Michel et al., 2014, Ribeiro et al., 2007). The hydrolytic cleavage of substituents located at C-3 and C-7 of the cephalosporin structure (Table 6.1) is indicated as the main degradation process in natural waters (Yamaha and Tsuji, 1976). Depending on the ion concentrations present in the used organisms media, nucleophilic attack may also integrate cephalosporin depletion processes due to the presence of other nucleophiles such as NO_3^-, SO_4^{2-} and HCO_3^- (Schwarzenbach et al., 2003).

6.4.2. Baseline toxicity

Methodologies used both for acute and chronic tests fulfilled all validation criteria. In the case of daphnia tests, survival and mobility rates in control vessels were always above 90%. Besides, pH values were constant and oxygen was always > 4 mgO_2 L^{-1} in all samples. Sensitivity control test of *Daphnia magna* with potassium dichromate resulted in 24h EC_{50} values of 1.8 mg L^{-1}, which were in accordance to both the DaphToxKit F^{TM} and ISO 6341 (2010) acceptability range, 0.6 to 2.1 mg L^{-1}. The reliability of algae tests was also assessed. The average biomass increasing factor in negative control cultures was 19.3 ± 1.5 fold and the mean coefficient of variation for section-by-section specific growth rates was 8.5 ± 4%. Therefore, the OECD 201 (2011) and ISO 8692 (2012) validation criteria were match. However, a high background was observed in all tests, with growth inhibitions around 10% in all tested concentration. Considering this intra-test limitation, were

considered as significant only responses higher than 20% of inhibition and showing clear dose-dependence. Test reproducibility was checked by using positive controls (potassium permanganate, 15.8 μM), which demonstrated uniform responses measured by mean inhibition calculation, I = 50 ± 3.5% (N = 8). Detailed validation data are provided in Supplementary Information.

Table 6.3

Daphnia magna acute toxicity tests. 48 hours mortality and immobilisation percentages (LC and $EC_{20/50/80}$) calculated using nominal and corrected values. Corrections were carried out by subtraction of the measured decay (average values*) of each drug

	48 h, CEF, μM							
	NOMINAL				CORRECTED			
x	Immobility, EC_x		Mortality, LC_x		Immobility, EC_x		Mortality, LC_x	
20	133	(152 - 114)[a]	168	(193 - 143)	70	(80 - 60)	89	(102 - 76)
50	260	(288 - 232)	336	(375 - 297)	139	(153 - 125)	179	(200 - 158)
80	510	(608 - 412)	675	(819 - 561)	272	(323 - 221)	358	(435 - 281)

Notes:

[a] 95% confidence interval

For CEPA, 48h LC and $EC_{20/50/80}$ > 1000 μM (nominal) and >510 μM (corrected).

*Average degradation after 48 h (in %) 50 ± 8 for CEPA and 47 ± 4 for CEF.

Under our test conditions only CEF and its primary hydrolysis products presented acute toxicity to *Daphnia magna*. Although immobilised organisms were observed in the first 24 hours of exposure, this response affected less than 10% of the experimental group. Therefore, only mortality and immobilisation observed after 48 hours were measured and fitted by probit analysis. Figure 6.2 illustrates dose-responses obtained for both drugs, referring to nominal concentrations. In the end of testing time, cephalosporins were quantified in two tested concentrations (187.8 and 17.67 μM for CEF and 10 and 100 μM for CEPA) in all four replicates. CEPA samples showed 42 to 58% of the nominal concentration. In the case of CEF, 49 to 57% were the obtained values. These results agreed

with the decay observed in the preliminary tests of stability, confirming hydrolysis as the main degradation process of CEPA and CEF during ecotoxicological experiments. Real exposure values for both cephalosporins were calculated considering that all tested concentrations presented similar decay. LC and $EC_{20/50/80}$ values and confidence intervals calculated using nominal and corrected concentrations are compiled in Table 6.3.

As expected, cladocera immobilisation was the more sensitive endpoint, presenting higher incidence than lethality (Figure 6.2). For CEPA, no abnormal response was observed, indicating that both parental and primary hydrolysis by-products have no toxicity to *D. magna*.

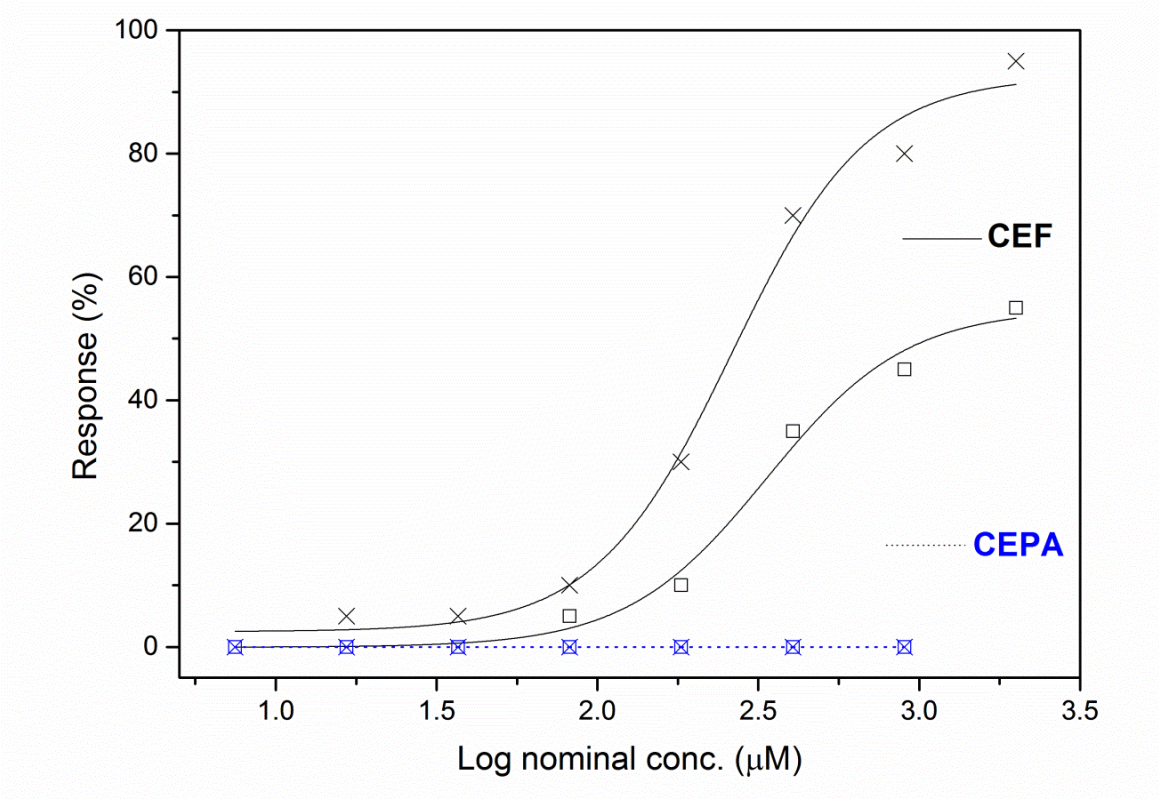


Figure 6.2. Cephalosporins acute toxicity (48h), comparative dose-response curves. Mortality (square) and Immobilisation (cross) of *Daphnia magna* exposed to CEPA (dotted line) and CEF (full line).

Chronic toxicity tests with *Scenedesmus spec.* did not indicate any significant adverse effects for both tested drugs and their primary hydrolysis products. As presented in Figure

6.3, the observed growth inhibition was always below 20%, showing no dose-dependence and moderated scattering. For both compounds, neither increased nor decreased toxicity with time was observed since the calculated average growth rates were steady during the full experimental period (Supplementary Information). CEPA and CEF were also quantified at the end of exposure in two tested concentrations (10 and 100 µM). After 72 hours, 80 to 85% of remaining CEF was detected. For CEPA, the values varied from 51 to 63%. These results are close to the expected decay (Table 6.2), confirming no other contribution in the cephalosporin degradation than hydrolysis. The resulted 72h NOAEC values are 57 ± 6 for CEPA and 82.5 ± 2.5 for CEF, both in µM and considering corrected concentrations, taking into account the observed drug degradation in the system.

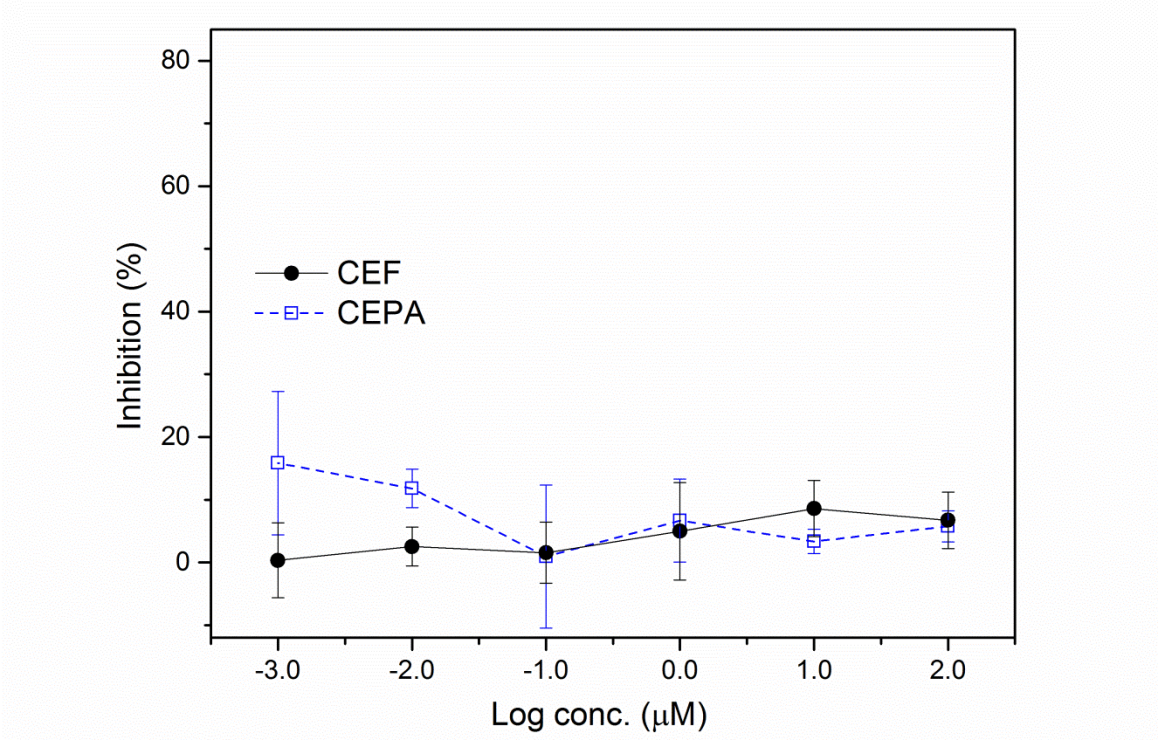


Figure 6.3. Cephalosporins chronic toxicity tests (72 hours), comparative dose-response curves. Growth of the green algae *Scenedesmus spec.* exposed to CEPA (open square) and CEF (closed circle).

The responses of *Daphnia magna* and *Scenedesmus spec.* to CEPA and CEF, observed in our study, corroborate the available data about cephalosporins' aqueous toxicity. Wang and Lin (2012) did not observe acute toxicity of CEPA to the luminescent bacteria *Vibrio fischeri* in all tested concentrations (15min EC_{50} > 450 mg L^{-1}). The same was observed by Li and Lin (2015), where CEPA 96h LC_{50} for the fish *Cyprinus carpio* was > 60.68 mg L^{-1}. Transforming CEPA corrected 48h LC $EC_{20/50/80}$ value (Table 6.3) from μM (>510) to mg L^{-1} (>227), one can observe that our result with *Daphnia magna* agrees with the available data. The corrected CEF 48h $EC_{20/50/80}$ values in mg L^{-1} are respectively 38, 79 and 148, respectively. To the best of our knowledge, these are the first reported ecotoxicological data for this antibiotic. Besides the cephem ring characteristic of all cephalosporins, CEF and cefepime share the same C-7 aminothiazole and imine groups (Table 6.1). For this similar drug, Bristol-Myers (2016) reported on *Daphnia magna* acute toxicity, being 640 mg L^{-1} the obtained 48h LC_{50} value. Similarly, Suárez (2013) reported on cefepime ecotoxicity, where no deleterious effects were observed (LC_x and EC_x >100 mg L^{-1}) for several organisms and endpoints (i.e. *Lactuca sativa*, *Eisenia foetida*, *Poecilia reticulate* and *Artemia salina*). Ecotoxicological investigations of cefotaxime (Wang and Lin, 2012; Suárez, 2013, Rahul et al., 2015), cefalexin (Brain et al., 2004; Wang and Lin, 2012; Li and Lin, 2015), cefradine (Chen and Guo, 2012), cefazolin (Eguchi et al., 2004; Zhang et al., 2010; Wang and Lin, 2012; Suárez, 2013; Li and Lin, 2015), cefalotin (Magdaleno et al., 2015), cefuroxime (GlaxoSmithKline, 2013; Suárez, 2013), cefotian (Al-Ahmad et al., 1999), cefazedone (Zhang et al., 2010), ceftaroline (EMA, 2012), ceftobiprole (Vestel et al., 2015), ceftolozane (EMA, 2015), ceftazidine (Suárez, 2013), and cefovecin (Zoetis, 2014) show low toxicity of cephalosporins. In general, no effects were observed or if observed, the response was only reported in concentrations of milligrams per liter, far from environmental values (Cha et al., 2006; Lin et al., 2008; Tamura et al., 2017; Biel-Maeso et al., 2018). Among the employed organisms, green blue algae showed the most restrictive values (EMA, 2012; EMA, 2015; Vestel et al., 2015).

Our results with CEF and CEPA corroborate the fact that apparently parental cephalosporins present low ecotoxicity to aquatic organisms. However, two points need to be highlighted. First, the available data about this antibiotic group is too scarce considering

the whole 53 drugs present in this group (WHO, 2017a; WHO, 2017b), the need of testing different trophic levels, and all possible toxicity interactions. Second, few authors investigated the toxicity of cephalosporin degradation products (Zhang et al., 2010; Wang and Lin, 2012) or drug stability during toxicity tests (Eguchi et al., 2004; Magdaleno et al., 2015).

6.4.3. Cephalosporins photo-transformation: acute and chronic toxicity

CEF photo-transformation products formed after three different radiation doses did not present acute toxicity to *Daphnia magna*. During all tests, neither immobility nor mortality was observed. On the other hand, photo-transformation of CEPA led to deleterious effects. As presented in Figure 6.4, once the radiation doses increased, the observed responses also increased. Both immobility and mortality were observed, being very similar (Figure 6.4). Daphnia responses slightly increased with exposure time. Even reporting the 48 h observation, immobility and mortality never exceeded 60% of the experimental group (Figure 6.4). Therefore, the generated data did not fit probit analysis and LC and EC values as well as confidence intervals could not be determined. Graphical analysis indicates a toxicity plateau after 150 mJ cm^{-2} dose, especially for the most concentrated samples. Therefore, even increasing test concentration, it is very unlikely to observe higher response. Observing the curves corresponding to 300 and of 150 mJ cm^{-2} (Figure 6.4), the tendency of toxicity stabilization is more evident. Cephalosporin quantification after cladocera tests was impeded due to the low starting drug concentration and their expected hydrolysis. CEPA and CEF values were below HPLC-DAD quantification and detection limits in all tested samples and any other pick was observed in the chromatography method used. Therefore, Daphnia tests were more sensitive than chemical analysis.

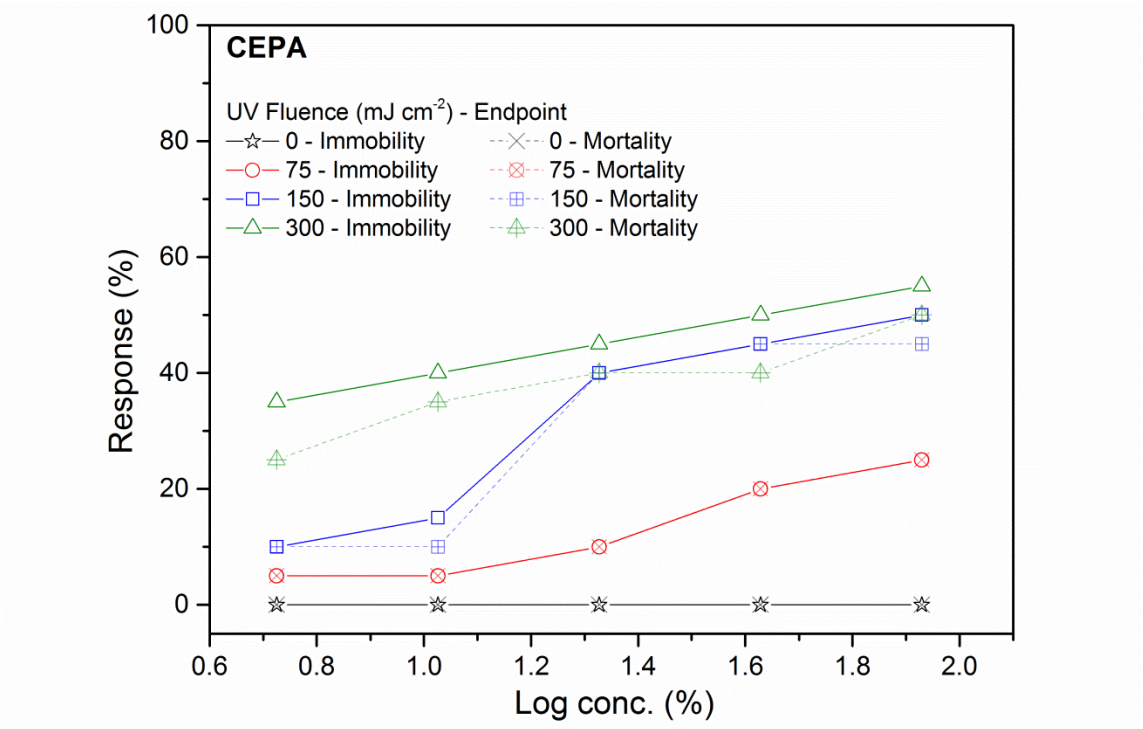


Figure 6.4. Effects of different UV radiation doses in the acute toxicity of CEPA. Immobility (line) and mortality (dot line) of *Daphnia magna* exposed for 48 h are shown for several samples (%) of CEPA after 0 (star), 75 (circle), 150 (square) and 300 (triangle) UV-C doses (in mJ cm^{-2}). X-axis shows serially diluted samples from right to left (85 to 5% (v/v) of treated solution in SFW).

