Universidade de Aveiro Departamento de Biologia

2010

Nuno Emanuel Pinto Barcelos Martins

Efeitos ecotoxicológicos de ciprofloxacina em espécies aquáticas

Ecotoxicological effects of ciprofloxacin on aquatic species

Universidade de Aveiro Departamento de Biologia
2010

Nuno Emanuel Pinto Barcelos Martins

Efeitos ecotoxicológicos de ciprofloxacina em espécies aquáticas

Ecotoxicological effects of ciprofloxacin on aquatic species

dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia e Ecotoxicologia, realizada sob a orientação científica da Dr. Catarina Marques, Investigadora de Pós-Doutoramento do Departamento de Biologia da Universidade de Aveiro e do CESAM, e co-orientação científica da Dr. Ruth Pereira, Investigadora Auxiliar do Departamento de Biologia da Universidade de Aveiro e do CESAM

A ignorância gera confiança com mais frequência do que o conhecimento: são aqueles que sabem pouco, e não aqueles que sabem muito, que tão positivamente afirmam que esse ou aquele problema jamais será resolvido pela ciência.

Charles Darwin (1809-1882)

o júri

presidente

Prof. Doutor António José Arsénia Nogueira
Professor associado c/ Agregação do Departamento de Biologia da Universidade de Aveiro

Prof. Doutor Fernando José Mendes Gonçalves
Professor associado c/ Agregação do Departamento de Biologia da Universidade de Aveiro

Doutora Ruth Maria de Oliveira Pereira (co-orientadora)
Investigadora Auxiliar, CESAM e Departamento de Biologia da Universidade de Aveiro

Doutor Nelson José Cabeços Abrantes
Investigador de Pós-Doutoramento, CESAM e Departamento de Ambiente e Ordenamento da Universidade de Aveiro

Doutora Catarina Pires Ribeiro Ramos Marques (orientadora)
Investigadora de Pós-Doutoramento, CESAM e Departamento de Biologia da Universidade de Aveiro

agradecimentos

Gostaria primeiro de agradecer ao Professor Fernando Gonçalves, por me aceitar na sua equipa de trabalho e me permitir assim trabalhar na área que mais curiosidade me desperta.

Gostaria de agradecer à minha orientadora Catarina Marques e co-orientadora Ruth Pereira por conseguirem apoiar o meu trabalho e permitirem que este trabalho pudesse ser realizado.

Gostaria de agradecer a todos os meus colegas de laboratório, Ana Catarina, Ana Cuco, Ana Luisa, Carina, Catia, Cláudia, Fátima, Inês, Inês Verónica, João, Márcia, Sara, Serginho, Sofia, Rita, Tânia, Teresa e mais colegas que poderei me estar a falhar, pela constante boa disposição, ajuda e paciência.

Gostaria de agradecer ao Prof. Bruno Nunes, Nelson Abrantes, Joana Pereira pela sua colaboração no desenvolvimento deste trabalho.

Gostaria de agradecer ao Doutor Bruno Castro e Doutora Sara Antunes pela ajuda quando necessária.

Gostaria de agradecer a minha namorada Raquel por me aturar quando estou stressado com trabalho e mandar vir comigo quando escrevo frases demasiado longas sem vírgulas.

Gostaria de agradecer ao meu Pai e à minha Mãe por acreditarem nas minhas capacidades e manterem me neste percurso académico que levou até este momento.

Gostaria de agradecer à Davina e ao Daniel por servirem de público para treinar a minha apresentação, e me ajudarem a superar os nervos.

palavras-chave

fármacos, antibióticos, ciprofloxacina, ecotoxicologia, *Vibrio fischeri*, *Pseudokirchneriella subcapitata*, *Lemna minor*, *Daphnia magna*, *Gambusia holbrooki*, efeitos agudos e sub-letais, quocientes de risco

resumo

Os antibióticos têm sido detectados em amostras de água naturais, no entanto, os seus efeitos ecotoxicológicos em espécies aquáticas não-alvo ainda não foram estudados de forma extensiva. A sua actividade biológica pode constituir um perigo ambiental, quer pela actuação directa nos organismos que possuam receptores e vias metabólicas que possam ser alteradas pelo antibiótico, quer pelo desenvolvimento de resistência bacteriana. Este estudo avaliou os efeitos de ciprofloxacina na bioluminescência de *Vibrio fischeri*, no crescimento de *Pseudokirchneriella subcapitata* e *Lemna minor*, na sobrevivência e ciclo de vida de *Daphnia magna* e na sobrevivência de *Gambusia holbrooki*. Pretendeu-se assim avaliar efeitos a diferentes níveis tróficos, recorrendo também ao cálculo dos riscos associados à exposição a ciprofloxacina através da determinação de quocientes PEC/PNEC (PEC – concentração ambiental prevista; PNEC – concentração para a qual não se prevê a ocorrência de um efeito). Registou-se inibição da bioluminescência de *V. fischeri* ao fim de 30 minutos de exposição. O crescimento das espécies produtoras *P. subcapitata* e *L. minor* foi também significativamente inibido. A toxicidade aguda de ciprofloxacina em *D. magna* foi moderada, no entanto, verificou-se que exposições a longo prazo a concentrações mais baixas do antibiótico conseguem produzir alterações nos parâmetros de história de vida da espécie, principalmente no tamanho de neonatos da primeira ninhada e nas taxas de fecundidade. Por outro lado, a ciprofloxacina não apresentou toxicidade aguda para *G. holbrooki*.

De um modo geral, os valores de toxicidade obtidos (mg L^{-1}) foram superiores às concentrações ambientais apresentadas em estudos prévios. No entanto, a exposição a longo prazo a concentrações reduzidas de antibiótico podem representar um perigo directo para os organismos não alvo, afectando vias metabólicas a um nível de organização biológica inferior. Por outro lado, os efeitos assim produzidos podem indirectamente afectar o equilíbrio na cadeia trófica de ecossistemas dulçaquícolas, principalmente quando os danos recaem sobre a base da cadeia trófica (produtores e consumidores primários). Efectivamente a integração de dados de avaliação da exposição e de efeitos da ciprofloxacina através do cálculo de quocientes PEC/PNEC indicou que esta fluorquinolona representa um risco para espécies aquáticas sensíveis. Este resultado reforça a necessidade de refinar a avaliação de risco deste fármaco recorrendo a ferramentas e espécies sensíveis que permitiram uma caracterização de risco mais protectora do equilíbrio dos ecossistemas aquáticos.

keywords

pharmaceuticals, antibiotics, ciprofloxacin, ecotoxicology, *Vibrio fischeri*, *Pseudokirchneriella subcapitata*, *Lemna minor*, *Daphnia magna*, *Gambusia holbrooki*, acute and sub-lethal effects, risk quotients

abstract

Antibiotics have been detected in natural samples, but their ecotoxicological effects in aquatic wildlife have not been extensively studied yet. Their biological activity may pose an environmental threat, either due to their direct action on similar receptors and metabolic pathways present in non-target organisms or due to development of bacterial resistance. This study evaluated the effects of ciprofloxacin on the bioluminescence of *Vibrio fischeri*, the growth of *Pseudokircheneriella subcapitata* and *Lemna minor*, on the survival and life-cycle of *Daphnia magna* and on the survival of *Gambusia holbrooki*. This way, it was evaluated the effects of ciprofloxacin on different trophic levels, while determining its risks associated with environmental exposure of non-target organisms, through the derivation of PEC/PNEC ratios (PEC – predicted-environmental-concentration, PNEC – predicted-no-effect-concentration). The bioluminescence of *V. fischeri* was inhibited after 30 minutes of exposure. The growth of the producers' species *P. subcapitata* and *L. minor* was also significantly inhibited. The acute toxicity of ciprofloxacin to *D. magna* was moderate, however, long-term exposures to lower concentrations of the antibiotic led to negative changes on life-history endpoints of *D. magna*, especially regarding the size of neonates from the first brood and the fecundity rates. On the other hand, ciprofloxacin was not acutely toxic for *G. holbrooki*.

In general, the toxicity values obtained (mg L^{-1}) were higher than the environmental concentrations presented in previous studies. Nevertheless, long-term exposures to low concentrations of the antibiotic may be a direct hazard to non-target organisms, while affecting metabolic pathways at a lower biological level of organization. Besides, the effects produced can also indirectly affect the balance of trophic chains in freshwater ecosystems, especially when the impairments fall over basis of the trophic chains (*i.e.*, producers and primary consumers).

Actually, the integration of exposure and effect data of ciprofloxacin in the PEC/PNEC ratios indicated that this fluoroquinolone represents a risk for the most sensitive aquatic species. This outcome reinforces the need of performing a more refined risk assessment, using more sensitive ecotoxicological tools and species that allow a protective risk characterization hence promoting the integrity of aquatic ecosystems.

ÍNDICE

Chapter I

General Introduction

General Introduction

1. Pharmaceutical drugs

The pharmaceutical industry is a growing market, showing an increase in the production of new pharmaceutical drugs each year to face health problems namely related with the enhancement of human life span, though they have been also increasingly used for veterinary medicine (Glassmeyer et al., 2009; Naddeo et al., 2009). A study from the National Authority for Medicines and Health Products IP (Infarmed) reported on Medicines' Statistics from 2008 (Infarmed, 2008) pointed out for an increase on pharmaceutical sales during the last 5 years (Figure 1).

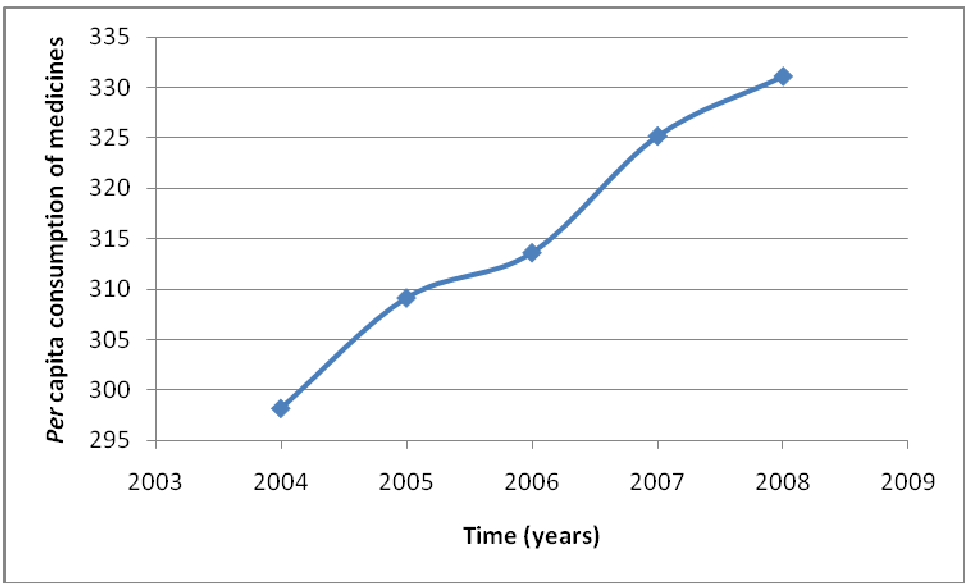


Figure 1. Annual *per capita* consumption of medicines in Portugal between 2004 and 2008 (Infarmed, 2008).

Pharmaceutical drugs are biologically active chemicals that are usually designed to act through a specific mode of action towards a certain target system. For this reason they may also interact with other biological systems, having similar metabolic pathways, receptors or biomolecules, promoting unwanted side effects. As such, when pharmaceutical residues (parental compound and its metabolites) are released to the environment, they can become a potential hazard for non-target flora and fauna species (Fent et al., 2006; Santos et al., 2010). In some cases, pharmaceuticals have showed to persist and bioaccumulate (Santos et al., 2010) and in other situations, though they may not

persist for long periods, they are continuously entering into the ecosystems, increasing the possibility of inducing chronic effects on exposed non-target individuals.

a. Sources and environmental pathways of pharmaceutical residues

Wastewater and effluents from sewage treatment plants (STPs) are the main point sources of pharmaceuticals in the aquatic environment, hence representing specific concerns regarding their quality (Fent et al., 2006). The wastewaters acting as sources of pharmaceuticals can have three main origins: (i) urban/domestic wastewaters and hospital and health centre effluents which receive pharmaceutical residues applied on human medicine; (ii) wastewaters from intensive livestock carrying medicines from veterinary use and, (iii) wastewaters from industrial production (Figure 2).

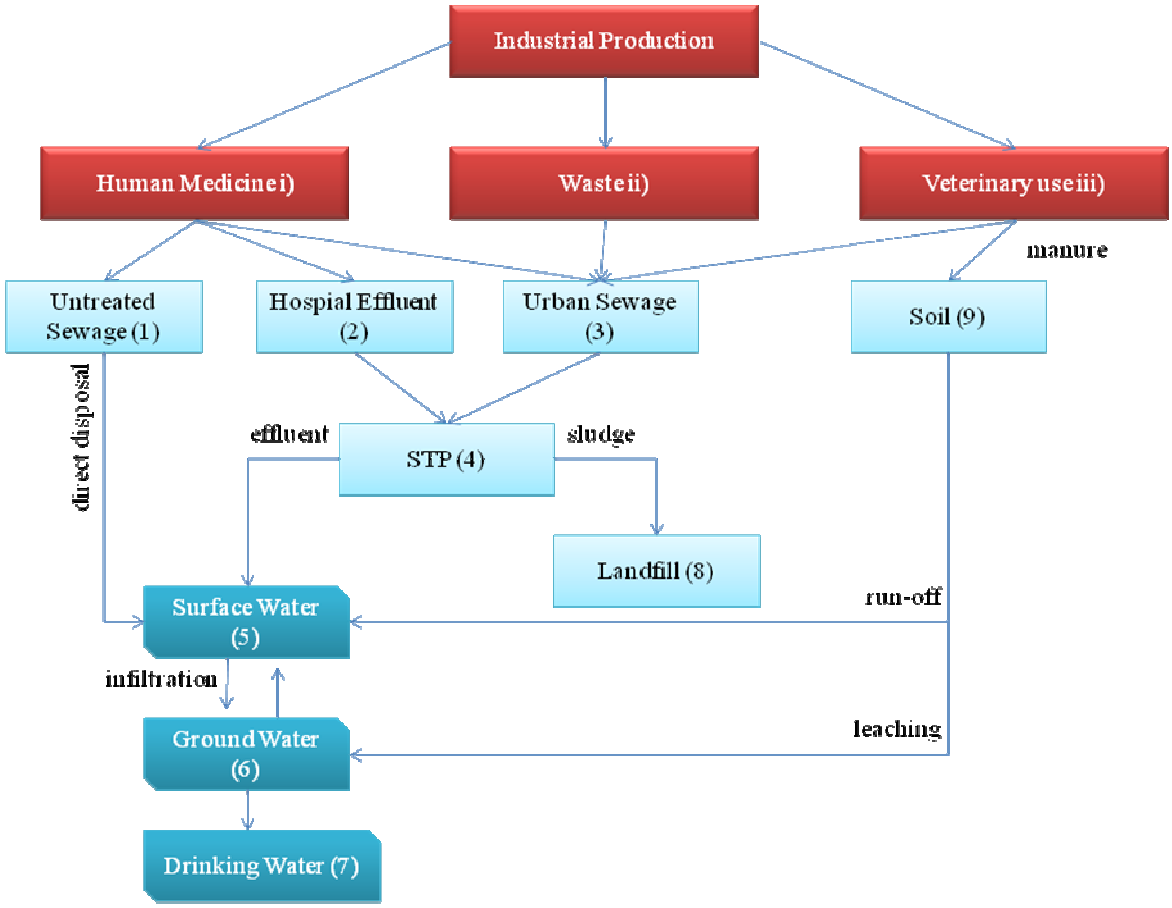


Figure 2. Sources and distribution of pharmaceutical drugs in the environment (based on Santos, 2010). STP stands for Sewage Treatment Plant.

Human pharmaceuticals (i) may enter the aquatic systems upon direct disposal of unwanted medicines or via excretion of the parent compound into urban sewage (3).

Furthermore, once ingested or administered the parent compound may follow different metabolic pathways, that may result in its absorption, bioaccumulation and/or biotransformation into more easily excreted polar metabolites which may also be transported into the aquatic systems through STPs (4) (Wagner, 1993; Heberer et al., 2002; Fent et al., 2006; Santos et al., 2010).

The veterinary pharmaceuticals (iii) administered and excreted by animals may also affect aquatic systems due to the application of contaminated manure on agricultural fields and subsequent percolation through soil (9) and/or run-off. The direct application of veterinary medicines on aquacultures is also an important and direct via of surface waters' contamination (5) (Fent et al., 2006; Li et al., 2009).

Production wastes, from the pharmaceutical industry, are discharged in sanitary landfills (8) or disposed on municipal STPs attaining, as well, superficial and groundwater resources (6, 7). In summary and independently of the origin, most pharmaceuticals reach STPs (4), where they may eventually not be properly degraded or removed thereby leading to the contamination of surface waters (*e.g.*, rivers, lakes, estuaries) (5) and groundwater (6), hence constraining the quality of drinking water (7).

b. Environmental Hazard: levels and possible impact

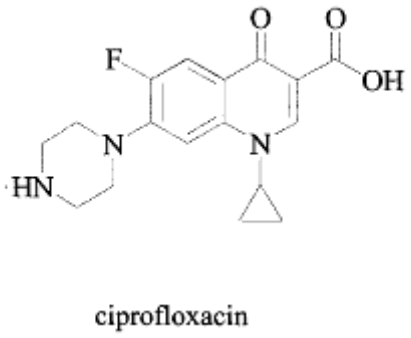
Fent et al. (2006) compiled information on environmental concentrations for different pharmaceuticals, and found values at the ng L^{-1} and µg L^{-1} level, in influent and effluents, as well as in freshwater systems. Minh et al. (2009) confirmed that there is a significant input of antibiotics (ng L^{-1} to µg L^{-1}) in receiving waters from the discharging of effluents without proper chemical/biological treatment. Although these low concentrations of pharmaceutical residues, found in several studies, may not be directly harmful for humans, they may affect the sustainability of the aquatic environment (Golet et al., 2002; Fent et al., 2006). Indeed, the environmental persistence and critical biological activity of pharmaceuticals raise concerns about their potential toxicological effects on on-target organisms (Grung et al., 2008; Minh et al., 2009). In particular, sub-lethal concentrations of antibiotics, have been reported to impact the cell function (*e.g.*, cellular growth and multiplication) (Santos et al., 2010) and to promote the resistance of bacteria to antibiotics. (Kümmer, 2009; Santos et al., 2010). In this context, this poses different kinds of perturbations on the environment besides antibiotic resistance, hence affecting various levels of the trophic chain.

c. Legislation of pharmaceutical drugs

Due to the increased environmental awareness related with the presence of pharmaceuticals and their residues in the aquatic systems, the European Community has published different directives to prevent, monitor and assess the hazard effects of these contaminants. The issue of environmental safety of medical products was first introduced by European legislation with the directives 93/39/EC and 93/40/EC (CEEC, 1993). Later, in 2001, the directives 2001/82/EC and 2001/83/EC stated the need of an environmental impact assessment, and established legislation regarding the rules for marketing authorization of pharmaceuticals. In the same year the application of Environmental Risk Assessment (ERA) schemes for human pharmaceutical products was discussed (CHMP, 2006). In 2004, the directives 2004/27/EC (amending directive 2001/83/EC) and 2004/28/EC (amending directive 2001/82/EC) established the requirement of an ERA process for the registration of new pharmaceuticals. In 2003 and 2005 were released the first and second guideline drafts for conducting an ERA (CHMP, 2006). The ERA of veterinary medicinal products was described in guidance documents produced by the European Medicines Agency (EMEA, 1998; EMEA, 2005). In 2008, it was released a Revised Guideline on Environmental Impact Assessment for Veterinary Medicinal Products, a new Guideline on Safety and Efficacy Follow-up-Risk Management of Advanced Therapy Medical Products and other regulatory guidelines (EMEA, 2008) to fulfill and update the legislation on pharmaceutical environmental risk assessment. In the national context the available legislation on pharmaceuticals includes regulations for the marketing authorization of new medicinal drugs that also establishes the need for describing potential hazards for the environment caused by their consumption or disposal. Recently, the REACH regulation (Registration, Evaluation, Authorization and Restriction of Chemical Substances) (EC, 2006) within the European Union, proposes a similar strategy for pesticides, biocides and pharmaceuticals, and demands information about: i) the physical and chemical properties of the substance; ii) the abiotic and biotic degradation; iii) the metabolism within biota; iv) the potential for bioaccumulation and persistence in the environment and v) the fate and mobility within the different environmental compartments.

2. The antibiotic ciprofloxacin

Ciprofloxacin [1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperaziny)-3-quinoline carboxylic acid] (Figure 3) is an antibiotic used for the treatment of bacterial infections, that presents a broad antibacterial spectrum and belongs to the chemical class of fluoroquinolones (Chin et al., 1984; Zeiler et al., 1984; Zeiler, 1985). It differs from the rest of quinolones derivates by having a cyclopropyl residue in position 1 of the molecule replacing the ethyl group (Zeiler et al., 1984) (Figure 3). Its action stops bacterial multiplication, by disrupting the DNA replication and repairing processes. Ciprofloxacin was approved in October 1987, by the FDA (Food and Drugs Administration), and it can be found in different forms (tablets, microcapsules and injection concentrate) (Streuff et al., 1987).



ciprofloxacin

Figure 3. Chemical structure of ciprofloxacin (Golet et al., 2008).

Infarmed (2008) evidenced that ciprofloxacin is the 43rd pharmaceutical with more packages sold by the SNS (National Health System) and the 3rd most sold generic antibiotic in Portugal (552,988 packages). A total of 655,694 different packages contain this active substance. Ciprofloxacin is usually prescribed to treat skin, lungs, airways, bones, and joints' infections caused by susceptible bacteria, urinary infections caused by some bacteria like *Escherichia coli,* and infectious diarrhoeas caused by *E. coli*, *Campylobacter jejuni* and *Shigella* sp. (Eliopoulos et al., 1984). The treatment may last from 7 to 21 days, but can be prolonged in severe cases (Krcmery et al., 1999).