Chronic tests with *Scenedesmus spec*. indicate no toxicity for both CEPA and CEF photo-degradation products. Independent of the radiation dose used, experiments presented moderated scattering and growth inhibitions always below 20%. As the inhibition curves of CEF (Figure 6.5) clearly shows, the two highest sample concentration (42.5 and 85%) led to a subtle increase of growth inhibition. The same is true for CEPA experiments (Figure 6.6), where all dose-response lines presented a slight increase in the highest tested concentration, except for 150 mJ cm^{-2} data. In fact, for both drugs the curves referring to samples before radiation (0 mJ cm^{-2}) show that samples dilution interferes in algal growth, mainly due to the resulting lack of nutrients. Although care was taken during dilution preparation, *Scenedesmus spec.* seems to be sensitive to lack of AM equal or higher than

56.5%. CEPA and CEF were quantified at the end of testing period. Similar to the observed in cladocera tests, final concentrations were below quantification and detection limits for both compounds in all tested samples.

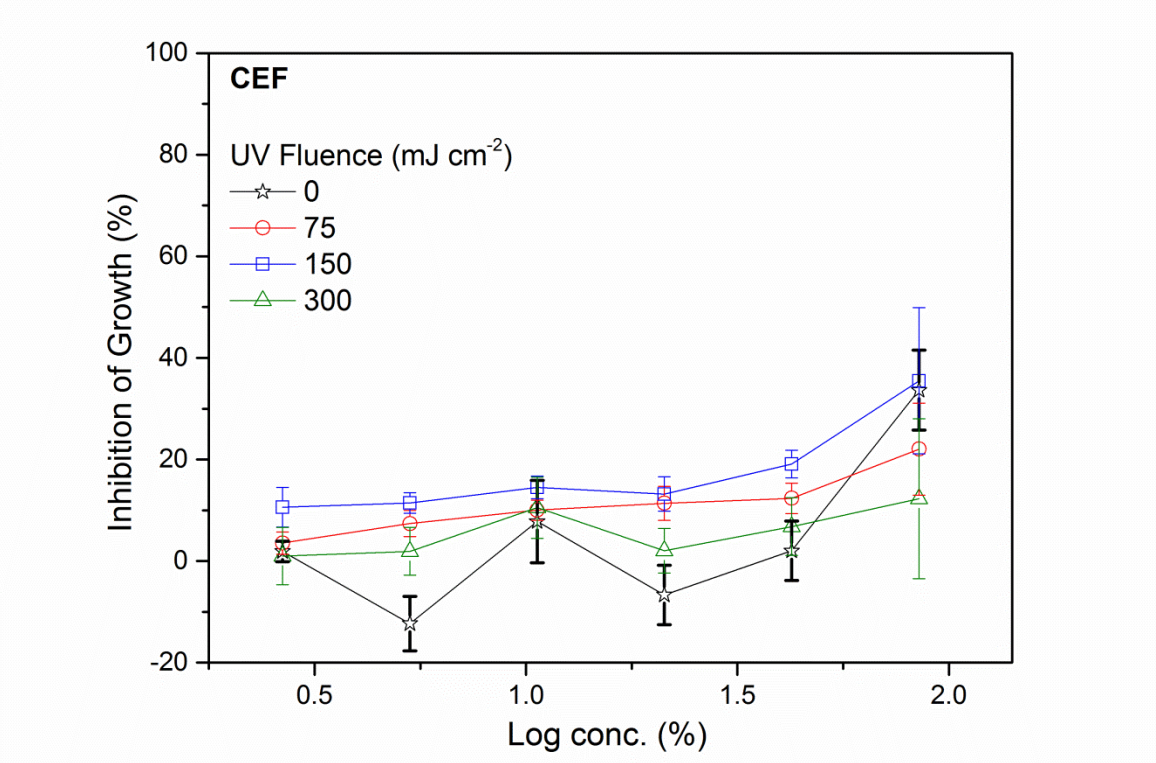


Figure 6.5. Effects of different UV radiation doses (in mJ cm^{-2}) on CEF chronic toxicity. Growth inhibition (72 hours) of *Scenedesmus spec.* exposed to diluted samples (%) of CEF after 0 (star), 75 (circle), 150 (square) and 300 (triangle) UV-C doses (in mJ cm^{-2}). X-axis shows serially diluted samples from right to left (85 to 2.65% (v/v) of treated solution in AM).

Despite sharing similar structure, cephalosporins may generate different photo-degradation products with different toxicities (Zhang et al., 2010; Wang and Lin, 2012). In our experiments, only CEPA photo-transformation led to acute toxicity to *Daphnia magna*. Moreover, toxic responses increased with UV-C doses increment (Figure 6.4). Indeed, Wang and Lin (2012) already reported relative increasing of CEPA acute toxicity after photo-degradation. Using *Vibrio fischeri* luminescence inhibition as endpoint, these authors

demonstrated that simulated-sunlight photolysis led to increased toxicity of several cephalosporins.

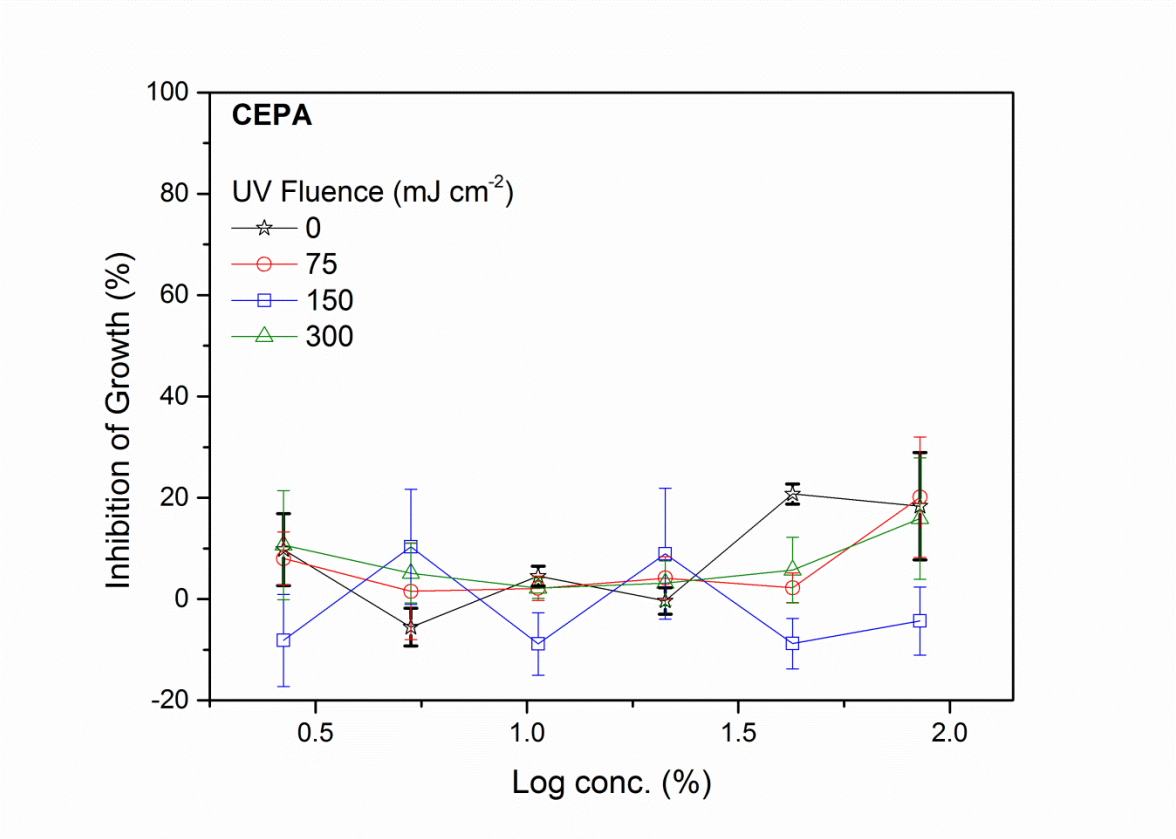


Figure 6.6. Effects of different UV radiation doses (in mJ cm^{-2}) on CEPA chronic toxicity. Growth inhibition (72 hours) of *Scenedesmus spec.* exposed to diluted samples (%) of CEPA after 0 (star), 75 (circle), 150 (square) and 300 (triangle) UV-C doses (in mJ cm^{-2}). X-axis shows serially diluted samples from right to left (85 to 2.65% (v/v) of treated solution in AM).

The toxic mechanisms involving cephalosporins' photoproducts are still unclear. After hydrolysis and photolysis, the cephem ring (cephalosporins core structure), may remain partially intact (Yamaha and Tsuji, 1976; Wang and Lin, 2012). The amide present in this moiety may integrate the process of reactive oxygen species generation (Rahul et al., 2015), which may lead to toxic effects. In living organisms, superoxide ion formation can cause oxidative stress as well as damage of DNA (Rahul et al., 2015). As demonstrated by Wang

and Lin (2012), the C-3 and C-7 substituents may also be involved in the formation of toxic photolysis products. Both CEPA and CEF photolysis products did not present toxicity to *Scenedesmus spec.*. The initial concentration of CEF employed for UV-C transformation was very small (i.e. 2 μM), near to environmental concentrations but far from the effective values observed for cladocera (Table 6.3). The growth of green algae cells is mainly affected by damage of the photosynthetic system, caused by chemical inhibition or interference in the chloroplast metabolism (Liu et al., 2011). Therefore, the effects observed with cladocera and CEPA photo-degradation products indicate a species-dependent response. Indeed, parental cefradine was more toxic to algae (Chen and Guo, 2012) than to bacteria (Wang and Lin, 2012). For ceftaroline, chronic toxicity tests with cladocera resulted in more restrictive values than green algae tests (EMA, 2012). This underlines the need of using different trophic level and species for cephalosporin investigations.

6.5. Conclusions

The present study demonstrates that CEPA and CEF would significantly hydrolyze under standardized toxicity test conditions. CEF and its first hydrolysis products presented acute toxicity to *Daphnia magna* but no chronic toxicity to *Scenedesmus spec.* in the tested conditions. However, effect concentrations are far away from values detected in aquatic environment. In the case of CEPA and its primary hydrolysis products, neither acute nor chronic toxicities were observed. Complementary, it was shown that UV-C radiation of CEPA generated toxic compounds, which affected the viability of *Daphnia magna* but not the growth of *Scenedesmus spec.*. Therefore, the need of assessing the aqueous toxicity of cephalosporins' transformation products as well as their removal from aquatic matrices is highlighted.

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7. Final conclusions and perspectives

This thesis should provide important reference data for the study of cephalosporin antibiotics as aquatic contaminants, with particular focus on the veterinary drugs ceftiofur and cefapirin. For the next years more investigations focusing on this group are expected.

Chapter 4 explored the lack of acid dissociation constant (pK_a) for many cephalosporins, fundamental physicochemical parameter that are involved in several biological and chemical processes. In fact, this issue was raised in the review provided in Chapter 3, where the need of discussing cephalosporins multifunctionality was demonstrated. The first experimental pK_a value for ceftiofur was reported (2.68 ± 0.05, carboxylic acid group deprotonation), as well as the previously reported pK_a values of cefapirin were confirmed (2.74 ± 0.01 for the carboxylic acid deprotonation and 5.13 ± 0.01 for the pyridinium ring deprotonation). Besides, the use of computational prediction (Marvin and ACD/Percepta packages) was shown as a promising tool for cephalosporins' pK_a determination. In Chapter 5, the environmentally relevant ionic species of cefapirin and ceftiofur, identified in Chapter 4, exhibited similar photo-degradation rates under UV-C (λ = 254 nm) radiation, presenting respectively the average k^{app} values of 0.0092 ± 0.001 and 0.0095 ± 0.0004 mJ cm^{-2}. Therefore, researchers are expected to further investigate the effects of cephalosporins ionic states during other environmental relevant processes. Moreover, the proper report of cephalosporins real charge used in environmental investigation is also expected in the future.

This thesis also presented the high importance of hydrolysis in the aqueous persistence of ceftiofur and cefapirin, which can be extended to other cephalosporins. Therefore, the possibility of hydrolysis needs to be accounted for during technical transformation (i.e. induced biodegradation, photolysis, chlorination, electrolysis, etc.); especially, when cephalosporins are exposed to alkaline environment or if long-term remediation technics/residence times are used. For example, Alexandrino et al. (2017) recently illustrated the contribution of abiotic degradation during the removal of ceftiofur in wetlands. Around 40% of the observed antibiotic decay after one month treatment was attributed to abiotic degradation (Alexandrino et al., 2017). The data generated in Chapter 5

also question the results presented by Wang and Lin (2012) concerning the photolysis of cefapirin during 45 hours under sunlight radiation. For this compound and this type of investigation, it is a prerequisite to present the decay in dark controls and the observed hydrolysis rates, which were not provided by the authors for this antibiotic even in the supplementary material.

Among the hot topics depicted in Chapter 3, the need of ecotoxicological assessment of both parental and transformed cephalosporins can be highlighted. In fact, Chapter 6 successfully illustrated that parental ceftiofur and cefapirin presented neither acute nor chronic toxicities respectively to *Daphnia magna* and *Scenedesmus spec*. However, after disinfection radiation (UV-C, λ = 254 nm) toxic cefapirin transformation products were formed, which led to deleterious effects to *Daphnia magna*. Recently, Serna-Gavis et al. (2017) showed the antimicrobial inactivation of cefalexin and cefadroxyl after UV-C, λ = 254 nm and UV-C/sulfate radical. Similar assessment of antimicrobial activity after cefapirin and ceftiofur UV-C photolysis has to be done. Furthermore, the ecotoxicological effects of parental and transformed cefapirin and ceftiofur on cyanobacteria, recently presented as more susceptive and more environmentally relevant organisms to antibiotics, are also needed (Le Page et al., 2017; Välitalo et al., 2017). Complementary, in Chapter 6 it was observed that both ceftiofur and cefapirin decayed more than 20% during cladocera and green algae tests. Therefore, the report of measured concentrations under ecotoxicological procedures needs to be mandatory for publications involving cephalosporins, but probably also many other compounds. The lack of synergic and antagonistic data of cephalosporins ecotoxicity is another future challenge, since such data is necessary for the hazard assessment of mixtures, as typically present in hospital and urban wastewaters (Marx et al., 2015).

One important limitation in the present study was the lack of assessment of antibacterial activity of ceftiofur and cefapirin after UV-C, as well as after hydrolysis in surface water (refers to Chapters 5 and 6). As recently pointed out by O'Neill (2016), it is urgent to minimize or neutralize the presence of active antimicrobial substances in the environment in order to reduce the growth and spread of antimicrobial resistance (AMR). Therefore, for future investigations it is suggested to include growth inhibition assays with both resistant

and sensitive bacteria after ceftiofur and cefapirin photolysis and surface water hydrolysis, as performed recently for other cephalosporins (Mitchell et al., 2015; Coladam et al., 2017).