Its pharmacokinetic properties have been extensively studied, and two main modes of administration were suggested: oral and intravenous, not showing significant differences

in resulting serum concentrations between both. Absorption occurs in the gastrointestinal track and there is a proportional increase of the peak concentration in serum with an increase of dosage on both administration pathways (Bergan et al., 1988). Ciprofloxacin half-life, after a single administration, ranges from 3 to 5h; nevertheless multiple dose administration has not resulted in a statistically relevant increase of concentration in serum and tissues (Bergan et al., 1988). Relatively to drug distribution, ciprofloxacin shows a high capacity of penetration in tissues, reaching high distribution levels in the body regardless the administration route (Bergan et al., 1988). Ciprofloxacin is mainly excreted in its unchanged form by renal and extra-renal routes (transintestinal and biliary excretion), with only slight doses of metabolites being excreted by urine and faeces. Elimination is processed by three routes: renal excretion, transintestinal secretion and metabolism (Bergan et al., 1988; Sörgel et al. 1991). Renal clearance is mostly completed after 24h of administration, but some levels of the drug and its metabolites are still eliminated until approximately 72h (Bergan et al., 1988). Excretion through bile contributes to only 1% of total excretion and transintestinal elimination for 15% of total clearance. These alternative excretion pathways can compensate, in case of reduced renal function, where most of the clearance from renal function is replaced by transintestinal elimination through faeces (Bergan et al., 1988). All this pharmacokinetic characteristics have made ciprofloxacin a clinically attractive drug.

Hence, following excretion, ciprofloxacin may enter the aquatic environment through STP effluents. Indeed, Golet et al. (2008) found environmental concentrations in river waters from northern Switzerland around 15 ng L^{-1}. In Portugal (Coimbra) concentrations of ciprofloxacin ranged between 127 and 10962.5 ng L^{-1} in wastewaters from four different hospitals, reaching wastewater treatment plants at concentrations of 667.1 ng L^{-1} (influent) and leaving it at 309.2 ng L^{-1} (effluent) (Seifrtová et al., 2008). This data indicate that it would be important to improve the assessment of ciprofloxacin effects on non-target wildlife.

3. Battery of test species for ecotoxicological evaluations

For the hazard assessment of chemicals in the environment, one of the tools usually used involves acute and chronic ecotoxicological tests, which can be performed with species from different trophic levels, following standard protocols available. It is important to choose species that are sensitive to the effects of the toxic substances, while they also have a representative role in the ecosystem.

Vibrio fischeri is a marine bioluminescent bacteria used to assess either the toxicity of solutions of pure chemical substances, or contaminated water or soil samples, in a screening step of ecotoxicological evaluations, in alternative to more elaborated and time consuming aquatic species tests (Parvez et al., 2006). The *V. fischeri* Microtox® test has been used as a standard test in ecotoxicology (Kaiser, 1998; Qureshi et al., 1998). It does not require the rearing of organisms; instead, lyophilized bacteria are used, after activation in a saline water suspension. The reduction of the luminescence emitted by the bacteria, after the exposure to toxicants/toxic matrices, reflects their toxicity. Although the sensitivity of the test has been discussed (Qureshi et al., 1998), its effectiveness was proved for testing the acute toxicity of several chemicals (Van der Grinten et al., 2010).

Pseudokirchneriella subcapitata has been recommended as a standard species for the testing of chemicals, as it is an important species belonging to the autotrophic level of the aquatic environment (Labra et al., 2007). They are very sensitive to different chemicals, easy and cheap to rear under laboratorial conditions, and have a short life cycle; such features make algal toxicity tests a good ecotoxicological tool for the risk assessment of chemicals (Mayer et al., 1997).

Lemna minor (duckweed) belongs to a group of ubiquitous floating freshwater monocotyledons and it is one of the world's smallest flowering plants (Landolt, 1986). The duckweed has diverse genetic populations, a fast reproductive cycle and is easy to maintain under laboratorial conditions, making it a relevant and important model for ecotoxicological tests (Kanoun-Boulé et al., 2009). The role of this species as refuge, habitat and food for a variety of herbivorous species also justifies their importance for the sustainability of freshwater food chains (Lewis et al., 1995).

Daphnia magna is a crustacean, belonging to the order Cladocera. They are also a key-group helping on the sustaining of freshwater trophic chains, either by controlling

algae blooms or by being a food supply for fish (Alonso, 1996). *D. magna* can be found in freshwaters or low salinity waters (Alonso, 1996). This cladoceran has a short life cycle, high fecundity rates, and low genetic variability (Terra et al., 2003). This low variability is related to its asexual reproduction (parthenogenesis) that is more common during its life cycle than the sexual one (Alonso, 1996). As a test subject, the asexual reproduction of *D. magna* helps to eliminate the effect of genetic variability in the response to toxic substances, and this may guarantee the reproducibility and comparability of test results among laboratories, further its short life cycle allows quick evaluation of chronic responses. *D. magna* is also easy to rear in laboratory with low costs and low equipment and material requirements (Terra et al., 2003).

Gambusia holbrooki (mosquitofish) is a small and aggressive fish species, originated from southern United States and Mexico (Garcia-Berthou et al., 2005). Nowadays it has a worldwide distribution, since it was introduced in different systems under temperate climate for mosquitoes' controlling (Nunes et al., 2005; Pyke, 2005). *G. holbrooki* is now reported to be the most widely distributed freshwater fish (Pyke, 2005), establishing them as an important representative of secondary consumers (Alcaraz and Garcia-Berthou et al., 2007). This, together with their easy capture, natural abundance and stability in laboratory conditions, makes this specie appropriate for ecotoxicological tests (Nunes et al., 2005).

4. Objectives and structure of the thesis:

The main objective of this thesis was to contribute with sound scientific ecotoxicological data for ciprofloxacin, in order to provide information to be used in future derivation of a protection value for the aquatic compartment. This protection values will be useful to regulate emissions of this compound to the environment as well as for risk assessment of effluents/wastewaters. To attain this general aim, more specific objectives were defined:

- to perform a general evaluation of the ecotoxicity of the antibiotic ciprofloxacin, carrying out a series of acute and chronic ecotoxicological tests, with a battery of freshwater species, always following standard protocols;
- to analyse these effects in different trophic levels, comparing their sensitivity;
- to fill the gap of information for sub-lethal effects caused by ciprofloxacin;

This dissertation is divided in three chapters.

Chapter I – General Introduction.

Chapter II – Ecotoxicological effects of ciprofloxacin on non-target freshwater species.

Chapter III – Concluding Remarks

In chapter 1 background information about the following aspects was presented: i) concerns related with the release of pharmaceutical substances on aquatic environments. the usage, sources, environmental fate of pharmaceutical products; iii) European legislation regulating the release of pharmaceutical substances into the environment and current frameworks for their risk assessment; iv) the chemical characterization and pharmacokinetics of ciprofloxacin in particular (the object of study in this thesis. Chapter II - describes the lethal and sub-lethal evaluation of the ecotoxicity of ciprofloxacin, with species from different throphic levels (*Vibrio fischeri*, *Pseudokirchneriella subcapitata, Lemna minor, Daphnia magna* and *Gambusia holbrooki*). The ecotoxicological data obtained is presented and discussed, in terms of species sensitivity, and a first attempt was made to derive a PNEC values (predicted-no-effect-concentration) with all the data available, following European Guidelines. In Chapter III, after a final and integrative conclusion, future perspectives were presented.

Chapter II

Ecotoxicological effects of ciprofloxacin on non-target freshwater species: data integration and derivation of toxicity thresholds for risk assessment

Ecotoxicological effects of ciprofloxacin on non-target freshwater species: data integration and derivation of toxicity thresholds for risk assessment

Martins N., Pereira R., Abrantes N., Pereira J., Gonçalves F., Marques C.R.
CESAM & Department of Biology, University of Aveiro, Campus Universitário de Santiago, 3810-056 Aveiro, Portugal

Abstract Although antibiotics have been increasingly used and detected in natural samples, their ecotoxicological effects on aquatic wildlife are not yet extensively studied. Considering the environmental threat posed by the biological activity of antibiotics it is quite relevant to assess the resulting impact, especially on sub-lethal endpoints. As such, this study has evaluated the effects of ciprofloxacin on *Vibrio fischeri* luminescence, *Pseudokirchneriella subcapitata* and *Lemna minor* growth, on the survival and reproduction of *Daphnia magna* and on *Gambusia holbrooki* survival. The risks associated with ciprofloxacin effects on non-target organisms were quantified through the calculation of the PEC/PNEC ratio. Overall, the toxicity values obtained (at the mg L^{-1} level) were higher than the environmental concentrations. *P. subcapitata* and *L. minor* were more sensitive under short-term exposures than *V. fischeri*, *D. magna* and *G. holbrooki.* No acute toxicity was observed for fish. The chronic assay with *D. magna* evidenced that long-term exposure to lower concentrations of this antibiotic induced impairment on its life history parameters. Such outcome may pre-empt potential damages on the long-term maintenance of natural populations continuously exposed to the input of antibiotics. Indeed, the PEC/PNEC ratios showed that ciprofloxacin represents a risk for the most sensitive aquatic organisms, since the defined threshold of an acceptable risk was considerably surpassed.

Key words: pharmaceuticals, *Vibrio fischeri*, *Pseudokirchneriella subcapitata*, *Lemna minor*, *Daphnia magna*, *Gambusia holbrooki*, acute and chronic effects, risk quotients.

Introduction

The high production and ubiquitous presence of pharmaceutical residues in different aquatic environments together with their biologically active nature had recently raised an environmental concern about the impacts of these micropollutants on non-target wildlife (Ferrari et al. 2004; Fent et al., 2006; Santos et al., 2010). Usually they enter the aquatic systems through wastewater and sewage treatment plants (STP's) that receive human disposal medicines often not effectively removed or biodegraded (Daughton and Ternes, 1999). Additionally, pharmaceutical residues may also derive from veterinary use (namely on aquacultures), industrial production wastes and from run-off and leaching processes of arable fields receiving contaminated manure or sludge (Halling-Sørensen et al., 2000; Santos et al., 2010).

In particular, antibiotics represent a special threat for environmental health due to the potential development of antibacterial resistance (Kümmerer, 2003). Antibiotics were the second most detected class (15%) of pharmaceutical residues in the environment between 1997 and 2009 (Santos et al., 2010). They have been quantified in hospital and STPs effluents (*e.g.*, Seifrtová et al., 2008; Santos et al., 2009), surface (Pena et al. 2007) and groundwaters (Kümmerer, 2003) from the ng L^{-1} to the µg L^{-1} level, what indicates that they are not easily degraded and tend to persist. Nevertheless, their ecotoxicological effects on non-target organisms have not been largely investigated (Santos et al., 2010).

Ciprofloxacin is a broad spectrum antibiotic used in human and veterinary medicine that belongs to the chemical class of fluoroquinolones (Chin et al., 1984; Zeiler et al., 1984; Zeiler, 1985). The National Authority for Medicines and Health Products (Infarmed) placed it as the 43rd pharmaceutical with more packages sold by the SNS (National Health System), with 655,694 different packages containing this active substance (Infarmed, 2008). In the European context, this is the most prescribed fluoriquinolone (Ferech et al., 2006).

The mode of action of ciprofloxacin relies on the inhibition of bacterial multiplication by disrupting DNA replication and repairing processes (Streuff et al., 1987). The absorption of the compound is generally rapid (Turnidge, 1999), being its half-life between 3 to 5 hours (Bergan el., 1988) and it is eliminated mainly through renal excretion (Bergan et al., 1988; Sörgel et al. 1991), hence entering the aquatic systems through STP effluents. Actually, it was reported that fluoriquinolones usually persist in the aquatic

compartment (Huang et al., 2001). Studies worldwide have found concentrations of ciprofloxacin of 0 – 10,962.5 ng L^{-1} in hospital effluents (*e.g.*, Brown et al., 2006; Seifrtová et al., 2008); 7 – 309.2 ng L^{-1} in wastewater treatment plant effluents (*e.g.*, Lindberg et al., 2005; Seifrtová et al., 2008); and 79.6 – 119.2 ng L^{-1} in river water (*e.g.*, Pena et al., 2007). Previous works have already demonstrated that ciprofloxacin is extremely genotoxic (Hartmann et al., 1998) and acutely toxic for *P. subcapitata* (Grung et al., 2008), nevertheless it is needed to fulfill the gap of ecotoxicological information about this antibiotic. Since the environmental concentrations found are quite low, the ecotoxicological analysis should include not only acute data, but also the assessment of sub-lethal effects on non-target organisms.

Thereby, this paper seeks to provide a general evaluation of the toxicity of ciprofloxacin, acquiring new values and diminishing the gap of information for sub-lethal effects. According to European legislation and guidance (CHMP, 2006; EC, 2006) the environmental risk assessment of pharmaceuticals is compulsory for their marketing authorization. However, as far as authors are aware, the assessment of effects of ciprofloxacin is not very extensive yet. As such, a battery of acute and sub-lethal ecotoxicological tests was performed in this work, using individuals of different aquatic trophic levels (bacteria - *Vibrio fischeri* -, microalgae - *Pseudokirchneriella subcapitata* -, macrophytes - *Lemna minor* -, crustaceans - *Daphnia magna* -, and fish - *Gambusia holbrooki*). At the end, differences of sensitivity between trophic levels were compared, and the PEC/PNEC ratio (*i.e.*, predicted-environmental-concentration and predicted-no-effect-concentration ratio) will be evaluated to ascertain potential environmental risks of ciprofloxacin to the aquatic environment (CHMP, 2006). The PNEC value was estimated based on the ecotoxicological data generated in this study and on the data available on literature, following the Technical Guidance Document on Risk Assessment (EC, 2003).

Material and Methods

1. Test species and culturing conditions

V. fischeri was used as freeze-dried reagent after being reconstituted according to the methods established on the Microtox® protocols supplied by Microbics Inc. Protocols.

P. subcapitata was kept in unialgal cultures in 250 ml erlenmeyer with Woods Hole MBL medium (Stein, 1973) in an orbital shaker (100 rpm). Every week the cultures were renewed.

The macrophyte *L. minor* was maintained in glass vessels of 150 mL with STEINBERG medium (OECD, 2006). The cultures were renewed weekly.

New born females of *D. magna* were maintained in 800 mL glass recipients with ASTM hard water (ASTM, 1980) and a seaweed extract (organic additive made of *Ascophylum nodosum*; Baird et al., 1989), being fed with the microalgae *P. subcapitata* (3.0×10^5 cell/mL/*Daphnia*). The cultures were changed every two days.

G. holbrooki was collected in the field and acclimated under laboratorial conditions for 2 weeks before test beginning. They were kept in tanks with continuous oxygenation and were fed with commercially available fish food (Aquapex® pond flakes) at least three times *per* week. Water was renewed every 2-3 weeks. The cultures were daily monitored for dead and/or sick animals.

All cultures of algae, macrophytes, daphnids and fish were kept under 20ºC±1°C and a photoperiod of $16h^L$: $8h^D$.

2. Ciprofloxacin and stock solutions

Ciprofloxacin was obtained from Fluka Analytical at 98% [1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-piperazin-1-ylquinoline-3-carboxylic acid hidrochloride; CAS 85721-33-1]. The solubility of ciprofloxacin is > 2 g L^{-1} (Halling-Sørensen et al., 2000), although it varies with the pH value of the solution medium (Yu et al., 1994). Its partitioning coefficient octanol-water (Log Kow) is 1.24±0.86 (Halling-Sørensen et al., 2000) and presents a half-life of about 1.5 h in water due to photodegradation (Cardoza et al., 2005) (Table 1).

The stock solutions of ciprofloxacin were prepared in the respective culture media of each organism (*i.e.*, MBL for *P. subcapitata*, STEINBERG for *L. minor*, ASTM for *D. magna*, and dechlorinated tap water for *G. holbrooki*). All solutions were thoroughly mixed and sonicated until total solubility of the compound.

Table 1. Physical, chemical and environmental occurrence data available for ciprofloxacin. In bold are signed out the values used for the derivation of PEC values (Table 4).

Parameter	Data	Reference
CAS #	85721-33-1	-
Therapeutic class	Fluoroquinolone antibiotic	-
Molecular weight (g mol^{-1})	331.34	-
Solubility (g L^{-1})	>2	Halling-Sørensen et al., 2000
Log Kow	1.24 ± 0.86	Halling-Sørensen et al., 2000
Koc (soil)	61000	Tolls, 2001
Biodegradability (half-life)		
activated sludge (days)	1.6 - 2.5	Halling-Sørensen et al., 2000
photodegradation in water (h)	1.5	Cardoza et al., 2005
Occurrence in aquatic environment (ng L^{-1})		
hospital effluents	0 – **10,962.5**	Brown et al., 2006; Seifrtová et al., 2008
STP effluents	7 – **309.2**	Lindberg et al., 2005; Seifrtová et al., 2008
river water	79.6 – **119.2**	Pena et al., 2007

3. Ecotoxicological assays

3.1. Microtox® test

Microtox® test (Microbics Corporation Inc. Protocols, 1988) uses the bioluminescent properties of the bacteria *V. fischeri* to assess the toxicity of xenobiotics (Kaiser, 1998). The principle of the assay is based on the chemical reaction in which the enzyme luciferase oxidise long chain aldheyde and reduces flavin in the presence of oxygen, thereby releasing chemical energy into blue-green light (Hernando et al., 2007). For this, a Basic Test Protocol was applied, exposing *V. fischeri* to several dilutions of a ciprofloxacin stock solution (made with distilled water) of 60 mg L^{-1}, being the readings of bioluminescence made 5, 15 and 30 minutes after exposure.

3.2. Microalgae growth inhibition

The experimental design was based on the OECD guideline (2006). Algae were exposed in 100 mL erlenmeyers to three replicates of each test concentration (final range: 0.273, 0.547, 1.09, 2.19, 4.38, 8.75, 17.5, and 35.0 mg L^{-1}) and then maintained for 96h at a 16^{L}:8^{D} h photoperiod, 20±1ºC and 100 rpm in an orbital shaker. At the end, the algae growth rate was determined based on the cell density parameter by microscopic (Olympus CKX41) counting of algae cells in a Neubauer chamber.

3.3. Macrophyte growth inhibition

The test followed the OECD guidelines for *Lemna* sp. growth inhibition (OECD, 2006). Three replicates *per* test concentration (final range: 0.050, 0.137, 0.412, 1.24, 3.70, 11.1, 33.3 and 100 mg L^{-1}) were prepared in 100 mL flasks and three plants with three fronds were placed in each one. The test was run at a 16^{L}:8^{D} h photoperiod and 20±1°C during seven days. The medium was renewed every three days. The effects of ciprofloxacin on *L. minor* growth rate were determined based on the frond number (OECD, 2006).

3.4. *Daphnia* sp. assays

All experiments were performed with <24h neonates from the third to the fifth offspring, in order to avoid maternal influence (Barata and Baird, 1998). The exposures were carried out under a 16^{L}: 8^{D} h photoperiod and 20±1°C.

3.4.1. *Daphnia sp*. acute immobilization test

The neonates were exposed to a range of six concentrations (final range: 6.25, 12.5, 25.0, 50.0 and 100 mg L^{-1}) for 48 hours (OECD, 2004). Five animals were placed randomly in each 180 mL glass flasks with 50 mL of the respective test solution up to four replicates. The animals were not fed during the test. pH (pH 330 WTW) and oxygen (Oxi 330 WTW) levels were measured in the beginning and at the end of the assay. At the end of the exposure the number of immobilized organisms was recorded.

3.4.2. *D. magna* reproduction test

The test was performed for six concentrations (final range: 1.79, 3.05, 5.19, 8.82, 15.0 and 25.5 mg L^{-1}) in an individual ten replicate design, during 21 days (OECD, 1998). The medium was renewed every two days. The exposure conditions and feeding (at least five times a week) of animals were carried out according to what was done during their culture. The endpoints analyzed along the test were the fecundity of females, the age at first reproduction, number of broods *per* female, the size of neonates from the first brood, the somatic growth rate and the rate of population increase. Parent animals' mortality was recorded daily. The somatic growth rate of females was assessed by measuring the length (Olympus SZX9 Stereo Microscope) of the first exopodite of the second antennae in the beginning and in the end of the test. Body length was measured through a formula that

establishes allometric relations between *D. magna* body length (BL) and exopodite length (EL) (Pereira et al., 2004):

$$BL = 10.499 \times EL - 0.329 \text{ (mm)}.$$

Somatic growth rate was then determined by:

$$SGR = [\ln(BL_f) - \ln(BL_i)] / \Delta t \text{ (days}^{-1}\text{)},$$

where, BL_f is the final body length, BL_i is the initial body length and Δt is the exposure time (21 days) (Sobral, 1997; Burns, 2000).

Intrinsic rate of population increase (r) integrates parameters at the individual level (*e.g.*, survival, fecundity, age at the first brood). The calculation involves various iterations to determine the r value for the Euler-Lotka equation (Meyer et al., 1986; McCallum, 2000):

$$1 = \Sigma (e^{-rx} . l_x . m_x)$$

where x is the age class (days), l_x is the probability of survival at age x and m_x is the fecundity at age x. The standard deviation was determined according to jackknife technique (Meyer et al., 1986).

3.5. Fish acute test

The test was performed according to the OECD guideline for fish acute test (1992). Male and non-pregnant female of *G. holbrooki* were placed in 750 mL plastic containers containing 500 mL of the test solution. Before beginning the test the solubility of ciprofloxacin was tested for 60, 70, 80 and 100 mg L^{-1}. The compound was totally dissolved only at 60 mg L^{-1}, thereby, this was the maximum concentration tested. The test was run with ten individual replicates during 96 h without aeration, no feeding, with a 16^L:8^D h photoperiod and a temperature of 20±1°C. The test solution was replaced each 24 h. Dead organisms, pH and oxygen values were verified at the 24, 48, 72 and 96 h.