7.1. References

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8. Annexes

8.1. Supplementary Information of Chapter 3

Cephalosporin antibiotics in the aquatic environment: A critical review on fate, occurrence, ecotoxicity and removal efficiency

Table S3.1

Representatives of each cephalosporins generation, non-ionic structures

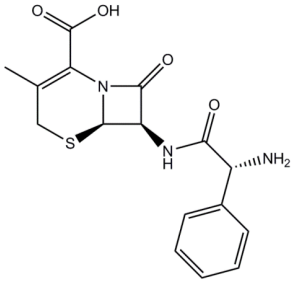
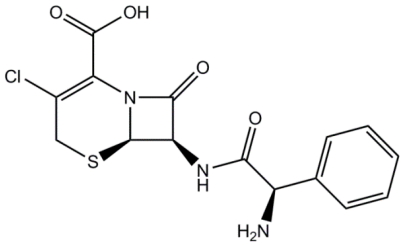
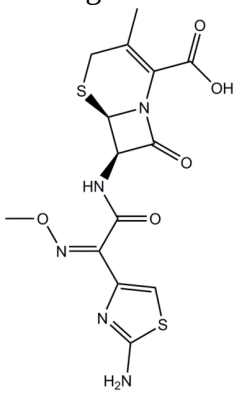
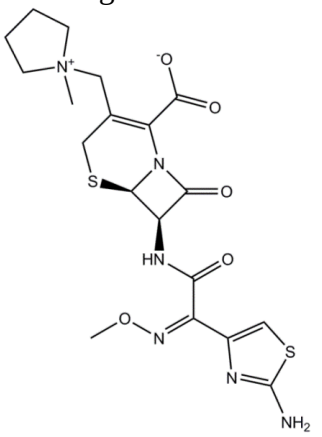
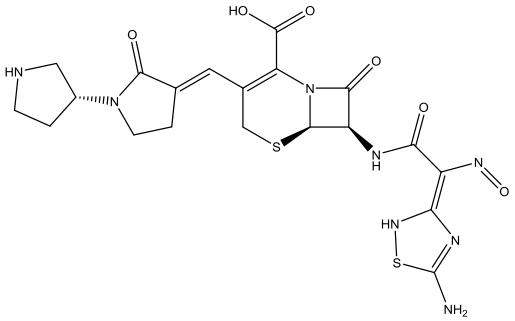
First-generation	Second-generation
	
cefalexin	**cefaclor**
Third-generation	Fourth-generation
	
cefotamet	**cefepime**
Others	
	
ceftobiprole	

Table S3.2

Reported ecotoxicological data of cephalosporin antibiotics

Cephalosporin	*Specie*	Taxon	Endpoints	Toxicity effect	Value in mg/L	Reference
Cefotaxime						
	Drosophila melanogaster	Diptera larvae	ß-Galactosidase activity, Lipid peroxidation, Glutathione content, Glutathione-S-transferase activity, Protein carbonyl content, Caspase 3 and 9 activities, midgut cell apoptosis, midgut cell tail length (Comet assay)	48h NOEC	20	Rahul et al., 2015
	Drosophila melanogaster	Diptera larvae	ß-Galactosidase activity, Lipid peroxidation, Glutathione content, Glutathione-S-transferase activity, Protein carbonyl content, Caspase 3 and 9 activities, midgut cell apoptosis, midgut cell tail length (Comet assay)	48h LOEC	40	Rahul et al., 2015
	Drosophila melanogaster	Diptera larvae	Total protein content	48h NOEC	10	Rahul et al., 2015
	Drosophila melanogaster	Diptera larvae	Total protein content	48h LOEC	20	Rahul et al., 2015
	Vibrio fischeri	Proteobacteria	Luminescence inhibition	15min EC50	>450	Wang and Lin, 2012
	Lactuca sativa	Plantae	Growth inhibition	120h EC50	>100	Suárez, 2013
	Eisenia foetida	Earthworm/ Annelida	Mortality	48h LC50	>100	Suárez, 2013
	Poecilia reticulata	Fish	Mortality	96h LC50	>100	Suárez, 2013
	Artemia salina	Crustacea	Mortality	24h LC50	>100	Suárez, 2013
	Physa cubensis	Mollusca	Mortality	96h LC50	>1000	Suárez, 2013
Cefalexin						
	Cyprinus carpio	Fish	Mortality	96h LC50	>60	Li and Lin, 2015
	Lemna gibba	Macrophyte	Wet mass, number of fronds, carotenoids, chlorophylls *a* and *b*	7d EC10	>1	Brain et al., 2004
	Lemna gibba	Macrophyte	Wet mass, number of fronds, carotenoids, chlorophylls *a* and *b*	7d EC25	>1	Brain et al., 2004
	Lemna gibba	Macrophyte	Wet mass, number of fronds, carotenoids, chlorophylls *a* and *b*	7d EC50	>1	Brain et al., 2004

	Vibrio fischeri	Proteobacteria	Luminescence inhibition	15min EC50	>450	Wang and Lin, 2012
Cefradine						
	Cyprinus carpio	Fish	Mortality	96h LC50	>60	Li and Lin, 2015
	Microcystis aeruginosa	Algae	Growth inhibition	72h EC50	1.38	Chen and Guo, 2012
	Scenedesmus obliquus	Algae	Growth inhibition	72h EC50	1.77	Chen and Guo, 2012
	Vibrio fischeri	Proteobacteria	Luminescence inhibition	15min EC50	>450	Wang and Lin, 2012
Cefapirin						
	Cyprinus carpio	Fish	Mortality	96h LC50	>60	Li and Lin, 2015
	Vibrio fischeri	Proteobacteria	Luminescence inhibition	15min EC50	>450	Wang and Lin, 2012
Cefazolin						
	Cyprinus carpio	Fish	Mortality	96h LC50	>60	Li and Lin, 2015
	Selenastrum capricornutum	Algae	Growth inhibition	72h EC50	>1000	Eguchi et al., 2004
	Selenastrum capricornutum	Algae	Growth inhibition	72h NOEC	>1000	Eguchi et al., 2004
	Vibrio fischeri	Proteobacteria	Luminescence inhibition	15min IC50	>450	Wang and Lin, 2012
	Danio rerio	Fish	Embryonic development-Teratogenic rate	72h NOEC	50	Zhang et al., 2010
	Danio rerio	Fish	Embryonic development-Teratogenic rate	72h LOEC	100	Zhang et al., 2010
	Lactuca sativa	Plantae	Growth inhibition	EC50	>100	Suárez, 2013
	Eisenia foetida	Earthworm/ Annelida	Mortality	48h LC50	>100	Suárez, 2013
	Poecilia reticulata	Fish	Mortality	96h LC50	>100	Suárez, 2013
	Artemia salina	Crustacea	Mortality	24h LC50	>100	Suárez, 2013
	Physa cubensis	Mollusca	Mortality	96h LC50	<1000£	Suárez, 2013
Cefepime						
	Daphnia magna	Cladocera	Mortality and Immobility	48h EC50	640	Bristol-Myers Squibb, 2016
	Lactuca sativa	Plantae	Growth inhibition	120h EC50	>100	Suárez, 2013
	Eisenia foetida	Earthworm/ Annelida	Mortality	48h LC50	>100	Suárez, 2013
	Poecilia reticulata	Fish	Mortality	96h LC50	>100	Suárez, 2013
	Artemia salina	Crustacea	Mortality	24h LC50	>100	Suárez, 2013
	Physa cubensis	Mollusca	Mortality	96h LC50	1000¢	Suárez, 2013
Cefalotin						
	Pseudokirchneriella subcapitata	Algae	Growth inhibition	72h EC10	76 ± 2	Magdaleno et al., 2015
	Pseudokirchneriella subcapitata	Algae	Growth inhibition	72h EC20	107 ± 5	Magdaleno et al., 2015
	Pseudokirchneriella	Algae	Growth inhibition	72h	>600	Magdaleno et al.,

	subcapitata			EC50		2015
Cefuroxime						
	Selenastrum capricornutum	Algae	Growth inhibition	72h EC50	>91	GlaxoSmithKline, 2013
	Daphnia magna	Crustacea	Mortality	48h EC50	>876	GlaxoSmithKline, 2013
	Oncorhyncus mykiss	Fish	Mortality	96h EC50	>105	GlaxoSmithKline, 2013
	Lactuca sativa	Plantae	Growth inhibition	120h EC50	>100	Suárez, 2013
	Eisenia foetida	Earthworm/ Annelida	Mortality	48h LC50	>100	Suárez, 2013
	Poecilia reticulata	Fish	Mortality	96h LC50	>100	Suárez, 2013
	Artemia salina	Crustacea	Mortality	24h LC50	>100	Suárez, 2013
	Physa cubensis	Mollusca	Mortality	96h LC50	>1000	Suárez, 2013
Cefazedone						
	Danio rerio	Fish	Embryonic development-Teratogenic rate	72h NOEC	50	Zhang et al., 2010
	Danio rerio	Fish	Embryonic development-Teratogenic rate	72h LOEC	100	Zhang et al., 2010
Ceftaroline						
	Anabaena flosaquae	Cyanobacteria	Grown inhibition	72h NOEC	0.0012	EMA, 2012
	Pseudokirchneriella subcapitata	Algae	Grown inhibition	72h NOEC	33	EMA, 2012
	Daphnia sp.	Cladocera	Reproduction	NOEC	7.9	EMA, 2012
	Fathead minnow(Pimephales promelas)	Fish	Early life stage toxicity	NOEC	5	EMA, 2012
Ceftobiprole						
	Daphnia	Cladocera	Mortality and Immobility	48h EC50	>46	Vestel et al., 2016
	Daphnia	Cladocera	*NA*	NOEC	>37	Vestel et al., 2016
	NA	Fish	Mortality	96h EC50	>50	Vestel et al., 2016
	NA	Fish	*NA*	96h NOEC	>5.6	Vestel et al., 2016
	NA	Cyanobacteria	*NA*	72h EC50	0.00105	Vestel et al., 2016
	NA	Cyanobacteria	*NA*	72h NOEC	0.00022	Vestel et al., 2016
Ceftolozane						
	Anabaena flosaquae	Cyanobacteria	Growth inhibition	72h EC10	0.15	EMA, 2015
	Daphnia sp.	Cladocera	Reproduction Test	NOEC	7,4	EMA, 2015
	Fathead minnow(Pimephales promelas)	Fish	Early life stage toxicity	NOEC	7,4	EMA, 2015
Ceftazidime						
	Eisenia foetida	Earthworm/Annelida	Mortality	48h LC50	0.032	Suárez, 2013
	Lactuca sativa	Plantae	Growth inhibition	120h EC50	>100	Suárez, 2013
	Poecilia reticulata	Fish	Mortality	96h	>100	Suárez, 2013

				LC50		
	Artemia salina	Crustacea	Mortality	24h LC50	>100	Suárez, 2013
	Physa cubensis	Mollusca	Mortality	96h LC50	>1000	Suárez, 2013
Cefovecin						
	Daphnia magna	Cladocera	Mortality and immobility	48h EC50	>1000	Zoetis, 2014
	Mysidopsis bahia	Shrimp	Mortality	48h LC50	580	Zoetis, 2014
	Cyprinodon variegatus	Fish	Mortality	48h LC50	770	Zoetis, 2014

Notes:
NA = not available; ¢ = at this concentration was observed 53.33% mortality; £ = at this concentration was observed 86.66% mortality

Table S3.3

Occurrence of cephalosporin antibiotics in aqueous matrices – reported data

Cephalosporin	Detected concentrations (ng/L)	Sampling site and details	Country	Reference
Cefalexin	2.0 ± 0.3*	Toucian River, upstream an hospital WWTP	Taiwan	Li and Lin, 2015
	22.6 ± 2.7*	Toucian River, downstream an hospital WWTP	Taiwan	Li and Lin, 2015
	2228 ± 205*	Raw wastewater of Zhudong hospital	Taiwan	Li and Lin, 2015
	2457	Hospitals wastewater	Taiwan	Lin et al., 2008
	27	Wastewater of pharmaceutical manufacturer	Taiwan	Lin et al., 2008
	283	Sewage Treatment Plants	Taiwan	Lin et al., 2008
	610	Regional Sewage discharges	Taiwan	Lin et al., 2008
	12	Wastewater of aquacultures	Taiwan	Lin et al., 2008
	1564 – 4367 $ (2965*)	Influent WWTPs	Taiwan	Lin et al., 2009
	10 - 994 $ (502*)	Effluent WWTPs	Taiwan	Lin et al., 2009
	2000	Influent STP	Australia	Costanzo et al., 2005
	78.2	Effluent STP	Australia	Costanzo et al., 2005
	15.4	0m Downstream STP	Australia	Costanzo et al., 2005
	20.4	50m Downstream STP	Australia	Costanzo et al., 2005
	26.8	500m Downstream STP	Australia	Costanzo et al., 2005
	15.1	Seawater sampling	China	Gulkowska et al., 2007
	10.8	Seawater sampling	China	Gulkowska et al., 2007
	10	Seawater sampling	China	Gulkowska et al., 2007
	47	Seawater sampling	China	Gulkowska et al., 2007
	182	Seawater sampling	China	Gulkowska et al., 2007
	34.5	Seawater sampling	China	Gulkowska et al., 2007
	41.2	Seawater sampling	China	Gulkowska et al., 2007
	4100	Hospitals effluent	Australia	Watkinson et al., 2009
	2800	Influent WWTPs	Australia	Watkinson et al., 2009
	1200 ± 18*	Influent Wan Chai STP	China	Gulkowska et al., 2008
	980 ± 6*	Effluent Wan Chai STP	China	Gulkowska et al., 2008
	670 ± 13*	Influent Tai Po STP	China	Gulkowska et al., 2008
	270 ± 5.7*	Effluent Tai Po STP	China	Gulkowska et al., 2008
	2900 ± 3.5*	Influent Shatin STP	China	Gulkowska et al., 2008

330 ± 13*	Effluent Shatin STP	China	Gulkowska et al., 2008
1900 ± 8*	Influent Stonecutters Island STP	China	Gulkowska et al., 2008
1800 ± 3*	Effluent Stonecutters Island STP	China	Gulkowska et al., 2008
1080 ± 72*	Influent Stonecutters Island STP	China	Leung et al., 2012
1290 ± 159*	Effluent Stonecutters Island STP	China	Leung et al., 2012
1290 ± 15*	Influent Shatin STP	China	Leung et al., 2012
178 ± 10*	Effluent Shatin STP	China	Leung et al., 2012
1130 ± 28*	Influent Tai Po STP	China	Leung et al., 2012
295 ± 12*	Effluent Tai Po STP	China	Leung et al., 2012
3750 ± 894*	Influent Central STP	China	Leung et al., 2012
3600 ± 976*	Effluent Central STP	China	Leung et al., 2012
5040 ± 856*	Influent Wan Chai East STP	China	Leung et al., 2012
4890 ± 262*	Effluent Central East STP	China	Leung et al., 2012
4540 ± 587*	Influent Wan Chai West STP	China	Leung et al., 2012
2990 ± 85*	Effluent Central West STP	China	Leung et al., 2012
4750 ± 198*	Influent North Point West STP	China	Leung et al., 2012
5000 ± 28*	Effluent Central West STP	China	Leung et al., 2012
0.55	Surface water Liobregat River campaign 1	Spain	Osório et al., 2016
0.40	Surface water Liobregat River campaign 2	Spain	Osório et al., 2016
0.52	Surface water Ebro River campaign 1	Spain	Osório et al., 2016
0.63	Surface water Ebro River campaign 2	Spain	Osório et al., 2016
0.40	Surface water Júcar River campaign 1	Spain	Osório et al., 2016
1.40	Surface water Júcar River campaign 2	Spain	Osório et al., 2016
0.40	Surface water Guadalquivir River campaign 1	Spain	Osório et al., 2016
0.40	Sediment Llobregat River campaign 1	Spain	Osório et al., 2016
0.40	Sediment Ebro River campaign 1	Spain	Osório et al., 2016
0.40	Sediment Júcar River campaign 1	Spain	Osório et al., 2016
0.40	Sediment Guadalquivir River campaign 1	Spain	Osório et al., 2016
1539 – 28889 $	Influent WWTPs	Taiwan	Lin et al., 2010
1290	Effluent Stonecutters Island STP	China	Minh et al., 2009
220	Effluent Shatin STP	China	Minh et al., 2009
294	Effluent Tai Po STP	China	Minh et al., 2009
3600	Effluent Central STP	China	Minh et al., 2009
4890	Effluent Wan Chai East STP	China	Minh et al., 2009
3000	Effluent Wan Chai West STP	China	Minh et al., 2009
5000	Effluent North Point STP	China	Minh et al., 2009
7	Seawater sampling - Stonecutters Island	China	Minh et al., 2009
6.1	Seawater sampling - Stonecutters Island	China	Minh et al., 2009
8.6	Seawater sampling - Central	China	Minh et al., 2009
139 ± 52*	Seawater sampling – Wan Chai	China	Minh et al., 2009
19.8 ± 0.2*	Seawater sampling – Wan Chai	China	Minh et al., 2009
6.6	Seawater sampling – Wan Chai	China	Minh et al., 2009
23	Seawater sampling – Wan Chai	China	Minh et al., 2009
28	Seawater sampling – Wan Chai	China	Minh et al., 2009
57	Seawater sampling – Causeway Bay	China	Minh et al., 2009
63	Seawater sampling – North Point	China	Minh et al., 2009
14.5	Seawater sampling – North Point	China	Minh et al., 2009