4. Statistical analysis

The EC_{50} (concentration inducing 50% effect) point estimates and respective 95% confidence limits were calculated for the growth of microalgae and macrophyte, the immobilization and fecundity of *D. magna* and the survival of *G. holbrooki* through the Probit regression analysis (Finney, 1971). For *V. fischeri* the EC_{50} was retrieved by the software.

The determination of NOEC (no-observed-effect-concentration) and LOEC (low-observed-effect-concentration) point estimates for microalgae and macrophyte growth rates, and for life-history parameters of *D. magna* was made by a one-way ANOVA test

followed by the Dunnett's multiple comparison test, which allowed to find out significant differences between the control and the concentrations tested ($p < 0.05$).

5. PEC/PNEC ratio

This ratio represents a preliminary approach for the risk analysis of ciprofloxacin to the aquatic wildlife. The PEC used corresponds to the highest environmental concentration of ciprofloxacin available in literature. The PEC was measured for different aquatic matrices, from hospital effluents to river waters, in order to account for worst-case situations in which the effluents could be directly disposed on surface waters. The PNEC value was calculated based on the lowest NOEC and EC_{50} obtained for algae, macrophyte, crustacean and fish exposed to ciprofloxacin according to standard procedures. The point estimates used were retrieved from this study and/or from other published studies (*cf.,* Table 2). Both point estimates were calculated because the NOEC value is dependent on the concentration range tested, while the EC_{50} is derived from a regression analysis of the data, thereby providing a more accurate estimation (Isidori et al., 2005). The NOEC and EC_{50} values were divided by an assessment factor of 50 and 1000, respectively, to obtain the PNEC value (EC, 2003). The Technical Guidance Document recommends the application of assessment factors, in detriment to probabilistic methods, to derive the PNEC when less than 10 NOEC/EC_{50} values are available for less than 8 different taxonomic groups (EC, 2003). If values >1 are obtained for the PEC/PNEC ratio, then there is a risk for the aquatic environment and further testing will be needed.

Results

All pH and oxygen values were maintained under the recommended range by the guidelines that were followed to perform the assays.

Table 2 summarizes the point estimates for each endpoint and species exposed to ciprofloxacin, while table 3 presents the summary of the one-way ANOVA test. The antibiotic was acutely toxic for *V. fischeri* (EC_{50} = 11.5 mg L^{-1}) and *D. magna* (EC_{50} = 65.3 mg L^{-1}), and not toxic for *G. holbrooki*. For *P. subcapitata* was determined an EC_{50} of 4.83 mg L^{-1}, being the LOEC of 2.19 mg L^{-1} (Figure 4, Tables 2 and 3). The growth rate of *L. minor* was significantly depleted at a LOEC of ≤0.050 mg L^{-1} (Figure 5, Tables 2 and 3).

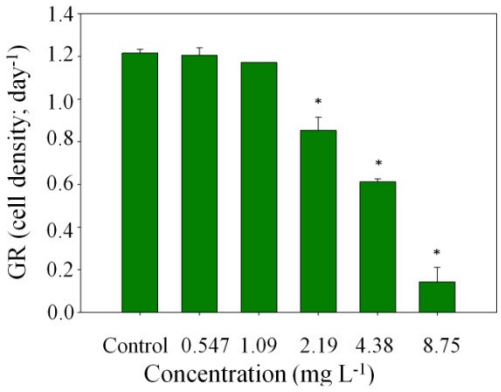


Figure 4. Growth rate (GR) of *P. subcapitata* exposed to increasing concentrations of ciprofloxacin. Standard error is indicated above each bar. * refers to a significant difference from the control ($p < 0.05$).

Table 2. Resume of point estimates [(NOEC - no-observed effect concentration -, LOEC – low observed effect concentration -, and EC_{50} – effect concentration at a 50% level (mg L^{-1})] determined for all the tests and species used in this study. The available ecotoxicological information on literature for ciprofloxacin is also presented (EC_{50}; mg L^{-1}). In brackets are presented the 95% confidence limits of EC_{50} values, whenever available. The values in bold were used to estimate PNECs (*cf.*, Table 4).

	Test Organism	Parameters	EC_{50}	NOEC	LOEC
Data from the current study	*V. fischeri*	Luminescence inhibition - at 30 min	11.5	ND	ND
	P. subcapitata	Growth inhibition (cell density) - 96 h	4.83 (3.44 -7.32)	**1.09**	2.19
	L. minor	Growth inhibition (no. of fronds) - 7 d	3.75	<0.050	≤0.050
	D. magna	Immobilisation - 48 h	65.3 (54.9-79.1)	ND	ND
		Fecundity	12.8 (10.8-15.3)	5.19	8.82
		Age at first reproduction	ND	15	25.5
		Number of broods *per* female	ND	8.82	15
		Size of neonates from the first brood	ND	1.8	3.05
		Somatic growth rate	ND	8.82	15
		Intrinsic rate of population increase	ND	8.82	15
	G. holbrooki	Mortality - 96 h	>60	ND	ND

	Test Organism	Parameters	EC_{50}	Reference
Data available in literature	*V. fischeri*	Luminescence inhibition - at 30 min	>5.9	Hernando et al., 2007
	P. subcapitata	Growth inhibition (cell density) -72 h	2.97 (2.41-3.66)	Halling-Sørensen et al., 2000
			18.7 (16.2-21.2)	Robinson et al., 2005
	Chlorella vulgaris	Growth inhibition (cell density) - 96 h	20.6	Nie et al., 2008
	Microcystis aeruginosa	Growth inhibition (cell density) - 120 h	0.017 (0.014-0.020)	Robinson et al., 2005
		Growth inhibition (cell density)	**0.005** (0.004-0.006)	Halling-Sørensen et al., 2000
	L. gibba	Growth inhibition (no. of fronds) - 7 d	0.697 (0.554-0.861)	Brian et al., 2004
	L. minor (1)	Growth inhibition (no. of fronds) - 7 d	0.203 (0.041-0.364)	Robinson et al., 2005
	D. magna	Immobilisation - 48 h	> 60	Halling-Sørensen et al., 2000
		Immobilisation - 48 h	>10	Robinson et al., 2005
	Brachydanio rerio	Mortality - 72 h	>100	Halling-Sørensen et al., 2000

ND - not determined.

(1) Assays not performed according to standard guidelines.

Table 3. One-way ANOVA outcome summary for the growth of microalgae and macrophyte, and for the chronic endpoints of daphnids.

Species	Endpoints	*F*	d.f.	P
P. subcapitata	Growth rate (cell density)	103.009	5	<0.001
L. minor	Growth rate (no. of fronds)	58.269	8	<0.001
D. magna	Fecundity of females	37.172	6	<0.001
	Age at first reproduction	16.347	6	<0.001
	Number of broods *per* female	32.467	6	<0.001
	Size of neonates from the first brood	11.511	6	<0.001
	Somatic growth rate	6.141	6	<0.001
	Rate of population increase	32.888	6	<0.001

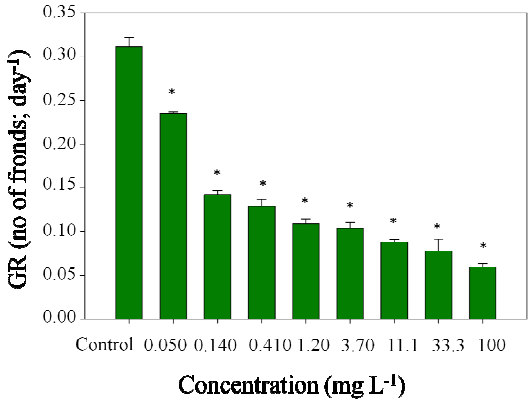


Figure 5. Growth rate (GR) *L. minor* exposed to increasing concentrations of ciprofloxacin. Standard error is indicated above each bar. * refers to a significant difference from the control ($p < 0.05$).

Ciprofloxacin induced chronic effects on *D. magna* life-history parameters (Figure 6, Tables 2 and 3). The most affected parameter was the size of neonates from the first brood as it retrieved the lowest LOEC value (3.05 mg L^{-1}; Table 2). Nevertheless, the fecundity of females was also inhibited though to a higher LOEC (8.82 mg L^{-1}), while the EC_{50} was of 12.8 mg L^{-1}. The somatic growth rate, the number of broods produced *per* female and the intrinsic rate of population increase were depleted at a 15.0 mg L^{-1} LOEC. A significant delay in the age at first reproduction was noticed at the highest concentration of ciprofloxacin (25.5 mg L^{-1}).

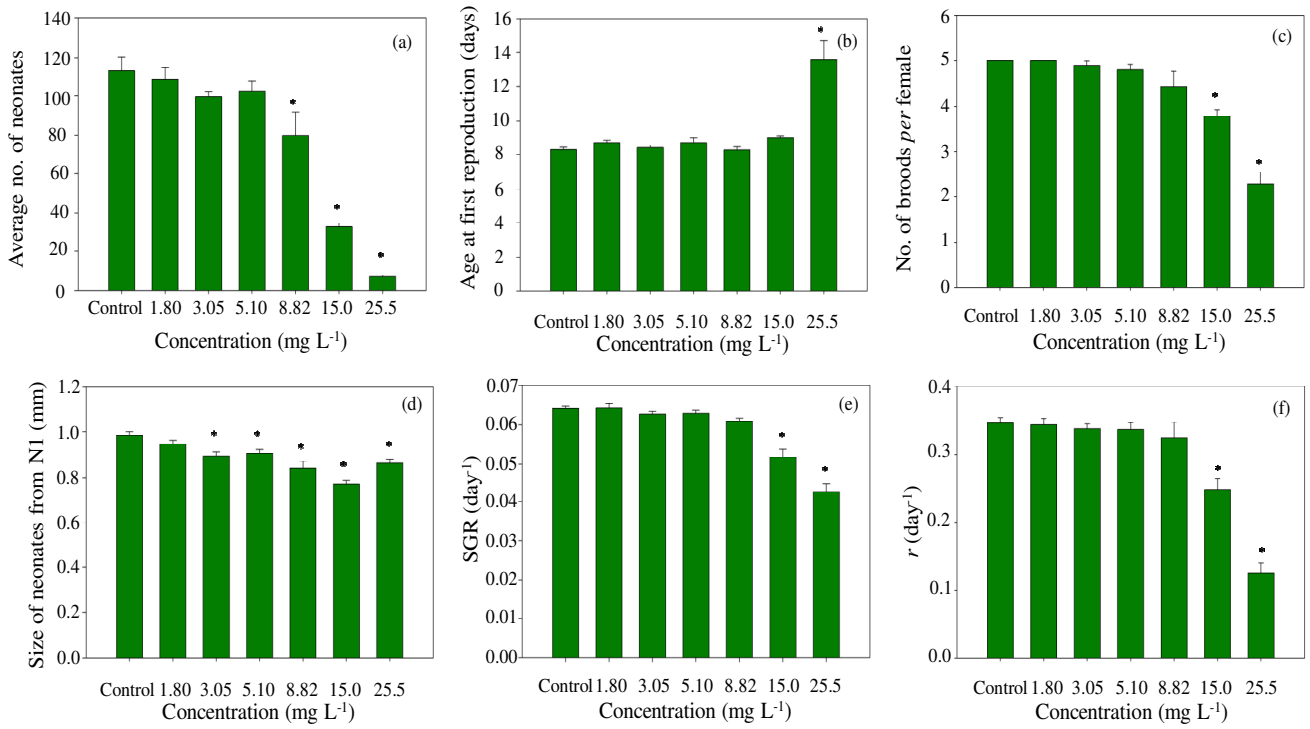


Figure 6. *Daphnia magna* reproduction test results of exposure to increasing concentrations of ciprofloxacin. a) fecundity; b) age at first reproduction; c) number of broods *per* female; d) size of neonates from the first brood (N1); e) somatic growth rate (SGR); f) intrinsic rate of population increase (*r*). Standard error is indicated above each bar. * refers to a significant difference from the control ($p < 0.05$).

The quotient risks determined for different aquatic samples and using two toxicity point estimates are presented on table 4. The ciprofloxacin $PEC/PNEC_1$ ratios based on the NOEC value obtained in this study for *P. subcapitata* were below 1. However, the $PEC/PNEC_2$ estimated with the lowest EC_{50} obtained for *M. aeruginosa* by Halling-Sørensen et al. (2000) (Table 2) assumed values much higher than 1 (Table 4).

Table 4. Risk quotients (PEC/PNEC) based on the PEC estimated for different aquatic matrices and PNEC estimated for NOEC (*i.e.,* $PNEC_1$) and EC_{50} (*i.e.,* $PNEC_2$) values (*cf.,* Table 2). The quotients indicating a risk are in bold. NOEC – no-observed-effect-concentration; EC_{50} – concentration inducing 50% of effect; PEC – predicted-environmental-concentration; PNEC – predicted-no-effect-concentration.

Aquatic matrices	NOEC (mg L^{-1})	Assessment factor	$PNEC_1$ (µg L^{-1})	PEC (µg L^{-1})	$PEC/PNEC_1$	EC_{50} (mg L^{-1})	Assessment factor	$PNEC_2$ (µg L^{-1})	PEC (µg L^{-1})	$PEC/PNEC_2$ ratio
Hospital effluent	1.09	50	21.8	10.96	0.503	0.005	1000	0.005	10.96	**2192.6**
STP effluent	1.09	50	21.8	0.309	0.014	0.005	1000	0.005	0.309	**61.8**
River water	1.09	50	21.8	0.119	0.005	0.005	1000	0.005	0.119	**23.8**

Discussion

The outcome of this study evidenced that ciprofloxacin caused lethal and/or sub-lethal effects in most of the tested species. Notwithstanding, the effect concentrations determined are generally above the levels detected in different aquatic matrices (*cf.*, Pena et al., 2007; Seifrtová et al., 2008).

Among the short-term tests performed, the bioluminescence inhibition of *V. fischeri* was the most sensitive parameter to ciprofloxacin (EC_{50} = 11.5 mg L^{-1}) (Table 2). Hernando et al. (2007) could not determine a toxicity value (EC_{50} > 5.9 mg L^{-1}) but attained 28% effect of ciprofloxacin on *V. fischeri* bioluminescence after 30 min of exposure. Previous studies with other antibiotics have shown that Microtox® test has low sensitivity to these pharmaceutical drugs (*e.g.*, Ferrari et al., 2004; Isidori et al., 2005; Christense et al., 2006; Van der Grinten et al., 2010), what was linked to the short exposure time hence preventing a noticeable damage on biosynthetic pathways (Backhaus and Grimme, 1999; Froehner et al., 2000; Isidori et al., 2005; Van der Grinten et al., 2010). On the other hand, ciprofloxacin was moderately to non-toxic for *D. magna* and *G. holbrooki*, respectively. Halling-Sørensen et al. (2000) found that this antibiotic was not toxic for *D. magna* (NOEC-48h = 60 mg L^{-1}) and *Brachydanio rerio* (NOEC-72h = 100 mg L^{-1}) (Table 2).

In the literature is often referred the need for generating protective and precautionary ecotoxicological data for integrating the risk assessment of pharmaceuticals (*e.g*. Grung et al., 2008; Santos et al., 2010). This way, the present study also aimed to identify sub-lethal effects at low ciprofloxacin concentrations.

The chronic toxicity of ciprofloxacin on *P. subcapitata* growth was similar to the one obtained by Halling-Sørensen et al. (2000) (EC_{50} = 2.97 mg L^{-1}). Notwithstanding, the toxicity value obtained by Robinson et al. (2005) for this species was almost one order of magnitude higher (EC_{50} = 18.7 mg L^{-1}), similarly to the one determined for *Chlorella vulgaris* (EC_{50}-96h = 20.6 mg L^{-1}) by Nie et al. (2008) (Table 2).

L. minor was the most sensitive organism to ciprofloxacin phytotoxicity, presenting the lowest EC_{50} and LOEC values comparatively to algae and daphnids under sub-chronic exposures (Table 2). Robinson et al. (2005) found a similar trend in which *L. minor* growth was more affected (EC_{50} of 0.203 mg L^{-1}) by ciprofloxacin than that of microalgae *P.*

subcapitata. Even though, the toxicity value they determined was about one order of magnitude lower than the one calculated in the current study (3.75 mg L^{-1}), similarly to what was published by Brain et al. (2004) for *L. gibba* (EC_{50} = 0.653 mg L^{-1}) (Table 2). Consistently with these two latter studies, it was observed the occurrence of chlorosis in new fronds of *Lemna* exposed to higher concentrations of ciprofloxacin. Brain et al. (2004) explained that the mode of action of ciprofloxacin inhibits the chloroplastic activities of *Lemna* cells.

Although *D. magna* life-history traits were not as sensitive as was the growth of the macrophyte *L. minor*, they were significantly impaired under increasing concentrations of ciprofloxacin (Figure 6, Tables 2 and 3). Among the endpoints tested, the size of neonates from the first brood was impaired at lower concentrations of ciprofloxacin. The first brood of daphnid females actually represents an important role in terms of the maintenance of a population under unstable conditions (Stibor and Lampert, 1993). Hence, the reduced size of neonates may constrain the fitness of newborn organisms to withstand deleterious conditions and to guarantee the balance of population dynamics. Fecundity was the second most affected parameter, followed by the number of broods *per* female, the somatic growth rate, the intrinsic population increase and the age at first reproduction (Figure 6, Tables 2 and 3). This latter parameter (*r*) integrates individual-level traits - survival, reproductive output, and period between successive reproduction, namely the age at first reproduction - to provide an overview of toxicant effects at the population level, hence making it an improved ecotoxicological endpoint that gives a more ecologically-sound assessment of the toxicant impact (Forbes and Calow, 1999). Notwithstanding, in the present study, this endpoint did not provide the most conservative information about ciprofloxacin effects. Ferrari et al. (2004) retrieved a NOEC value for the fluoroquinolone ofloxacin of 10 mg L^{-1} for the 7-day reproduction test with *Ceriodaphnia dubia*. For the same species and antibiotic, Isidori et al. (2005) determined a chronic EC_{50} of 3.13 mg L^{-1} (Table 2). Thereby, fluoriquinolones do affect the reproduction of cladocerans, although chronic data is not available for ciprofloxacin.

Comparing the responses of the different trophic levels to ciprofloxacin it was clear that the producers were the most sensitive trophic level while consumers were more tolerant, being the overall decreasing order of sensitivity *L. minor* > *P. subcapitata* > *V. fischeri* > *D. magna* > *G. holbrooki*. Under the ecosystem level, however, bottom-up and

top-down effects may occur whenever the maintenance of natural populations from lower trophic levels is constrained (Relyea and Hoverman, 2006). Although the effect concentrations herein obtained are far above the levels of ciprofloxacin quantified in the aquatic systems, its ability to persist adsorbed onto particulate matter (*cf.,* Koc value in Table 1), jointly with the continual input to the environment (Robinson et al, 2005) may pose a risk to aquatic wildlife.

In fact, from the integration of exposure (PEC) and effect (PNEC) assessment data it was verified that ciprofloxacin may become a risk to the aquatic environment (Table 4), depending on the point estimates used for the calculation of PNEC. Usually the calculation of a PNEC based on long-term NOECs reduces the uncertainty of extrapolations from laboratory to field effects, comparatively to the PNECs estimated through EC_{50} values (EC, 2003; Isidori et al., 2005). Notwithstanding, the latter point estimate provides a more accurate outcome since it is generated from a regression analysis applied to the dataset, whilst the NOEC values are constrained by the concentration range tested. For these reasons, both toxicity values were used for the derivation of PNECs to ciprofloxacin.

The $PNEC_1$ calculated with the lowest NOEC obtained for the most sensitive species in the current study – *P. subcapitata* – resulted in an acceptable risk of ciprofloxacin to aquatic species. On the other hand, the ratios estimated from the $PNEC_2$ based on the lowest EC_{50} available, had exceeded the threshold outlined for an acceptable risk, especially for a worst-case PEC scenario related with the discharge of a hospital effluent in surface waters (Tables 1 and 2). This risk characterization was actually in agreement with the outcome observed by Halling-Sørensen et al. (2000), Robinson et al. (2005) and Grung et al. (2008) that attained PEC/PNEC ratio values between 5.88 and 1401. It should be noticed that in these latter studies, the PEC values were based on the consumption of pharmaceuticals *per* inhabitant (Grung et al., 2008) or *per* year (Halling-Sørensen et al., 2000), or based on hypothetic protective concentration scenarios (Robinson et al., 2005). Similar approaches are recommended for the predictive risk assessment of human and veterinary medicals (CHMP, 2006; EMEA, 2008). However, whenever effective environmental concentrations of pharmaceutical residues are available, they should be preferably used for the derivation of PEC/PNEC ratios, as it was done in this study, since they already integrate the role of biotic and abiotic factors in the fate of these

chemicals in the environment, thereby improving the ecological relevance of risk characterization.

Conclusion

The fluoroquinolone ciprofloxacin induced acute and sub-lethal effects on most species of producers and consumers. The lower trophic level comprising bacteria, microalgae and a macrophyte was more sensitive to this antibiotic, especially *L. minor*, what strengthens the phytotoxic effect of ciprofloxacin mentioned by different authors. On the other hand, it was moderately toxic to acute exposures of *D. magna* and non-toxic for *G. holbrooki* survival. This study presents the first data related with ciprofloxacin chronic effects on *D. magna* reproduction. It was particularly observed a significant impact on the size of neonates from the first brood and on the fecundity rates. Overall, the obtained effect concentrations were generally above the levels detected in the aquatic systems. However, the integration of exposure and effect data in the PEC/PNEC ratios, showed that ciprofloxacin may pose a risk for the most sensitive aquatic species, particularly when aquatic samples with higher loads of this antibiotic are considered, *e.g.*, in hospital effluents. Such worst-case situation pre-empts a potential threat for ecosystem integrity and functioning. The risk characterization herein performed thus indicated that the risk assessment of ciprofloxacin should proceed to a more refined assessment tier. One strategy will be to increase the baseline dataset available for ciprofloxacin and its metabolites, what may include the testing of different species, the assessment of other sensitive sub-lethal endpoints at different biological levels of organization.