	72	Seawater sampling – Kwun Tong	China	Minh et al., 2009
	88	Seawater sampling – Kwun Tong	China	Minh et al., 2009
	300	Seawater sampling – Kwun Tong	China	Minh et al., 2009
	493 ± 27*	Seawater sampling – Kwun Tong	China	Minh et al., 2009
	63	Seawater sampling – Kwun Tong	China	Minh et al., 2009
	15	Seawater sampling – Kwun Tong	China	Minh et al., 2009
	15.5	Seawater sampling – Shau Kei Wan	China	Minh et al., 2009
	15	Seawater sampling – Shau Kei Wan	China	Minh et al., 2009
	17	Seawater sampling – Chai Wan	China	Minh et al., 2009
	1718 ± 130*	Influent Shatin WWTP	China	Li and Zhang, 2011
	658 ± 49*	Influent Shatin WWTP	China	Li and Zhang, 2011
	857 ± 25*	Influent Shatin WWTP	China	Li and Zhang, 2011
	1164 ± 6*	Influent Shatin WWTP	China	Li and Zhang, 2011
	1176 ± 110*	Effluent Shatin WWTP	China	Li and Zhang, 2011
	215 ± 7*	Effluent Shatin WWTP	China	Li and Zhang, 2011
	142 ± 5*	Effluent Shatin WWTP	China	Li and Zhang, 2011
	807 ± 19*	Effluent Shatin WWTP	China	Li and Zhang, 2011
	525 ± 30*	Influent Stanley WWTP	China	Li and Zhang, 2011
	65.7 ± 5*	Influent Stanley WWTP	China	Li and Zhang, 2011
	252 ± 1*	Influent Stanley WWTP	China	Li and Zhang, 2011
	434 ± 23*	Influent Stanley WWTP	China	Li and Zhang, 2011
	20.7 ± 1*	Effluent Stanley WWTP	China	Li and Zhang, 2011
	7.2 ± 1*	Effluent Stanley WWTP	China	Li and Zhang, 2011
	31 ± 0.8*	Effluent Stanley WWTP	China	Li and Zhang, 2011
	41.7 ± 2.4*	Effluent Stanley WWTP	China	Li and Zhang, 2011
	539.4 ± 20.2*	Influent Shatin WWTP	China	Li et al., 2009
	375.6 ± 19.7*	Effluent Shatin WWTP	China	Li et al., 2009
	175.4 ± 8.3*	Influent Stanley WWTP	China	Li et al., 2009
	4600	Influent WWTP	Australia	Watkinson et al., 2007
	10	Surface water of Qiantang River	China	Chen et al., 2012
	2905 ± 530*	Influent municipal wastewater -	Taiwan	Dutta et al., 2014
	166	Influent WWTP	China	Zhang et al., 2013
	29	Effluent WWTP	China	Zhang et al., 2013
	57 ± 4.5*	Influent WWTP	China	Zhang et al., 2013
	83 ± 7.5*	Influent WWTP	China	Zhang et al., 2013
	20	Influent WWTP	China	Zhang et al., 2013
	40 ± 4.4*	Influent WWTP	China	Zhang et al., 2013
	23	Sampling of an urban river in Dalian	China	Zhang et al., 2013
	30	Seawater sampling, 50 m away from WWTP discharge	China	Zhang et al., 2013
	18480 ± 4490	Influent cephalosporin factory WWTP	China	Yu et al., 2016
	920 ± 530	Effluent cephalosporin factory WWTP	China	Yu et al., 2016
Cefradine	6.0 ± 0.8*	Toucian River downstream of Zhudong hospital WWTP	Taiwan	Li and Lin, 2015
	166 ± 40*	Raw wastewater of Zhudong hospital	Taiwan	Li and Lin, 2015
	1	Wastewater of drug production facilities	Taiwan	Lin et al., 2008
	113	Hospitals wastewater	Taiwan	Lin et al., 2008
	12	Sewage Treatment Plants	Taiwan	Lin et al., 2008
	128	Regional Sewage discharges	Taiwan	Lin et al., 2008
	2	Wastewater of aquacultures	Taiwan	Lin et al., 2008
	819	Influent Municipal WWTP	Korea	Sim et al., 2011
	353	Effluent Municipal WWTP	Korea	Sim et al., 2011
	486	Influent Hospital WWTP	Korea	Sim et al., 2011
	353	Effluent Municipal WWTP	Korea	Sim et al., 2011
	486	Influent Hospital WWTP	Korea	Sim et al., 2011

	ND-2889$	Influent WWTPs	Taiwan	Lin et al., 2010
	51.2 ± 6*	Influent municipal wastewater - Anaerobic membrane bioreactor	Taiwan	Dutta et al., 2014
	125	Influent WWTP	China	Zhang et al., 2013
	34	Effluent WWTP	China	Zhang et al., 2013
	78 ± 4.7*	Influent WWTP	China	Zhang et al., 2013
	99 ± 9.8*	Influent WWTP	China	Zhang et al., 2013
	39	Influent WWTP	China	Zhang et al., 2013
	101 ± 13*	Influent WWTP	China	Zhang et al., 2013
	90	Sampling of an urban river in Dalian	China	Zhang et al., 2013
	30	Seawater sampling, 50 m away from WWTP discharge	China	Zhang et al., 2013
	5.3	Seawater sampling, 50 m away from WWTP discharge	China	Zhang et al., 2013
	51.2 ± 6*	Influent municipal wastewater	Taiwan	Dutta et al., 2014
	20160 ± 4650	Influent cephalosporin factory WWTP	China	Yu et al., 2016
	1020 ± 610	Effluent cephalosporin factory WWTP	China	Yu et al., 2016
Cefotaxime	0.3 ± 0.1*	Raw wastewater of Zhudong hospital	Taiwan	Li and Lin, 2015
	413	Hospitals wastewater	Taiwan	Lin et al., 2008
	7	Sewage Treatment Plants	Taiwan	Lin et al., 2008
	24 ± 0.1*	Influent Tai Po STP	Hong Kong	Gulkowska et al., 2008
	34 ± 3.2*	Effluent Shatin STP	Hong Kong	Gulkowska et al., 2008
	1100 ± 16*	Influent Shenshen Nan Shan STP	China	Gulkowska et al., 2008
	89	Wastewater of Josep Trueta hospital	Spain	Gros et al., 2013
	40 ± 5*	Influent Shatin STP	Hong Kong	Leung et al., 2012
	83-8793	Influent WWTPs	Taiwan	Lin et al., 2010
	93 ± 4.7*	Influent Shatin WWTP	China	Li and Zhang, 2011
	55.5 ± 2.4*	Influent Shatin WWTP	China	Li and Zhang, 2011
	38.4 ± 1.3*	Influent Shatin WWTP	China	Li and Zhang, 2011
	63.6 ± 1.6*	Influent Shatin WWTP	China	Li and Zhang, 2011
	56.7 ± 3.5*	Effluent Shatin WWTP	China	Li and Zhang, 2011
	36.1 ± 2.3*	Effluent Shatin WWTP	China	Li and Zhang, 2011
	24.6 ± 0.9*	Effluent Shatin WWTP	China	Li and Zhang, 2011
	24.1 ± 0.2*	Effluent Shatin WWTP	China	Li and Zhang, 2011
	1.88	Influent Almadinah Almunawarah STP	Saudi Arabia	Shraim et al., 2016
	1.53	Effluent Almadinah Almunawarah STP	Saudi Arabia	Shraim et al., 2016
	18080 ± 4200*	Influent cephalosporin factory WWTP	China	Yu et al., 2016
	550 ± 620*	Effluent cephalosporin factory WWTP	China	Yu et al., 2016
	85	Influent STP	Germany	Rossmann et al., 2014
	217 (max)	Effluent STP	Germany	Rossmann et al., 2014
Cefapirin	5	Hospitals wastewater	Taiwan	Lin et al., 2008
	9	Poudre River, agricultural area	USA	Cha et al., 2006
Ceftiofur	1.6	Effluent WWTP in Kyoto	Japan	Tamura et al., 2017
	0.9	Effluent sept tank in Tokushima	Japan	Tamura et al., 2017
	1.4	Coastal water – Sancti-Petri Channel	Spain	Biel-Maeso et al., 2018
	1.7	Coastal water – Sancti-Petri Channel	Spain	Biel-Maeso et al., 2018
Cefazolin	6221	Hospitals wastewater	Taiwan	Lin et al., 2008
	27	Sewage Treatment Plants	Taiwan	Lin et al., 2008
	5892	Regional Sewage discharges	Taiwan	Lin et al., 2008
	53	Wastewater of animal husbandries	Taiwan	Lin et al., 2008
	15	Aquacultures	Taiwan	Lin et al., 2008
	1186.3	Hospital wastewater	China	Chen et al., 2012

	130.6	Hospital wastewater	China	Chen et al., 2012
	10	Surface water of Qiantang River	China	Chen et al., 2012
	4905 ± 1236*	Raw wastewater of Zhudong hospital	Taiwan	Li and Lin, 2015
	12850 ± 1560*	Influent cephalosporin factory WWTP	China	Yu et al., 2016
	2730 ± 1430*	Effluent cephalosporin factory WWTP	China	Yu et al., 2016
Cefaclor	500	Influent WWTPs	Australia	Watkinson et al., 2009
	500	Influent WWTP	Australia	Watkinson et al., 2007
	9.4	Coastal water – Sancti-Petri Channel	Spain	Biel-Maeso et al., 2018
	5.9	Coastal water – Sancti-Petri Channel	Spain	Biel-Maeso et al., 2018
Cefdnir	1	Coastal water – Sancti-Petri Channel	Spain	Biel-Maeso et al., 2018
	15.8	Coastal water – Sancti-Petri Channel	Spain	Biel-Maeso et al., 2018
Cefquinome	1.8	Coastal water – Sancti-Petri Channel	Spain	Biel-Maeso et al., 2018
Ceftriaxone	15150 ± 3170*	Influent cephalosporin factory WWTP	China	Yu et al., 2016
	2030 ± 1110*	Effluent cephalosporin factory WWTP	China	Yu et al., 2016
Cefuroxime	141550 ± 4248*	Influent cephalosporin factory WWTP	China	Yu et al., 2016
	24380 ± 8820*	Effluent cephalosporin factory WWTP	China	Yu et al., 2016
	1586	Influent STP	Germany	Rossmann et al., 2014
	599	Effluent STP	Germany	Rossmann et al., 2014
Cefoxitin	13150 ± 3170*	Influent cephalosporin factory WWTP	China	Yu et al., 2016
	50 ± 80*	Effluent cephalosporin factory WWTP	China	Yu et al., 2016

Notes:
*average value and standard deviation; $ maximun-minimun

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8.2. Supplementary Information of Chapter 4

Determination of acid dissociation constants (pKa) of Cephalosporin antibiotics: computational and experimental approaches

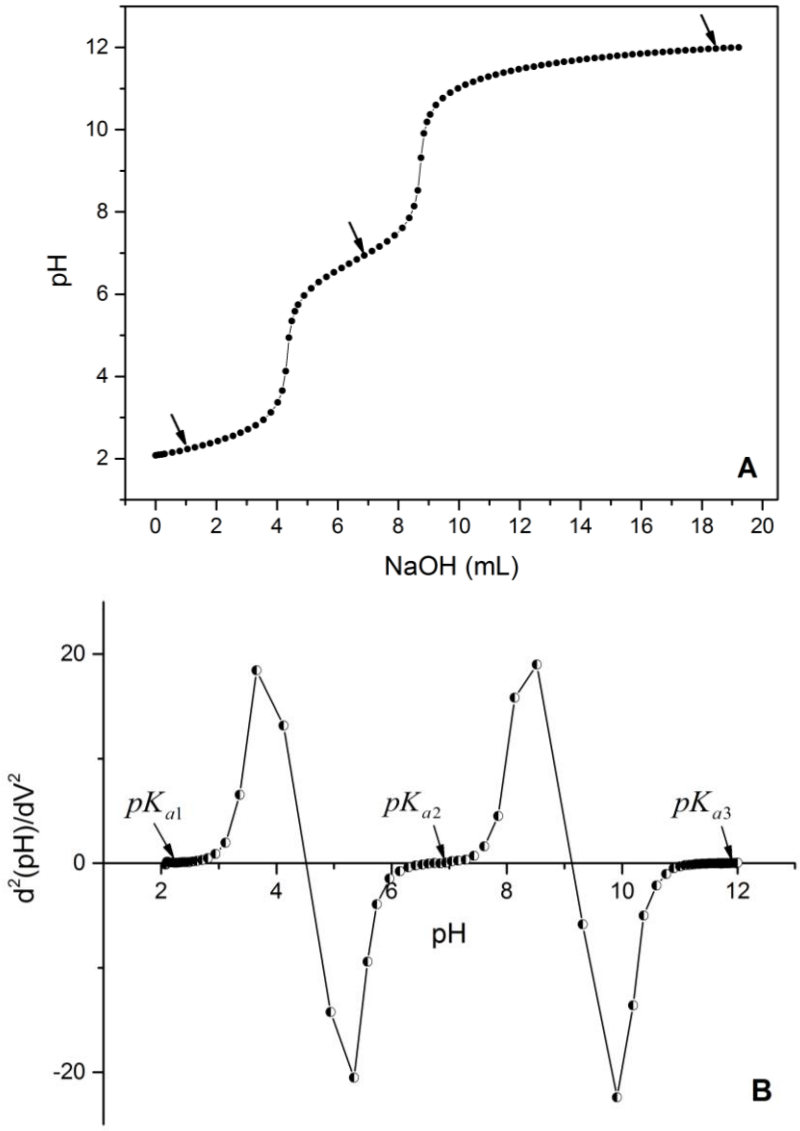


Figure S4.1. Example of the potentiometry titration of 1mM Phosphoric Acid. **A**) Poliprotic acid curve obtained after automatized titration and **B**) Second-Derivative curve, where each inflection point (f´(x)=0) represents the pK_a value.

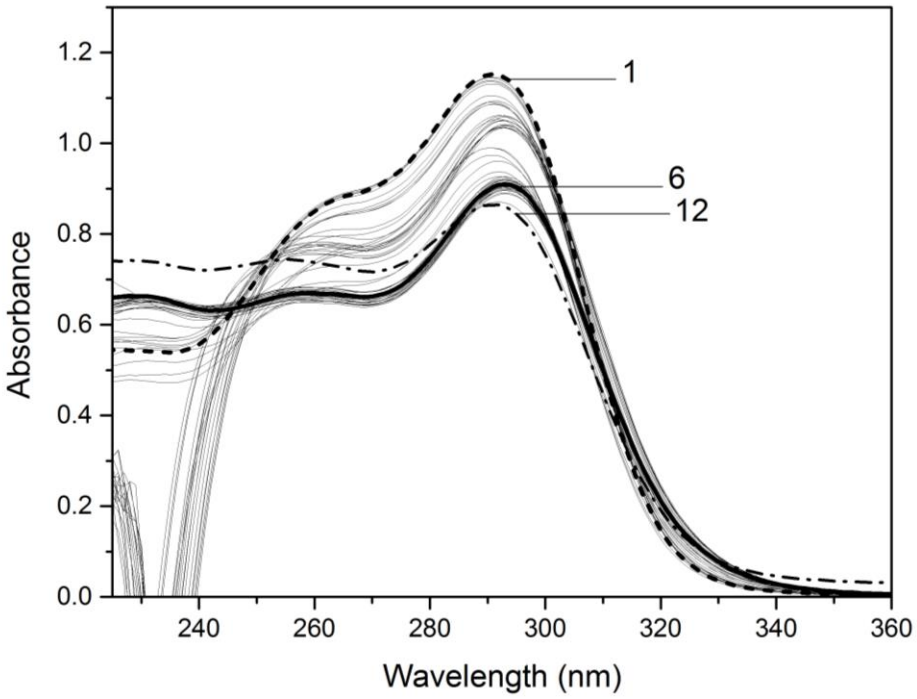


Figure S4.2. Spectrophotometric titration of ceftiofur. UV absorption spectra of $4.7x10^{-5}$M ceftiofur in the pH range 1-12 using two buffer systems (details see main paper). pH values 1, 6 and 12 are indicated in the figure.

Table S4.1

Obtained pK_a values and statistical parameters

	pK_{a1a}	pK_{a1b}	pK_{a1c}	pK_{a1d}	Mean pK_a	Error	Order of accuracy	Standard deviation
Cefapirin								
Carboxylic	2.746	2.757	2.738	--	2.747	0.0115	0.004	0.0078
Pyridine	5.142	5.167	5.081	--	5.131	0.0560	0.110	0.0361
Ceftiofur								
Carboxylic	2.733	2.718	2.598	2.673	2.684	0.0675	0.025	0.0526
Phosphoric Acid								
p*Ka* 1	2.277	2.291	2.310	--	2.293	0.02	0.009	0.0135
p*Ka* 2	7.062	7.002	7.008	--	7.052	0.056	0.008	0.0364
p*Ka* 3	12.002	12.013	12.010		12.008	0.008	0.003	0.014

Notes:
1a, 1b, 1c and 1d represent each replicate, -- No data

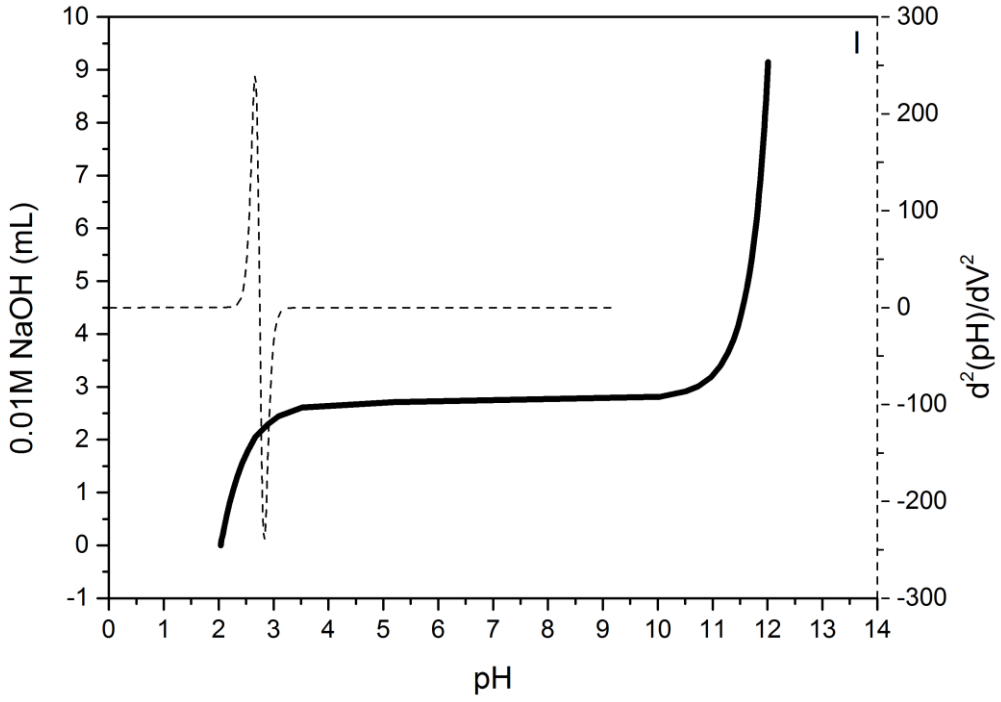


Figure S4.3. Potentiometric titration of 5.8×10^{-5} M cefapirin (solid curve) and second-derivative plot (dashed curve). The inflection point indicates the obtained mean pK_{a1} 2.74 ± 0.01.

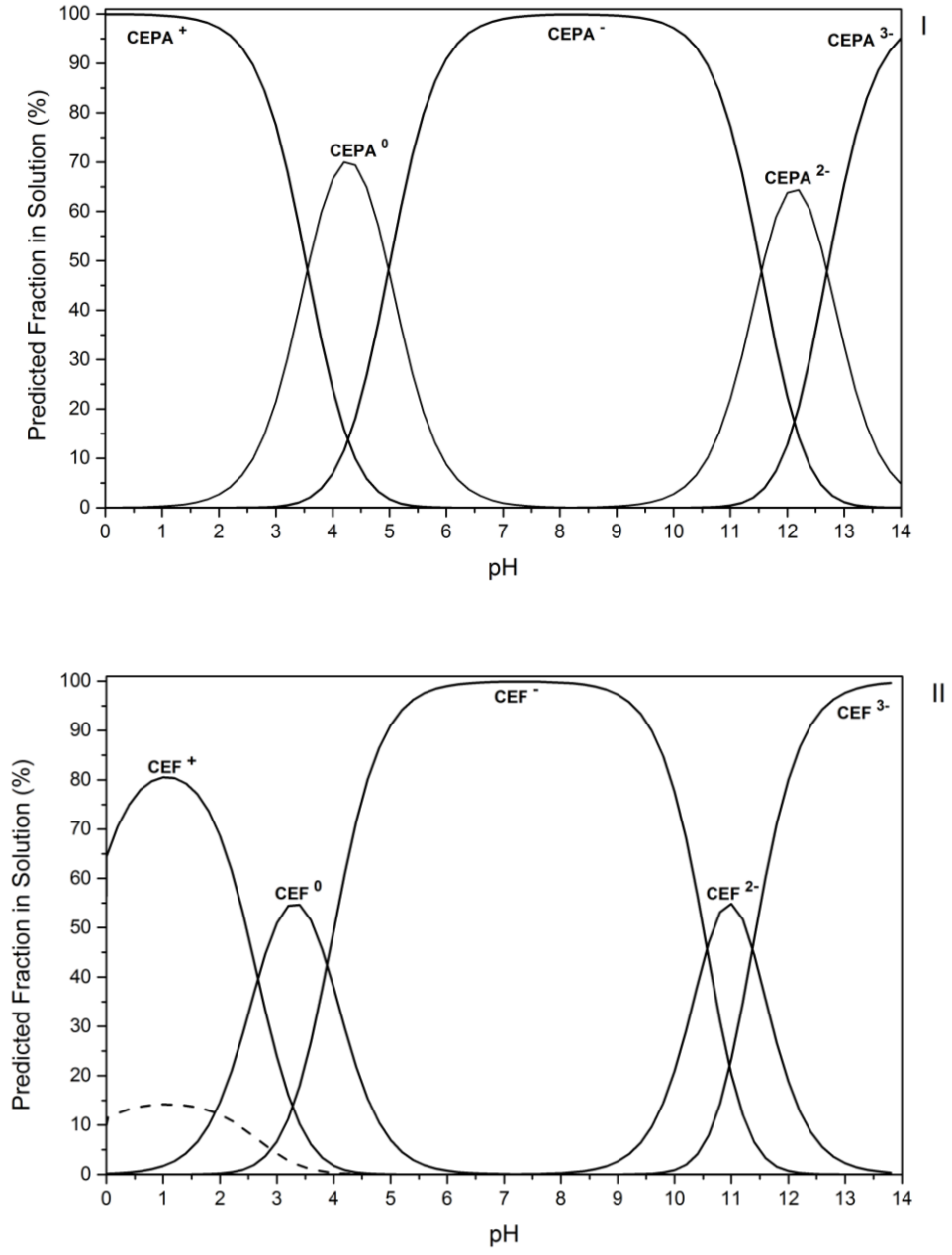
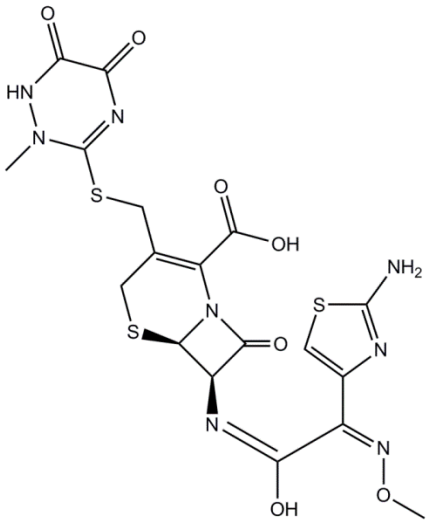


Figure S4.4. Marvin's predicted speciation diagram of Cefapirin (**I**) and Ceftiofur (**II**) in solution as a function of pH. Dashed curve in **II** represents the protonated aminothiazole specie fraction, indicated as microspecie in this software. **Note**: For further information how speciation diagrams are calculated, please see detailed equations in Streng (1977).

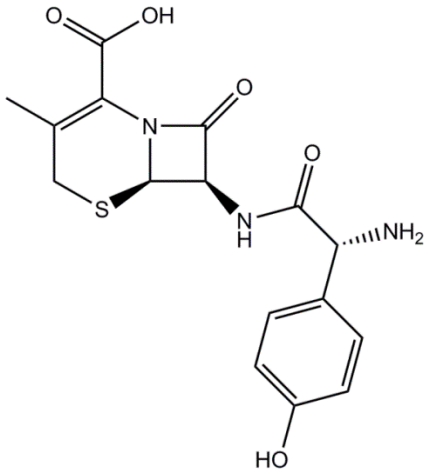
Table S4.2

Non-ionic species of cephalosporin antibiotics used for pK_a prediction

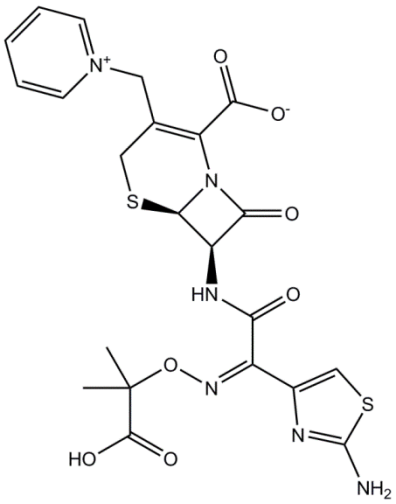
Ceftriaxone



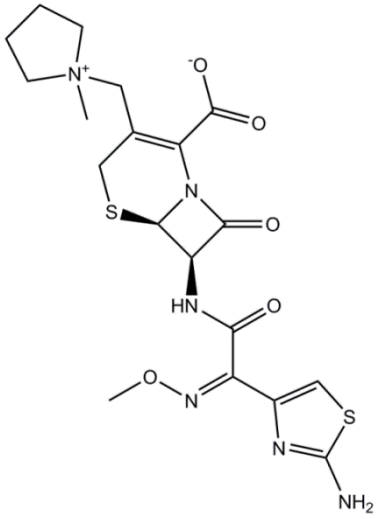
Cefadroxil



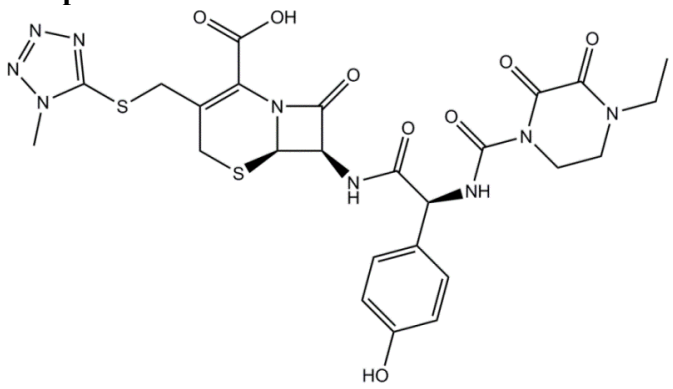
Ceftazidime



Cefepime

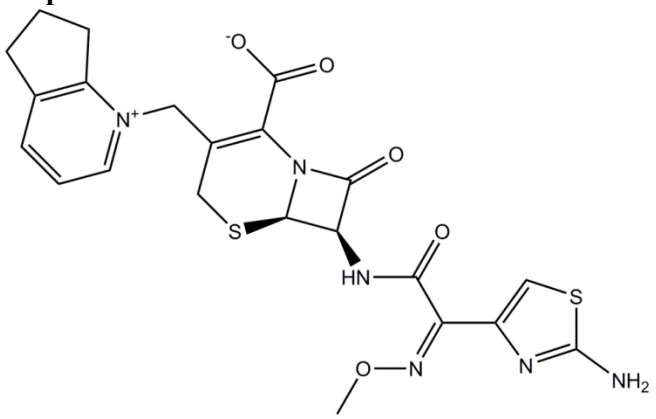


Cefoperazon

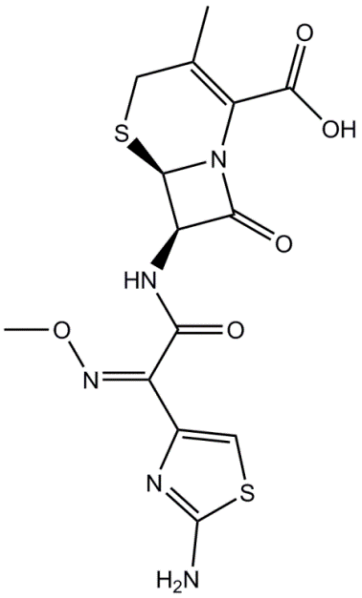


Continuation of Table S4.2

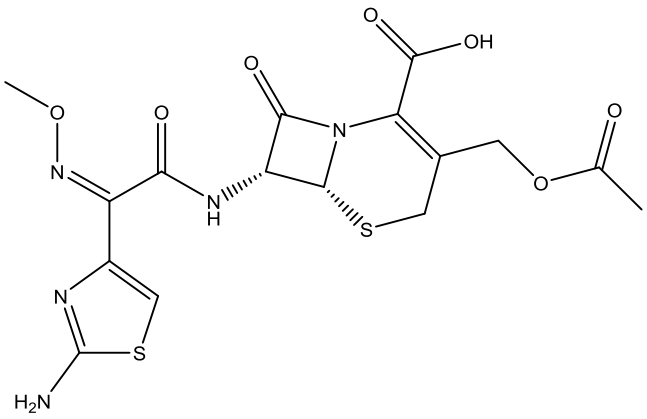
Cefpirome



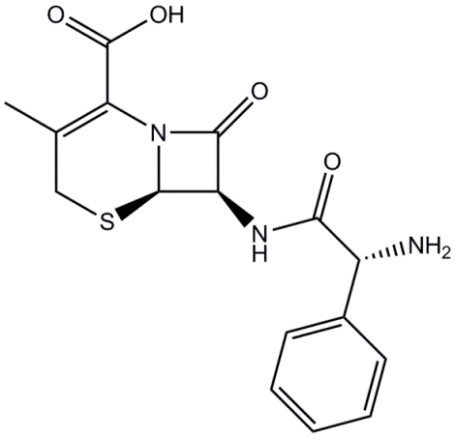
Cefetamet



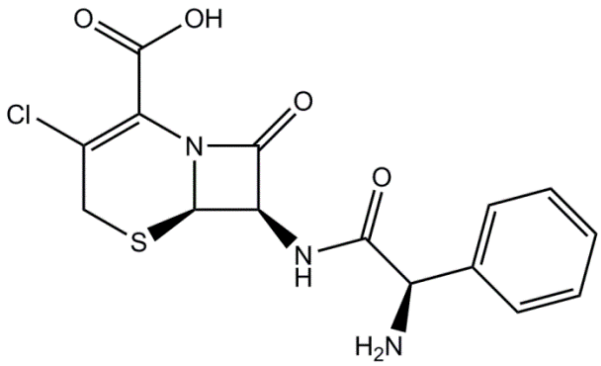
Cefotaxime



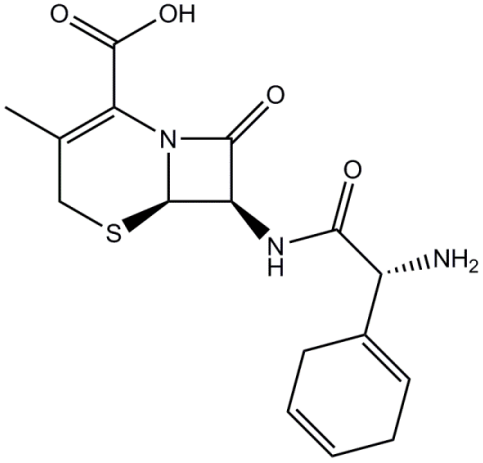
Cefalexin



Cefaclor

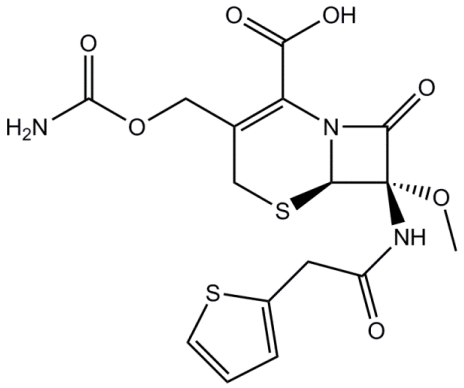


Cefradine

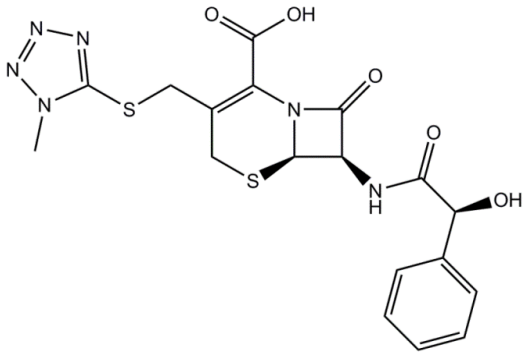


Continuation of Table S4.2

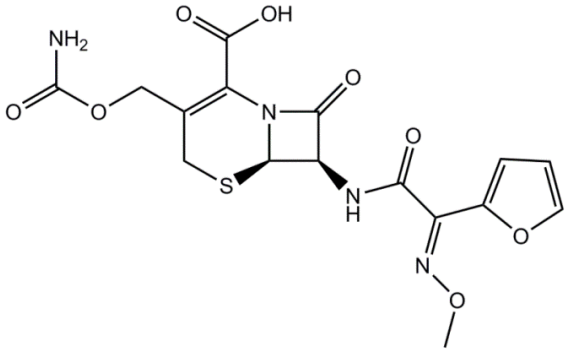
Cefoxitin



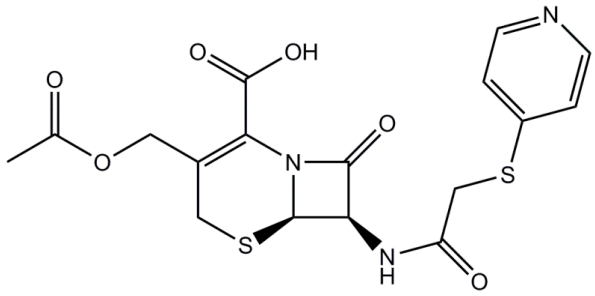
Cefamandole



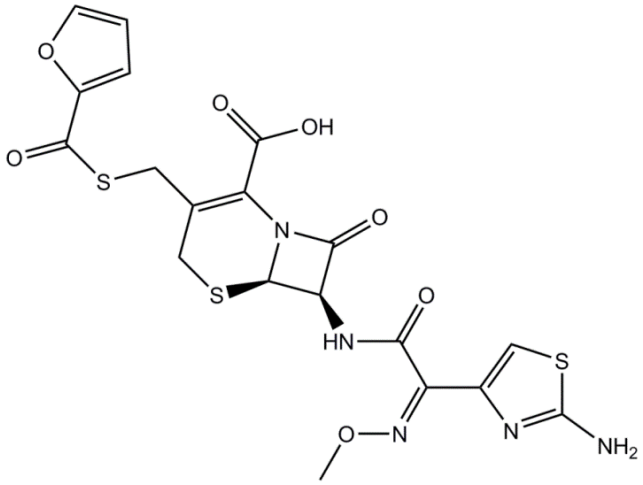
Cefuroxime



Cefapirin



Ceftiofur



8.3. Supplementary Information of Chapter 5

Base-catalyzed hydrolysis and speciation-dependent photolysis of two cephalosporin antibiotics, Ceftiofur and Cefapirin

Chemical quantification

Table S5.1

Parameters – High performance liquid chromatography, HPLC

Complete Shimadzu instrumentation composed of system controller, SCV-10-Avp; degasser unit, DGU-20 5R; liquid chromatograph, LC-10Atvp; auto injector, SIL-10Advp; diode array detector SPD-M10avp and column oven, CTO-10Asvp.
Column: 250 x 4 mm Prontosil 120-C_{18}, particle diameter of 5 µm, provided by Bischoff.
Temperature: room temperature of 22±1°C.