Chapter III

Concluding remarks

Concluding remarks

This study used a battery of ecotoxicological tests with different trophic levels in order to assess the potential risk of ciprofloxacin to the aquatic ecosystem, and discern which trophic levels are most affect by it. This was done while having in mind that antibiotics are chemical pollutants that raise many questions on their persistence and their action on non-target organisms. The results had shown that ciprofloxacin may represent a potential hazard for algae and aquatic plants' populations, endangering the stability of the food chain at the first trophic level, the producers. This poses a risk that must not be ignored, has it may affect populations of the next trophic levels, not only by diminishing their food supply, but also through the consumption of contaminated food, increasing their exposure to the chemical. Sub-lethal effects obtained for *D. magna* also demonstrated that populations may suffer from long-term exposure that can lead to the disruption of populations' viability and stability, proving that it is important to assess sub-lethal effects, as acute data may not represent an ecological relevant prediction of the potential risk for non-target organisms.

Overall, the obtained effect concentrations were generally above the levels detected in the aquatic systems. However, the integration of exposure and effect data in the PEC/PNEC ratios, it was observed that ciprofloxacin may pose a risk for the most sensitive aquatic species. Future research on the evaluation of the impacts this antibiotic may induce on the aquatic environment may need the application of more fine-assessment methodologies, as some standard tests proved to be ineffective to assess their contamination. Namely the use of biomarkers can help to determine early-warning signals that might facilitate the detection of ciprofloxacin contamination. Investigation on oxidative stress and anti-oxidative enzymes could be a good approach to fully understand the extension of the effects of ciprofloxacin in individual species at lower biological levels of organization. Furthermore, the investigation on methods for the removal of fluoroquinolones in sewage treatment plants might be an important step to mitigate the risks posed by antibiotic contamination on natural ecosystems.

References

- Alcaraz, C., García-Berthou, E., 2007. Life history variation of invasive mosquito fish (*Gambusia holbrooki*) along a salinity gradient. *Biol. Conserv.* 39: 83-92.
- Alonso, M., 1996. *Crustacea*, *Branchiopoda*. En: *Fauna Ibérica*, vol. 7. Ramos, M.A. et al. (Eds.). Museo Nacional de Ciencias Naturales. CSIC. Madrid. 486 pp.
- ASTM (American Society for Testing and Materials). 1980. Standard practice for conducting acute toxicity tests with fishes, macroinvertebrates and amphibians. Report E 729-80. American Society for Testing and Materials, Philadelphia, USA.
- Backhaus, T., Grimme, L.H., 1999. The toxicity of antibiotic agents to the luminescent bacterium *Vibrio fischeri*. *Chemosphere* 38: 3291–3301.
- Baird, D.J., Soares, A.M.V.M., Girling, A., Barber, I., Bradley, M.C., Calow, P., 1989. The long-term maintenance of *Daphnia magna* Straus for use in ecotoxicity tests: problems and prospects. In: Lokke, H., Tyle, H., Bro-Rasmussen F, editors. Proceedings of the First European Conference on Ecotoxicology. Denmark: Lyngby, pp. 144–8.
- Baird, D.J., Barata, C., 1998. Variability in the response of Daphnia clones to toxic substances: are safety margins being compromised? *Arch. Toxicol. Suppl.* 20: 399-406.
- Bergan, T., Dalhoff, A. & Rohwedder, R., 1988. Pharmacokinetics of ciprofloxacin. *Infection* 16 (Suppl. 1): S3–S13.
- Bergan, T., Thorsteinsson, S.B., 1986. Pharmacokinetics and bioavailability of ciprofloxacin. Neu, H. C., Weuta, H. (eds.): Proceedings of the First International Ciprofloxacin Workshop, Leverkusen 6-8. Nov. 1985, Excerpta Medica, Amsterdam, pp. 111-120.
- Brain, R.A., Johnson, D.J., Richards, S.M., Sanderson, H., Sibley, P.K., Solomon, K.R., 2004. Effects of 25 pharmaceutical compounds to *Lemna minor* using a seven-day static renewal test. *Environ. Toxicol. Chem.* 23: 371-382.
- Brown, K.D., Kulis, J., Thomson, B., Chapman, T.H., Mawhinney, D.B., 2006. Occurrence of antibiotics in hospital, residential, and dairy effluent, municipal wastewater, and the Rio Grande in New Mexico, *Sci. Total Environ.* 366: 772-783.

- Burns, C.W., 2000. Crowding-induced changes in growth, reproduction and morphology of *Daphnia*. *Freshwat. Biol.* 43: 19-29.
- Cardoza, L.A., Kanpp, C.W., Larive, C.K., Belden, J.B., Lydy, M., Graham, D.W., 2005. Factors affecting the fate of ciprofloxacin in aquatic field systems. *Water, Air, and Soil Pollution* 161: 383-398.
- CEEC, 1993. Council Directive 93/39/EC of 14 June 1993 amending Directives 65/65/EC, 75/318/EC in respect of medicinal products. Official Journal of the European Communities L214 (24/08/1993), 22-30. In: Straub, J.O., 2002. Environmental risk assessment for new human pharmaceuticals in the European Union according to the draft guideline/discussion paper of January 2001. *Toxicol. Lett.* 131: 137-143.
- Chin, N.-X., and Neu, H.C., 1984. Ciprofloxacin, a quinolone carboxylic acid compound active against aerobic and anaerobic bacteria. *Antimicrob. Agents Chemother.* 25: 319-326.
- Christensen, F.M., 1998. Pharmaceuticals in the environment – a human risk? *Regul. Toxicol. Pharm.* 28: 212–221.
- Christensen, A.M., Ingerslev, F., Baun, A., 2006. Ecotoxicity of mixtures of antibiotics used in aquacultures. *Environ. Toxicol. Chem.* 25: 2208–2215.
- CHMP, 2006, Guideline on the Environmental Risk Assessment of Medicinal Products for Human use. Doc. Ref. EMEA/CHMP/SWP/4447/00.
- Daughton, C.G., Ternes, T.A., 1999. Pharmaceuticals and personal care products in the environment: agents of subtle change? *Environ. Health Perspect.* 107: 907–937.
- EC (European Commission). 2003. Technical Guidance Document on Risk Assessment in support of Commission Directive 93/67/EEC on Risk Assessment for new notified substances, Commission Regulation (EC) No 1488/94 on Risk Assessment for existing substances, Directive 98/8/EC of the European Parliament and of the Council concerning the placing of biocidal products on the market.
  Part II, Joint research Centre, Ispra.
- EC (European Commission). 2006. Regulation (EC) No 1907/2006 of the European Parliament and of the Council concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European

Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. In: Communities. O.J L 396, 30.12.2006.

- Eliopoulos, G.M., Gardella, A., Moellering Jr, R.C., 1984. In vitro activity of ciprofloxacin, a new carboxyquinoline antimicrobial agent. *Antimicrob. Agents Chemother.* 25(3): 331-335.
- EMEA (European Medicines Agency), 1998. Note for Guidance: Environmental Risk Assessment for Veterinary Medicinal Products other than GMO containing and Immunological Products. EMEA, London (EMEA/CVMP/055/96).
- EMEA (European Medicines Agency), 2005. Note for Guidance on Environmental Risk Assessment of Medicinal Products for Human Use, CMPC/SWP/4447/draft. The European Agency for the Evaluation of Medicinal Products (EMEA), London.
- EMEA (European Medicines Agency), 2008. Revised Guideline on Environmental Impact Assessment for veterinary medical products in support of VICH guidelines GL6 and GL38. European Medicines Agency. Committee for Medicinal Products for Veterinary use (CVMP), EMEA/CVMP/ERA/418282/2005-Rev.1, 17 Nov.2008.
- EMEA (European Medicines Agency), 2008. Guildeline on Safety and Efficacy Follow-up-risk Management of Advanced Theray Medical Products. European Medicines Agency. Committee for Medical Products for Human use (CHMP), EMEA/CHMP/ERA/149995/2008.
- Fent, K., Weston, A.A., Caminada, D., 2006. Ecotoxicology of human pharmaceuticals Review, *Aquat. Toxicol.* 76: 122-159.
- Ferech, M., Coenen, S., Malhotra-Kumar, S., Dvorakova, K., Hendrickx, E., Suetens, C., Goossens, H., 2006. European surveillance of antimicrobial consumption (ESAC): outpatient quinolone use in Europe. *J. Antimicrob. Chemother.* 58: 423-427.
- Ferrari, B., Mons, R., Vollat, B., Fraysse, B., Paxéus, N., Lo Giudice, R., Pollio, A., Garric, J., 2004. Environmental risk assessment of six human pharmaceuticals: are the current environmental risk assessment procedures sufficient for the protection of the aquatic environment? *Environ. Toxicol. Chem.* 23: 1344–1354.

- Finney, D.J., 1971. Probit Analysis. 3rd Ed. Cambridge University Press, Cambridge.
- Forbes, V.E., Calow, P., 1999. Is the per capita rate of increase a good measure of population-level effects in ecotoxicology? *Environ. Toxicol. Chem.* 18(7): 1544-1556.
- Froehner, K., Backhaus, T., Grimme, L.H., 2000. Bioassays with *Vibrio fischeri* for the assessment of delayed toxicity. *Chemosphere* 40: 821–828.
- Garcı´a-Berthou, E., Alcaraz, C., Pou-Rovira, Q., Zamora, L., Coenders, G., Feo, C., 2005. Introduction pathways and establishment rates of invasive aquatic species in Europe. *Can. J. Fish. Aquat. Sci.* 62: 453–463.
- Glassmeyer, S.T., Hinchey, E.H., Boehme, S.E., Daughton, C.G., Ruhoy, I.S., Conerly, O., Daniels, R.L., Lauer, L., McCarthy, M., Nettesheim, T.G., Sykes K., Thompson, V.G., 2009. Disposal practices for unwanted residential medications in the United States, *Environ. Int.* 35: 566-572.
- Golet, M.E., Alder, C.A., Giger, W., 2002. Environmental Exposure and Risk Assessment of Fluoroquinolone Antibacterial Agents in Wastewater and River Water of the Glatt Valley Watershed, Switzerland. *Environ. Sci. Technol.* 36: 3645-3651.
- Grung, M., Källqvist, T., Sakshaug, S., Skurtveit, S., Thomas, K.V., 2008. Environmental assessment of Norwegian priority pharmaceuticals based on the EMEA guideline, *Ecotoxicol. Environ. Saf.* 71: 328-340.
- Halling-Sørensen, B., Lutzhoft, H., Andersen, H., Ingerslev, F., 2000. Environmental risk assessment of antibiotics: comparison of mecillinam, trimethoprim and ciprofloxacin. *J. Antimicrob. Chemother.* 46: 53–58.
- Hartmann, A., Alder, A.C., Koller, T., Widmer, R.M., 1998. Identification of fluoroquinolone antibiotics as the main source of *umuC* genotoxicity in native hospital wastewater. *Environ. Toxicol. Chem.* 17(3): 377-382.
- Heberer, T., 2002. Occurrence, fate, and removal of pharmaceutica residues in the aquatic environment: a review of recent research data. *Toxicol. Lett.* 131: 5-17.
- Hernando, M.D., De Vettori, S., Martínez Bueno, M.J., Fernández-Alba, A., 2007. Toxicity evaluation with *Vibrio fischeri* test of organic chemicals used in aquaculture. *Chemosphere* 68: 724-730.