		Mobile phase				Injection volume				
Chemical	Code	A	B	Ratio A:B	Flow (mL min^{-1})	(µL)	RT	LOQ	LOD	r^2
Cefapirin Sodium	CEPA	Acetonitrile	UW pH 2 adjusted with HCl	10:90	0.5	100	12.6	439	145	0.999
Ceftiofur Sodium	CEF	Acetonitrile	UW pH 2 adjusted with HCl	25:75	0.5	100	15.5	913	345	0.999
Atrazine	ATZ	Methanol	UW pH 6	50:50	0.8	50	14.2	840	270	0.999

Notes:

Due to the known significant spectral change between CEPA's ionic species and to improve detection and quantification, the detector was set at two wavelengths: 259 nm at pH > 5.5 and 291 nm at pH < 5.5. In the case of CEF, only 291 nm was used.
UW, ultrapure water
RT, average retention time, in minutes.
LOQ, limit of quantification, in nM
LOD, limit of detection, in nM

Table S5.2

Parameters – Ion chromatography, IC

Complete Metrohm instrumentation, model 883 Basic				
Detector	Conductivity			
Column	Metrosep A SUPP 4, 250 x 4 mm, particle diameter of 5 µm, provided by Metrohm			
Eluent	1.8 mM L^{-1} Na_2CO_3, 1.7mM L^{-1} $NaHCO_3$			
Flow	1 mL min^{-1}		Sample volume	10 µL
Anion	**RT**	**LOQ**	**LOD**	**r^2**
Cl^- (Chloride)	4.1	0.19	0.06	0.997
NO_3^- (Nitrate)	4.6	0.22	0.07	0.996
NO_2^- (Nitrite)	5.8	0.08	0.02	0.999
SO_4^{-2} (Sulfate)	8.9	0.13	0.04	0.998

Notes:

RT, average retention time, in minutes, LOQ, limit of quantification, in mM,
LOD, limit of detection, in mM

Hydrolysis

Table S5.3

Water quality parameters of Ruhr River (RK), Germany measured on March 2017

pH	7.8 (raw) 8 (filtered)	**HCO_3^-** [mM]	2.42 ± 0.01
DOC [mgC L^{-1}]	1.86 ± 0.16	**Cl^-** [mM]	0.663 ± 0.002
Total Carbon [mgC L^{-1}]	19.30 ± 0.13	**NO_3^-** [mM]	0.158 ± 0.0006
Inorganic Carbon [mgC L^{-1}]	17.44 ± 0.03	**NO_2^-** [mM]	<0.02
Total Nitrogen [mgN L^{-1}]	3.42 ± 0.03	**SO_4^{2-}** [mM]	0.229 ± 0.0004
[mM]	0.244 ± 0.001		
NPOC[a] [mgC L^{-1}]	1.57 ± 0.04		

Notes:
[a] Non-purgeable organic carbon

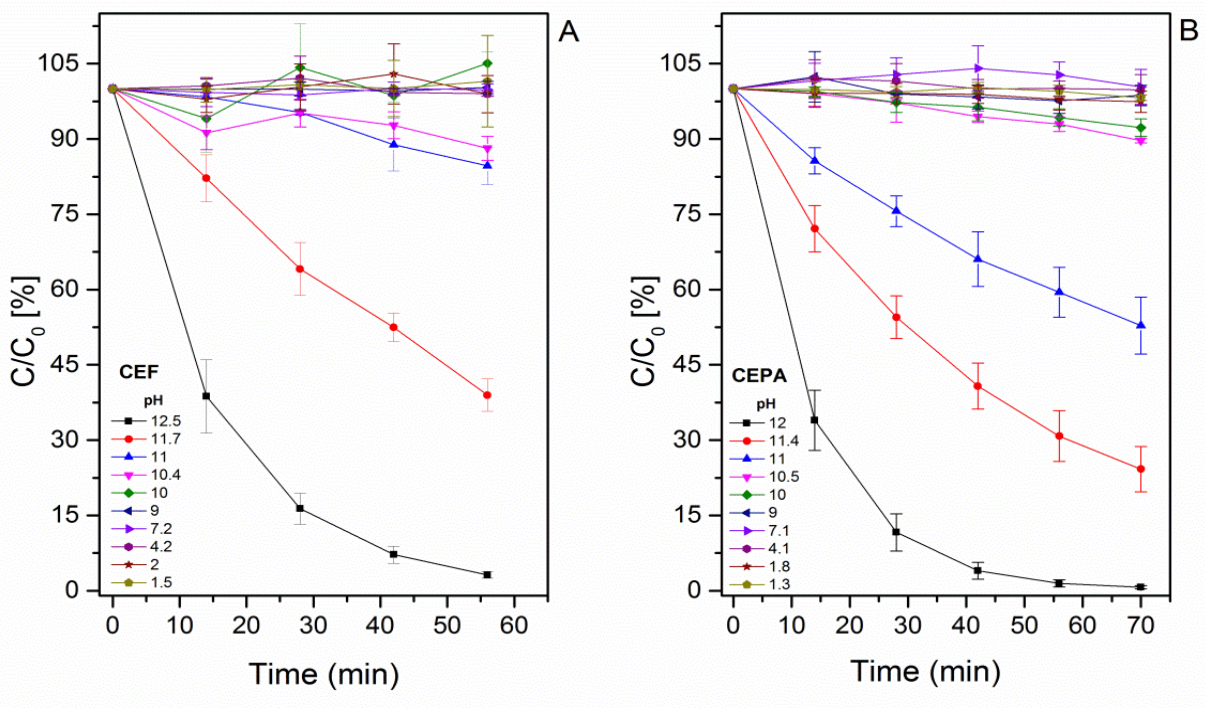


Figure S5.1. Time-based hydrolysis of CEF (A) and CEPA (B) under different pH values and controlled temperature (22 ± 1°C).

Table S5.4

Composition of the buffer solutions used in hydrolysis experiments

pH	Composition
1 to 2.3	Clark and Lubs (0.2 N HCl + 0.2 N KCl)
3.7 and 4.2	Universal buffer (H_3PO_4 0.1M + H_3BO_3 0.1M + CH_3COOH 0.1 M) adjusted with HCl 0.1M
7	Clark and Lubs (KH_2PO_4 0.1M + 0.1 N NaOH)
9	Phosphate buffer(KH_2PO_4 0.1M + K_2HPO_4 0.1M) adjusted with NaOH 0.1M
10	Phosphate buffer(KH_2PO_4 0.1M + K_2HPO_4 0.1M) adjusted with NaOH 0.1M
11 and 11.5	Universal buffer (H_3PO_4 0.1M +H_3BO_3 0.1M +CH_3COOH 0.1 M) adjusted with NaOH 0.1M
12	NaOH 0.1 M adjusted with HCl 0.1M
12.5	NaOH 0.1 M

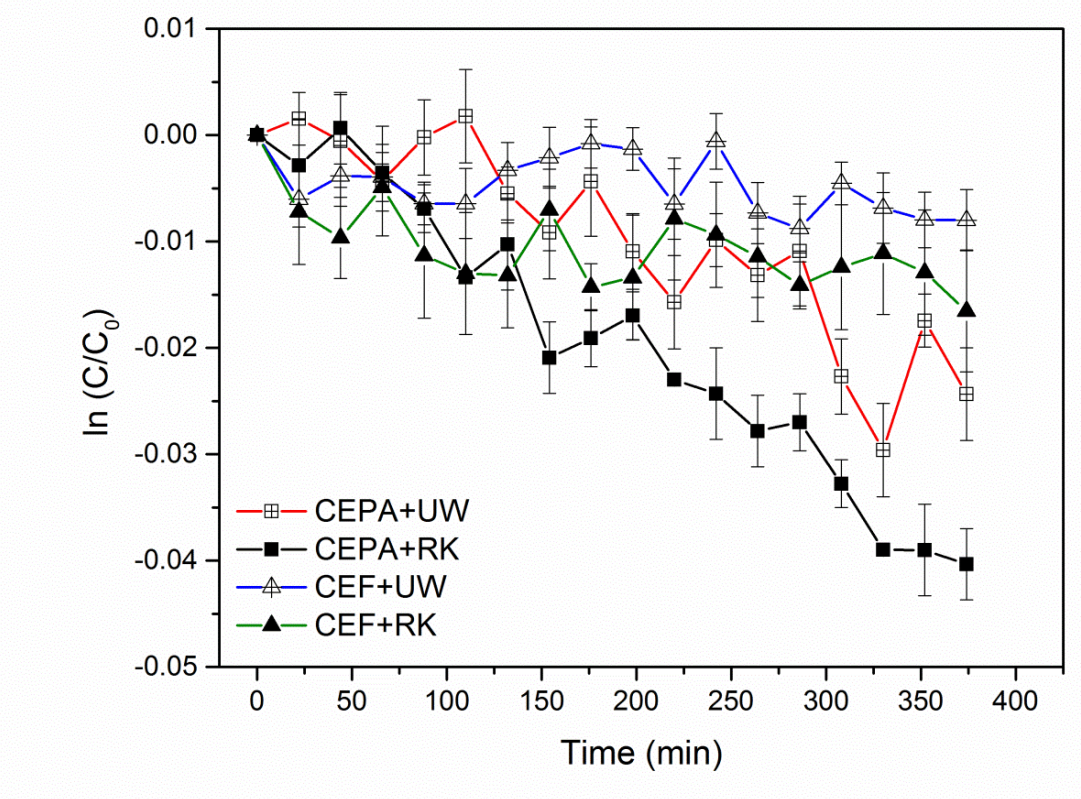


Figure S5.2. Observed hydrolytic time-course of CEPA and CEF in ultrapure (UW) and Ruhr River (RK) waters, after 6 hours of experiments (T = 22 ± 1°C).

Derivation of k_B, k_N and k_A

For the derivation of the base, neutral and acid-catalyzed hydrolysis rates, the following calculation was made:

First, the degradation rate observed at pH near to 7 was considered as neutral rate.

$$k_N = k_{obs}\ (pH\ 7.1)$$

As a result, the neutral-catalyzed rate constant for CEPA was derived, k_N = 0.027x10^{-4} s^{-1} (22 ± 1°C). Consequently, considering that only neutral and base-catalyzed hydrolysis were taking place at pH values above 9, the k_B value for CEPA was obtained using the given k_N and rearranging Eq. S5.1, as following:

$$k_{obs} = k_A[H^+] + k_N + k_B[OH^-] \qquad (S5.1)$$

$$k_B = \frac{k_{obs}[pH\ 12] - k_N}{[OH^-]}$$

To solve that, the ionization constant of water at 25°C ($K_W = 10^{-14.08}$) was used, along with the [H^+] calculated for pH 12 (i.e., 10^{-12}).

$$k_b = \frac{11.94\ x\ 10^{-4} - 0.027\ x\ 10^{-4}}{[10^{-2.08}]} = 0.143\ M^{-1}s^{-1}$$

Therefore, the base-catalyzed rate constant (k_B) calculated for CEPA at 22 ± 1°C was 0.143 M^{-1} s^{-1}.

Furthermore, Eq. S5.1 was similarly rearranged and the acid-catalyzed rate constant (k_A) for CEPA at 22 ± 1°C was also calculated, 79.8 x 10^{-7} M^{-1} s^{-1}.

Photolysis

Table S5.5

Photolytic time-based apparent pseudo-first-order rate constants

CEPA			**CEF**		
pH (charge)[a]	tk^{app} (s^{-1})	r^2	pH (charge)[a]	tk^{app} (s^{-1})	r^2
2 (+)	0.027	0.999	2 (+)	0.029	0.966
2.3 (+)	0.025	0.993	2.2 (+)	0.029	0.964
3.7 (+/-)	0.023	0.994	3.2 (+/-)	0.026	0.980
4.3 (+/-)	0.024	0.992	3.5 (+/-)	0.026	0.984
7 (-)	0.035	0.991	3.7 (-)	0.029	0.992
9 (-)	0.033	0.999	7.2 (-)	0.029	0.996
			9 (-)	0.030	0.997
	0.028 ± 0.004[b]			0.028 ± 0.001[b]	

Notes:
[a] Charge of the predominant specie in solution
[b] Average and Standard Deviation

To check internal fluence and to validate the experimental methodology, 5 μM of ATZ was spiked in RK plus 5 mM of phosphate buffer pH 7.2. Following, ATZ photo-degradation was performed and quantified using the procedures described in the manuscript. Finally, the known ATZ molar absorption coefficient (ε) of 3860 $M^{-1}cm^{-1}$ (Nick et al., 1992)[a] and its quantum yield (ϕ) of 0.046 mol $einstein^{-1}$ (Hessler et al., 1993) [b] (both calculated at 254

nm), along with the obtained time-based pseudo-first order photolysis rate (*k*) were used for internal fluence rate determination. As demonstrated in Figure S5.3, no significant difference was observed between the fluence rate obtained by atrazine actinometry and the one calculated by using uridine actinometry and the Morowitz correction factor. In addition, same photolytic degradation of ATZ was observed both in UW and RK, proving that our photolytic set-up was able to reproduce the known direct photolysis of this herbicide.

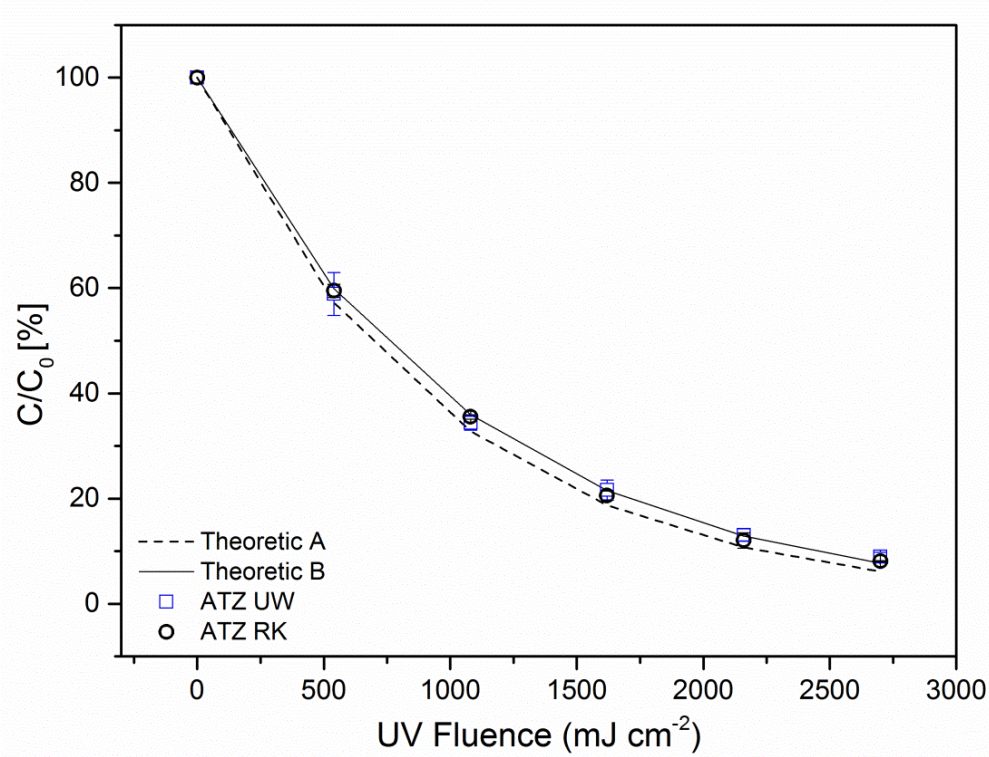


Figure S5.3. Predicted and observed photolysis of the model pollutant ATZ under different scenarios. The lines represent the expected ATZ photo-transformation calculated by using the fluence rates derived by URI (dashed line, absolute) and ATZ (straight line, internal) actinometry, respectively. Average (n=3) photo-transformation of ATZ in ultrapure water (UW, boxes) and in Ruhr River (RK, circles). T = 25± 0.2°C, pH =7.2

[a]Nick, K., Schöler, H,F., Mark, G., Söylemez, T., Akhlaq, M.S., Schuchmann, H.P., von Sonntag, C., 1992. Degradation of some triazine herbicides by UV radiation such as used in the UV disinfection of drinking water. L. Water Supply Res. Technol.-Aqua 41 (2), 82-87.

[b]Hessler, D.P., Gorenflo, V., Frimmel F.H., 1993. Degradation of aqueous atrazine and metazachlor solutions by UV and UV/H2O2 – influence of pH and herbicide concentration. Acta Hydrochim. Hydrobiol. 21 (4), 209-214.

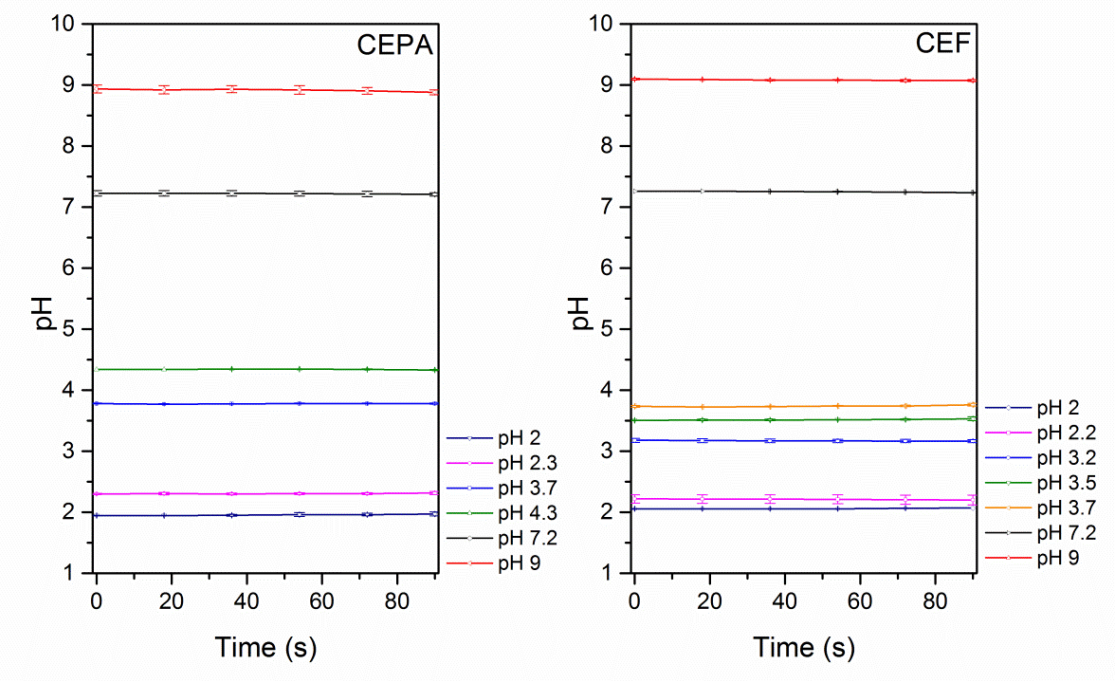


Figure S5.4. Measured pH values during photolytic transformation of the target cephalosporins

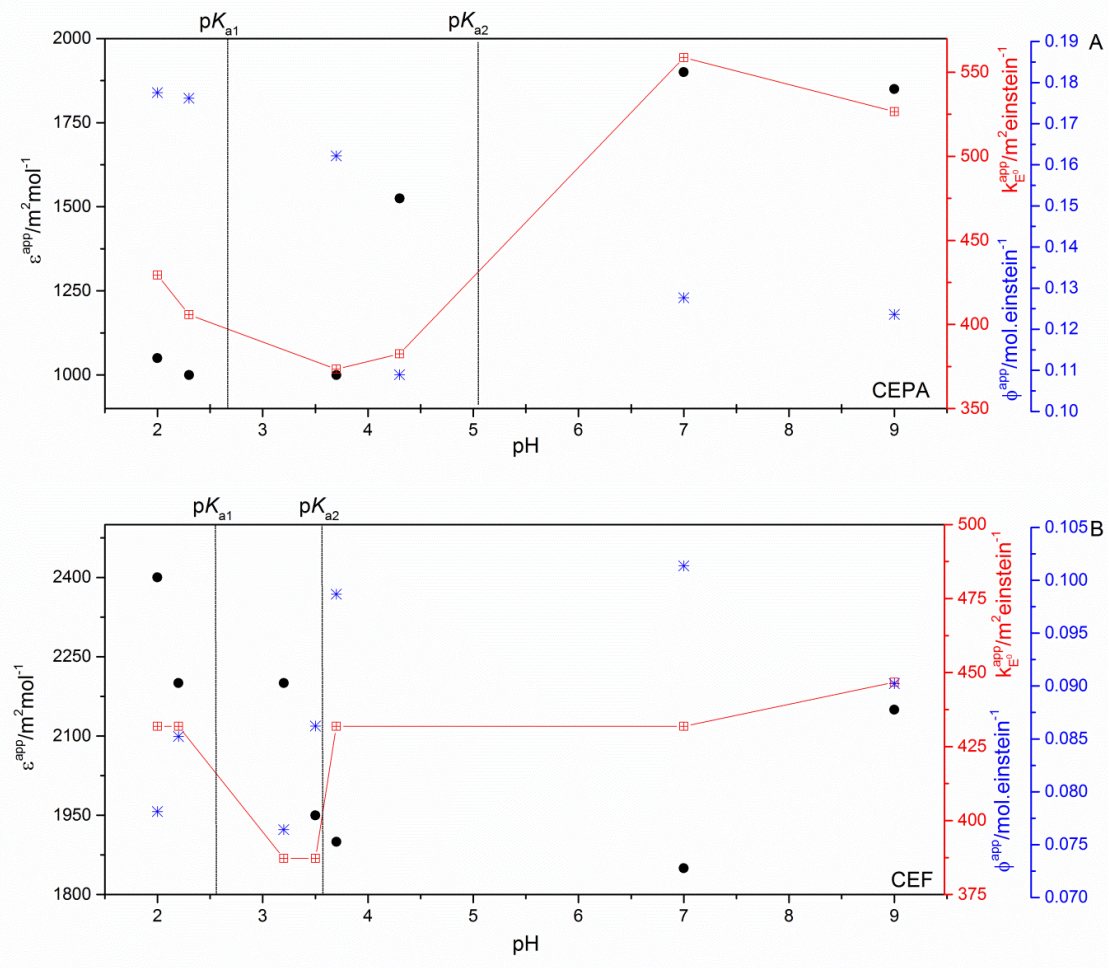


Figure S5.5. Graphical compilation of photo-kinetics parameters of Cefapirin (CEPA, A) and Ceftiofur (CEF, B) ionic species under UV-C (254 nm) irradiation. Where ε^{app} is the apparent molar absorption coefficient; ($k^{app}E^0$) is the photon fluence based apparent pseudo-first-order rate constant and ϕ^{app} is the apparent quantum yield.

8.4. Supplementary Information of Chapter 6

Ecotoxicity of the two veterinarian antibiotics ceftiofur and cefapirin before and after photo-transformation

Chemical stability

Preliminary stability studies were carried out according to Ribeiro et al. (2017). Figure S6.1 provides the hydrolysis curves of CEPA and CEF in ecotoxicological media along with kinetic parameters.

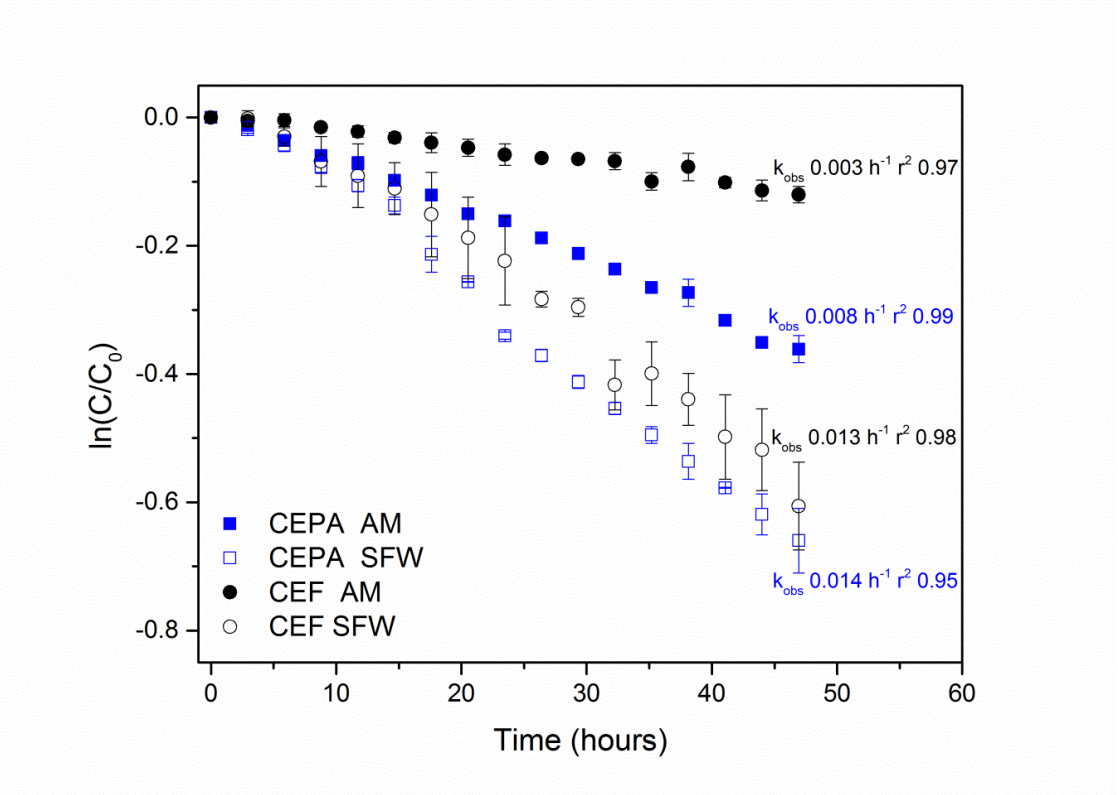


Figure S6.1. Hydrolysis kinetics of cephalosporins in ecotoxicological media. Squares (CEPA) and circles (CEF) represent respectively experiments in Modified Algal Media, AM (closed) and Standard Freshwater, SFW (open). k_{obs} represents the observed pseudo-first-order hydrolysis rate (in hours^{-1}). Linearity was expressed as r^2. Experiments carried out in triplicate.

Chromatographic data. CEPA and CEF concentrations after *Daphnia magna* and *Scenedesmus spec.* tests.

Table S6.1

Cephalosporin quantification (via HPLC-DAD) at the end of ecotoxicological tests

Test and duration (h)	Concentrations (µM)				Average degradation (%)	
	Nominal		Measured			
	CEF	CEPA	CEF	CEPA	CEF	CEPA
Daphnia, 48	187.8 17.67	100 10	110 - 119[a] 9 - 10	54 - 49 6 - 4	47 ± 4[b]	50 ± 8
Algae, 72	100 10	100 10	84 - 81 9 - 8	48 - 37 6 - 5	18 ± 3	43 ± 6

Notes:
[a] Maximum and minimum values, [b] Standard deviation

Calculation of expected photolytic degradation

Equation S6.1 describes the relation between antibiotic degradation (as the natural logarithm of $[C]_f/[C]_0$), the pseudo-first order degradation rate (k, in mJ cm^{-2}) and the UV dose (H, in mJ cm^{-2}) which was obtained multiplying the average fluence rate (E^0, mJ cm^{-2} s^{-1}) by the exposure time (t, in s^{-1}).

$$ln\frac{[C]_f}{[C]_0} = -k \times H \quad [S6.1]$$

According to Ribeiro et al. (2017), the k values of CEPA and CEF at pH ≈ 7 are 0.0116 and 0.0098 mJ cm^{-2}, respectively. The UV doses employed in this study were 75, 150 and 300 mJ cm^{-2}. Below, an example of expected degradation degree calculation is given. Scenario: CEPA after 75 mJ cm^{-2}

$$ln\frac{[C]_f}{[C]_0} = -0.0116 \times 75 = 100 - (e^{-0.87} \times 100) = 58\%$$

Daphnia tests

DaphToxKit F™, ephippia vials. Batch number used: DM 151216, DM210416 and DM 060417, all provided by MicroBioTests (Belgium)

Green algae growth inhibition tests

A modified Algae Medium (AM) was used both for *Scenedesmus spec.* cultivation and for chronic tests.

Table S6.2

Modified Algal Medium (AM), stock solutions preparation

Solution	Chemical	Mass (g)	Solution preparation
1	$Ca(NO_3)_2 . 4H_2O$	5.900	Make up to 1 liter with ultrapure water
	$NaNO_3$	46.700	
2	$K_2HPO . 3 H_2O$	4.100	Make up to 1 liter with ultrapure water
3	$MgSO_4 . 7 H_2O$	2.500	Make up to 1 liter with ultrapure water
4	$NaHCO_3$	16.800	Make up to 1 liter with ultrapure water
5	NaEDTA	1.145	Complete to 60 mL with ultrapure water, autoclave and make up to 1 liter with ultrapure water
	$FeSO_4 . 7 H_2O$	0.300	
6	H_3BO_3	3.100	Make up to 1 liter with ultrapure water
	$MnSO_4 . H_2O$	1.690	
	$(NH_4)_6Mo_7O_{24} . 4 H_2O$	0.090	
	$ZnSO_4 . 7 H_2O$	0.290	
	$Co(NO_3)_2 . 6 H_2O$	0.150	
	$CuSO_4 . 5 H_2O$	0.130	

To prepare 1 liter of AM, insert 2.50 mL of solutions 1, 2 and 3; 10 mL of solutions 4 and 5 and 0.08 mL of solution 6 in a volumetric flask (1L) and make up with ultrapure water.

Algae detectability

The calibration method was used for biomass measurement. For that, seven equidistant algal concentrations (from 18.6×10^3 to 18.6×10^5 Cell mL^{-1}) were prepared using visual counting (i.e., Neubauer's chamber method) and submitted to chlorophyll fluorescence

quantification. Accordingly, limits of detection (18 x 10^3 Cell mL^{-1}) and quantification (55 x 10^3 Cell mL^{-1}) as well as linearity (r^2, 0.999) were estimated as recommended by ISO 8692 (2012). Parameters used for fluorescence measurements are described below, in Table S6.3.

Table S6.3

Technical parameters used for fluorescence measurements

Device Infinite 200; Software Tecan i-Control version 1.2.7.0 Serial number 612000011				
Mode	Fluorescence Top Reading			
Excitation Wavelength	450	nm	Number of Reads	25
Emission Wavelength	685	nm	Integration Time	2000 µs
Excitation Bandwidth	9	nm	Lag Time	20 µs
Emission Bandwidth	20	nm	Settle Time	0 ms
Gain	100	Manual		

Tests were performed according to the experimental design observed in Figure S6.2 below.

	1	**2**	**3**	**4**	**5**	**6**
I	A	B	C	B	A	D
II	B	D	B	D	C	A
III	D	B	A	C	A	C
IV	A	D	C	B	C	D

Figure S6.2. Experimental design used for samples distribution in the 24-well microplates. Each letter corresponds to a tested concentration (4 per microplate/ 8 per single test, counting + and - controls). Numbers represent the columns while roman numerals indicate lines.

Table S4.4

Green algae tests, validation criteria applied in control cultures

Control type:	**Negative[a]**			**Positive[b]**
Identification	72-hour increasing factor (fold)	Average specific growth rate (day^{-1})	CV[c] (%)	Growth Inhibition (%)
CEPA baseline	17	1.04	13	53
CEF baseline	21	1.23	17	52
Photolysis CEF 75 mJ cm^{-2}	20	1.27	15	44
Photolysis CEF 150 mJ cm^{-2}	22	1.04	8	--
Photolysis CEF 300 mJ cm^{-2}	20	1.14	9	56
Photolysis CEPA 75 mJ cm^{-2}	17	0.96	4	42
Photolysis CEPA 150 mJ cm^{-2}	19	0.97	6	--
Photolysis CEPA 300 mJ cm^{-2}	19	1.03	7	50
Photolysis CEF 0 mJ cm^{-2}	17	1.33	3	52
Photolysis CEPA 0 mJ cm^{-2}	21	1.40	3	51
Mean value and Standard deviation	**19.3 ± 1.5**	**1.14 ± 0.13**	**8.5 ± 4**	**50 ± 3.5**

Notes

[a] Modified algal medium (described in Table S6.2)

-- no data,

[b] Potassium permanganate, 15.8 μM,

[c] Coefficient of variation calculated of daily/section-by-section average specific growth rates

Average growth rates curves obtained in all definitive chronic tests are presented below, from Figure S6.3 to Figure S6.5.