- Huang, C., Renew, J.E., Semby, K.L., Pinkston, K., Sedlak, D.L., 2001. Assessment of potential antibiotic contaminants in water and preliminary occurrence analysis. *Water Resour. Update* 120: 30-40.
- Infarmed, 2008. Medicines Statistic 2008, p. 1-95.
- Isidori, M., Lavorgna, M., Nardelli, A., Pascarella, L., Parrella, A., 2005. Toxic and genotoxic evaluation of six antibiotics on non-target organisms. *Sci. Total Environ.* 346: 87-98.
- Kaiser, K.L.E., 1998. Correlations of *Vibrio fischeri* bacteria test data with bioassay data for other organisms. *Environ. Perspect.* 106: 583-591.
- Kanoun-Boulé, M., Vicente, J.A.F., Nabais, C., Prasad, M.N.V., Freitas, H., 2009. Ecophysiological tolerance of duckweeds exposed to copper, *Aquat. Toxicol.* 91: 1-9.
- Krcmery, S., Naber, K.G., 1999. Ciprofloxacin once versus twice daily in the treatment of complicated urinary tract infections. *Int. J. Antimicrob. Agents* 11: 133-138.
- Kümmerer, K., 2003. Significance of antibiotics in the environment. *J. Antimicrob. Chemother.* 52: 5-7.
- Kümmer, K., 2009. Antibiotics in the aquatic environment – A review – Part II. Chemosphere 75: 435-441.
- Labra, M., Bernasconi, M., Grassi, F., Mattia, D.F., Sgorbati, S., Airoldi, R., Citterio, S., 2007. Toxic and genotoxic effects of potassium dichromate in *Pseudokirchneriella subcapitata* detected by microscopy and AFLP marker analysis, *Aquat. Bot.* 86: 229-335.
- Landolt E (1986). The family of Lemnaceae - a monographic study. In: Biosystematic investigations in the family of duckweeds (Lemnaceae), vol 2. Veröffentlichungen des Geobotanischen Institutes, Stiftung Rübel, ETH, No.71, Zürich.
- Lewis, M.A., 1995. Algae and vascular plant tests. In: Rand, G.M. (Ed.), Fundamentals of Aquatic Toxicology: Effects, Environmental fate, and Risk Assessment. *2nd ed. Taylor and Francis,Washington, DC*, pp. 135‑169.

- Li, Z.H., Randak, T., 2009. Residual pharmaceutically active compounds (PhACs) in aquatic environment – status, toxicity and kinetics: a review, *Veter. Medic.* 54: 295-314.
- Lindberg, R.H., Wennberg, P., Johansson, M.I., Tysklind, M., Andersson, B.A.V., 2005. Screening of human antibiotic substances and determination of weekly mass flows in five sewage treatment plants in Sweden, *Environ. Sci. Technol.* 39: 3421–3429.
- Mayer, P., Cuhel, R., Nyholm, N., 1997. A simple in vitro fluorescence method for biomass measurements in algal growth inhibition tests, *Wat. Res.* 31: 2525-2531.
- Meyer, J.S., Ingersoll, C.G., McDonald. L.L., Boyce, M.S., 1986. Estimating uncertainty in population growth rates: jackknife vs. bootstrap techniques. *Ecology* 67: 1156 –1166.
- Minh, T. B., Leung, H.W., Loi, I. H., Chan, W. H., So, M.K., Mao, J.Q., Choi, D., Lam, J.C.W., Zheng, G., Martin, M., Lee, J.H.W., Lam, P.K.S., Richardson, B.J., 2009. Antibiotics in the Hong Kong metropolitan area: Ubiquitous distribution and fate in Victoria Harbour, *Mar Pollut Bull.* 58: 1052-1062.
- McCallum, H., 2000. Population Parameters: Estimation for Ecological Models. Methods in Ecology, *Blackwell Science Ltd.* 5: 139-156.
- Naddeo, V., Meriç, S., Kassinos, D., Belgiorno, V., Guida, M., 2009. Fate of pharmaceuticals in contaminated urban wastewater effluent under ultrasonic irradiation, *Water Res.* 43: 4019-4027.
- Nie, X.P., Wang, X., Chen, J., Zitko, V., An, T., 2008. Response of the freshwater *Chlorella vulgaris* to trichloroisocyanuric acid and ciprofloxacin. *Environ. Toxicol. Chem.* 27(1): 168-173.
- Nunes, B., Carvalho, F., Guilhermino, L., 2005. Acute toxicity of widely used pharmaceuticals in aquatic species: *Gambusia holbrooki*, *Artemia parthenogenetica* and *Tetraselmis chuii. Ecotoxicol. Environ saf.* 61: 413-419.
- Oaks, J.L., Gilbert, M., Virani, M.Z., Watson, R.T., Meteyer, C.U., Rideout, B.A., Shivaprasad, H.L., Ahmed, S., Chaudhry, M.J.I., Arshad, M., Mahmood, S., Ali, A., Khan, A.A., 2004. Diclofenac residues as the cause of vulture population decline in Pakistan. *Nature* 427 (6975): 630–633.

- OECD, 1992, Fish, Acute Toxicity Test, nº203, Organization for Economic Cooperation.
- OECD, 1996. *Daphnia magna* Reproduction Test. Guidelines for Testing of Chemicals, nº 202, Part II, Organization for Economic Cooperation and Development.
- OECD, 2000. Daphnia sp., Acute Immobilization Test. Guidelines for Testing of Chemicals, nº202, Organization for Economic Cooperation and Development.
- OECD, 2006, Freshwater Alga and Cyanobacteria, Growth Inhibition Test, nº201, Organization for Economic Cooperation and Development.
- OECD, 2006, Lemna sp. Growth Inhibition Test, nº201, Organization for Economic Cooperation and Development.
- Parvez, S., Venkataraman, C., Mukherji, S., 2006. A review on advantages luminescence inhibition test (*Vibrio fischeri*) for acute toxicity prediction of chemicals. *Environ Int.* 32: 265-268.
- Pena, A., Chmielova, D., Lino, C.M., Solich, P., 2007. Determination of fluoroquinolone antibiotics in surface waters from Mondego River by high performance liquid chromatography using amonolithic column, *J. Sep. Sci.* 30: 2924–2928.
- Pereira, J.L., Marques, C.R., Gonçalvez, F., 2004. Allometric relations for *Ceriodaphnia* spp. and *Daphnia* spp., *Ann. Limnol. – Int. J. Lim.* 40 (1): 11-14.
- Pyke, G.H., 2005. A review of the biology of *Gambusia affinis* and *G. holbrooki*. *Rev. Fish Biol. Fish.* 15: 339–365.
- Qureshi, A.A., Bulich, A.A., Isenberg, D.L., 1998. Microtox toxicity test systems—where they stand today. In Wells PG, Lee K, Blaise C, eds, *Microscale Testing in Aquatic Toxicology: Advances, Techniques, and Practice.* CRC, New York, NY, USA, pp. 185–199.
- Relyea, R., and Hoverman, J., 2006. Assessing the ecology in ecotoxicology: a review and synthesis in freshwater systems. *Ecol. Lett.* 9: 1157-1171.
- Robinson, A.A., Belden, J.B., Lydy, M.J., 2005. Toxicity of fluoroquinolone antibiotics to aquatic organisms. *Environ. Toxicol. Chem.* 24: 423–430.

- Santos, J.L., Aparício, I., Callejón, M., Alonso, E., 2009. Occurrence of pharmaceutically active compounds during 1-year period in wastewaters from four wastewater treatment plants in Seville (Spain). *J. Hazard. Mater.* 164: 1509-1516.
- Santos, L.H.M.L.M., Araújo, A.N., Fachini, A., Pena, A., Delerue-Matos, C., Montenegro, M.C.B.S.M., 2010. Review Ecotoxicological aspects related to the presence of pharmaceuticals in the aquatic environment. *J. Hazard. Mater.* 175: 45-95.
- Seifrtová, M., Pena, A., Lino, C.M., Solich, P., 2008. Determination of fluoroquinolone antibiotics in hospital and municipal wastewaters in Coimbra by liquid chromatography with a monolithic column and fluorescence detection. *Anal Bioanal Chem.* 391: 799-805.
- Sobral, O.M.F., 1997. Ecotoxicidade de efluentes de indústrias de pasta de papel resultantes de diferentes processos de pré-branqueamento, Dissertação de Mestrado em Ecologia, Universidade de Coimbra, Coimbra. Portugal, 78pp.
- Sörgel, F., Naber, K.G., Kinzig, Martina, Mahr, G., Muth, P., 1991. Comparative pharmacokinetics of ciprofloxacin and temafloxacin in humans: A review. *Am. J. Med.* 91(6): 51-66.
- Stein, J.R., 1973. Handbook of Phycological Methods: Culture Methods and Growth Measurements. University Press, London, UK.
- Stibor, H., Lampert, W., 1993. Estimating the size at maturity in field populations of *Daphnia* (cladocera). *Freshwat. Biol.* 30: 433– 438.
- Streuff, B., Luchtemberg, H., 1987. Pharmaceutical formulations of ciprofloxacin. *United States Patent* 5286754: 1-6.
- Terra, N.R., Feiden, I.R., 2003. Reproduction and Survival of *Daphnia magna Straus*, 1820 (Crustacea: Cladocera) under different hardness conditions. *Acta limnol. Bras.* 15 (2): 51-55.
- Thomulka, K.W., McGee, D.J., Lange, J.H., 1993. Detection of biohazardous materials in water by measuring bioluminescence reduction with the marine organism *Vibrio harveyi*. *J. Environ. Sci. Health Part A* 28: 2153–2166.
- Tolls, J., 2001. Sorption of veterinary pharmaceuticals in soils: a review. *Environ. Sci. Technol.* 35: 3397-3406.

- Turnidge, J., 1999. Pharmacokinetics and pharmacodynamics of fluoroquinolones. *Drugs* 58: 29–36.
- Van der Grinten, E., Pikkemaat, M.G., Van den Brandhof, E., Stroomberg, G. J., Kraak, M.H.S., 2010. Comparing the sensitivity of algal, cyanobacterial and bacterial bioassays to different groups of antibiotics. *Chemosphe*: 1-6.
- Wagner, J.G., 1993. Pharmacokinetics for the pharmaceutical scientist. *Technomic Publishing Company, Inc*: 1-275.
- Yu, X., Zipp, G.L., Davidson, G.W.R., 1994. The effects of temperature and pH on the solubility of quinolone compounds: estimation of heat of fusion. *Pharmaceutical Research* 11(4): 522-527.
- Zeiler, H.-J., and K. Grohe. 1984. The in vitro and in vivo activity of ciprofloxacin. *Eur. J. Clin. Microbiol.* 3: 339-343.
- Zeiler, H.-J. 1985. Evaluation of the in vitro bactericidal action of ciprofloxacin on cells of *Escherichia coli* in the logarithmic and stationary phases of growth. *Antimicrob. Agents Chemother* 524-527.