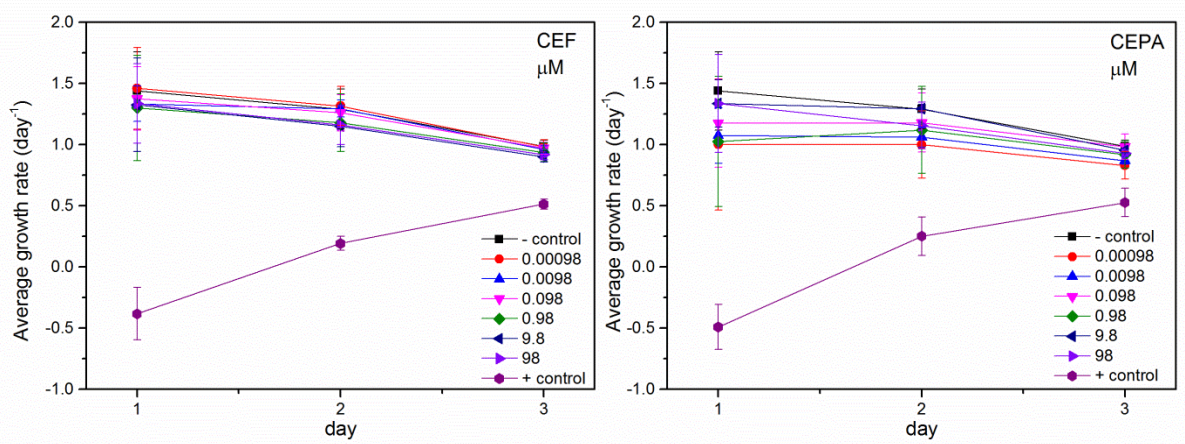


Figure S6.3. CEPA and CEF chronic toxicity tests with *Scenedesmus spec.*, daily determined average algal growth rate. Positive control was 15.8 µM of potassium permanganate.

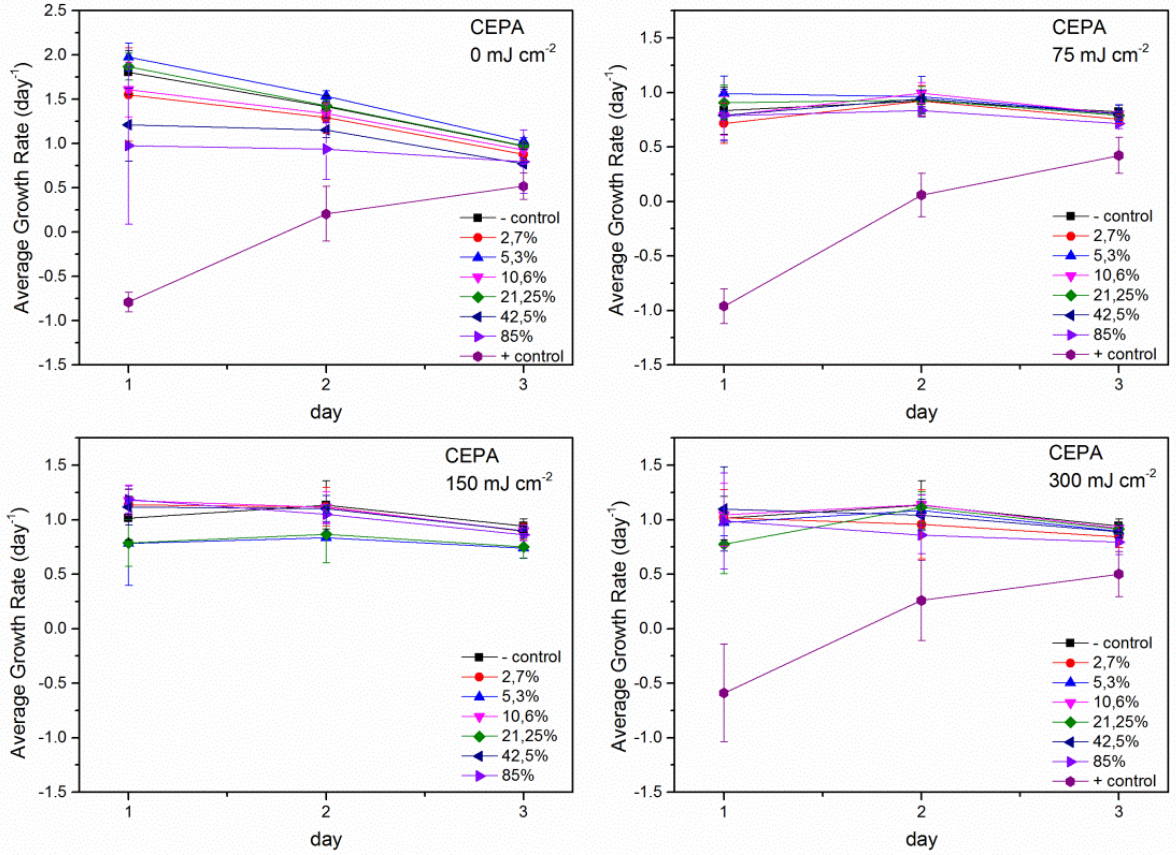


Figure S6.4. CEPA chronic toxicity tests with *Scenedesmus spec.*, daily determined average algal growth rate. Positive control was 15.8 µM of potassium permanganate.

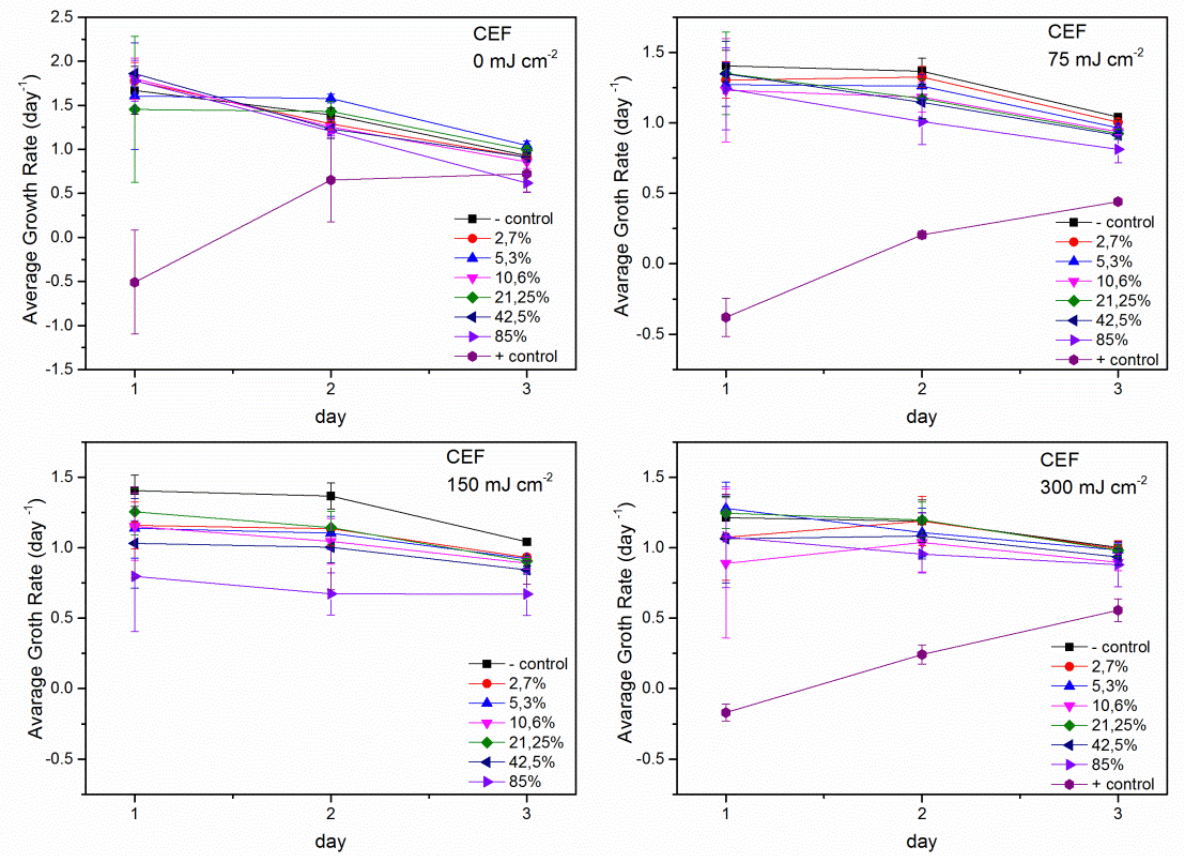


Figure S6.5. CEF chronic toxicity tests with *Scenedesmus spec.*, daily determined average algal growth rates. Positive control was 15.8 µM of potassium permanganate.

8.5. List of Symbols and Abbreviations

Abbreviations and symbols in alphabetic order, respecting the English orthography:

ACD – Advanced Chemistry Development Inc.

ACD/Percepta – ACD´s pK_a predictor

Ag_3PO_4 – silver phosphate

≈ – almost equals to

AM – algal modified cultivation medium

AOP – advanced oxidation processes

ATC – Anatomical Therapeutic Chemical

ATZ – atrazine

Au – gold

Bi_2CuO_4 – bismuth copper oxide

BiOBr – bismuth oxide bromide

°C – degree Celsius

$[C]_0$ – initial concentration

$[C]_t$ – posterior/final concentration

CAS# – Chemical Abstracts Service number

Cd^{2+} – cadmium ion

CEPA – cefapirin

$CEPA^+$ – cefapirin, pyridine ring protonated

$CEPA^{+/-}$ – cefapirin, pyridine ring protonated and carboxylic acid deprotonated

$CEPA^-$ – cefapirin, carboxylic acid deprotonated

$CEPA^{2-}$ – cefapirin, carboxylic acid and amide deprotonated

CEF – ceftiofur

CEF^+ – ceftiofur, protonated aminothiazole

$CEF^{+/-}$ – ceftiofur, pyridine ring protonated and carboxylic acid deprotonated

CEF^- – ceftiofur, carboxylic acid deprotonated

CEF^{2-} – ceftiofur, carboxylic acid and amide deprotonated

CH_3COOH – acetic acid

$C_{17}H_{17}N_3O_6S_2$ – cefapirin

$C_{19}H_{17}N_5O_7S_3$ – ceftiofur

Cl^- – chlorine

cm – centimeter

CNT – carbon nanotubes

CO_2 – carbon dioxide

conc. – concentration

Cu^{2+} – copper ion

CZE – Capillary Zone Electrophoresis

d – days

DAC – desacetyl cefapirin

DAD – diode array detector

DFC – desfuroyl ceftiofur

DOC – dissolved organic carbon

$d^2(pH)/dV^2$ - second-derivative of titration curve

Dr. rer. nat – Doctor rerum naturalium

E^0 – average fluence rate

EC_{50} – half maximal effective concentration

EF – Electro-Fenton

ϕ – quantum yield

ϕ^{app} – apparent quantum yield

EMA – European Medicines Agency

ε^{app} – apparent molar absorption coefficient

= – equal to

ERA – Environmental Risk Assessment

EU – European Union

g – gram

GMP – Good Manufacturing Practices

h – Hour

H – UV dose

H^+ – proton of hydrogen

$[H^+]$ – concentration of proton of hydrogen

– number of positive samples for this antibiotic

H_3BO_3 – Boric acid

HCl – hydrogen chloride

HCO_3^- – bicarbonate

Hg – mercury

H_2O_2 – hydrogen peroxide

HPLC – high performance liquid chromatography

H_3PO_4 – phosphoric acid

I – ionic strength

I – intersection points where hydrolysis reactions are equally important

I_{AB} – acidic-base intersection

I_{AN} – acidic-neutral intersection

IC – inorganic carbon

IC_{50} – half maximal inhibition concentration

I_{NB} – neutral-base intersection

In silico – computational approaches

J – Joule

k – pseudo-first order degradation rate

k_A – acid-catalyzed reaction

k^{app} – apparent pseudo-first order degradation rate

$k^{app}E^0$ – photon fluence based apparent pseudo-first-order rate constant

k_B – base-catalyzed reaction

kg – kilogram

k_N – neutral or pH-independent reaction

k_{obs} – observed pseudo-first-order hydrolysis rate constant

K_{ow} – octanol-water partition coefficient

K_W – ionization constant of water

L – Liter

λ – Wavelength

LC_{50} – half maximal lethal concentration

log – logarithm

LOEC – lowest observed effect concentration

LOQ – limit of quantification

LOD – limit of detection

LP – low pressure

M – mol

m – meter

Marvin – ChemAxom's pK_a predictor

mg – milligram

MgO – magnesium Oxide

min – minute

mJ – milijoules

mL – milliliter

mm – millimeter

mM – milimolar

MMTD – 2-mercapto-5-methyl-1,3,4-thiadiazole

MΩ – Megaohm

MRL – Maximum Residue Limits

MRSA – Methicillin-Resistant-Staphylococci

μeinstein – microeinstein

μg – microgram

μL – microliter

μM – micromolar

μm – micrometer

μs – microsecond

MW – Molecular Weight

N – nitrogen

NaCl – sodium chloride

NaH_2PO_4 – sodium dihydrogen phosphate

NaOH – sodium hydroxide

s – second

NCP – clinoptilolite nanoparticles

ND – not defined

ng – nanogram

NiO – nickel oxide

nM – nanomolar

nm – nanometer

NO_3^- – nitrate

NO_2^- – nitrite

NOAEC – no observed effect concentration

NOAEL – no observed adversed effect level

NPOC – non purgeable organic carbon

O – oral

O_2 – oxygen

OECD – Organization for Economic Co-operation and Development

OH^- – hydroxide ion

$[OH^-]$ – concentration of hydroxide ion

P – parenteral

pH – potential of hydrogen

pK_a – acid dissociation constants

Pot – potentiometry

r^2 – linear regression

RK – Ruhr River surface water collected in Kupferdreh (Germany)

RMSE – Root-Mean-Squared Error

rpm – revolutions per minute

RT – retention time

§ – amount of effect number (i.e. EC, LC, NOEC, NOAEL) available

7-ACA – 7-aminocephalosporanic acid

SFW – standard freshwater

SO_4^{2-} – sulfate

$SO_4^{\bullet-}$ – sulfate radical

$S_2O_8^{2-}$ – peroxy disulfate

Spec – Spectrophotometry

spec. – species

STP – Sewage Treatment Plant

T – Temperature

$t_{½}$ – Half-life

TC – total carbon

tE^0 – average fluence rate multiplied by the irradiation time

TiO_2 – titanium dioxide

tk^{app} – time-based apparent pseudo-first-order rate constant

TOC – total organic carbon

TP – transformation products

UK – United Kingdom

URI – uridine

USA – United States of America

UV – ultraviolet

UV-C – ultraviolet ($100 \geq \lambda \geq 280$ nm)

UW – ultrapure water

Vis – visible

VMP – veterinary medicinal products

W – Watts

WHO – World Health Organization

WWTP – Wastewater Treatment Plant

Zn^{2+} – zinc ion

8.6. List of Figures and Schemes

8.7. List of Tables

8.8. List of Publications

Peer-reviewed manuscripts:

Ribeiro, Alyson R., Sures, Bernd, Schmidt, Torsten C., 2017. Ecotoxicity of the two veterinarian antibiotics ceftiofur and cefapirin before and after photo-transformation. Submitted to Science of the Total Environment.

Ribeiro, Alyson R., Lutze, Holger V., Schmidt, Torsten C., 2017. Base-catalyzed hydrolysis and speciation-dependent photolysis of two cephalosporin antibiotics, Ceftiofur and Cefapirin. Submitted to Water Research.

Ribeiro, Alyson R., Schmidt, Torsten C., 2017. Determination of acid dissociation constants (pK_a) of cephalosporin antibiotics: Computational and experimental approaches. Chemosphere, v. 169, p. 524-533.

Conferences:

Poster - **Ribeiro, Alyson R**., Ruchter, N., Sures, B., Schmidt, T. C., (2016). Implementation of ecotoxicological tests using freshwater planarians: Choice of cultivation medium by using the fecundity mean index as viability marker. In: Setac Europe 26th Annual Meeting, 2016, Nantes, France. Abstract book.

Poster - **Ribeiro, Alyson R**., Schmidt, T. C., (2016). The experimental and computational determination of the acid dissociation constants (pK_a) of cephalosporins, an environmentally relevant antibiotic group. In: Setac German Language Branch Annual Meeting, 2016, Tübingen. Abstrack book.

Platform presentation - **Ribeiro, Alyson R**., Schmidt, T. C., (2016). Cephalosporin antibiotics in the environment: risk assessment for aquatic life and bacterial resistance. In: Setac German Language Branch Annual Meeting, 2016, Tübingen. Abstract Book.

8.9. Curriculum Vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

8.10. Acknowledgment

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8.11. Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit mit dem Titel

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selbst verfasst und keine außer den angegebenen Hilfsmitteln und Quellen benutzt habe, und dass die Arbeit in dieser oder ähnlicher Form noch bei keiner anderen Universität eingereicht wurde.

Essen, im November 2017.

Alyson Rogério Ribeiro