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Ecotoxicological Screening of the Select Drug II

(Ekotoxikologický screening vybraného léčiva II)

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DECLARATION

I hereby declare that the work presented in this master thesis is the result of my own investigations except where specifically stated in the text. I elaborated this work during the years 2008 – 2010 with the use of cited references.

Hradec Králové, 10.05.2010 Marianna Dubánková

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Abbreviation List

BOD	biological oxygen demand
COD	chemical oxygen demand
DDD	defined daily dose
DW	dry weight
EC_{50}	median effective concentration
ESAC	European Surveillance of Antimicrobial Consumption
IC_{50}	median inhibition concentration
LC_{50}	median lethal concentration
NSAIDs	non-steroid anti-inflammatory drugs
UV B	ultraviolet B radiation

Abstract

Quinolone antibacterial agents represent a chemically homogenous group of purely synthetic antibiotics which originated in the early 1960s and still play an important role in the antimicrobial chemotherapy.

Quinolones (or more specific, fluoroquinolones) are frequently used in human therapy for treatment of both common and serious diseases (they are irreplaceable also against bioterrorist weapons, such as anthrax). Some of the fluoroquinolones are also among the most used veterinary antimicrobials, including their large-scale usage in aquaculture. Only in the Czech Republic, their annual consumption is in the order of millions of defined daily doses (DDD).

Fluoroquinolones as entirely synthetic compounds do not have any natural source in the environment – therefore their occurrence in both terrestrial and aquatic ecosystems is the result of human activity. Excluding the direct application to the aquatic environment (prophylaxis and treatment of bacterial diseases in the aquaculture), the most common point of entry of fluoroquinolones into the environment is via the wastewaters. Numerous studies carried out in different countries have shown that even after several steps of wastewater cleaning process, fluoroquinolones can be found in the final effluent of the wastewater treatment plants (the amounts of fluoroquinolones are mostly in the order of tens or hundreds of ng L^{-1}). The effects of fluoroquinolones on the aquatic ecosystem are not known enough.

In this study, I performed an ecotoxicological screening of three fluoroquinolone antibiotics (ciprofloxacin, norfloxacin, and ofloxacin, that are most commonly used in the Czech Republic) on four freshwater organisms in order to determinate acute toxicity of the tested chemotherapeutical agents. The experimental organisms included a green algal species, *Pseudokirchneriella subcapitata*, a ciliated protozoan, *Tetrahymena pyriformis*, a rotifer, *Brachionus calyciflorus*, and an anostracan crustacean, *Thamnocephalus platyurus*. All tests were conducted as acute toxicity tests. The concentrations of fluoroquinolones started at 12 mg L^{-1}, 3 mg L^{-1}, and 0.3 mg L^{-1}, respectively and in each test at least eight different concentrations of the antibiotics were investigated.

The results suggest that the acute toxicity of the three fluoroquinolones is considerably different to various species. The fluoroquinolones exhibited low

acute toxicity on algal species *P. subcapitata* and crustacean *T. platyurus*. Significantly higher acute toxicity of the quinolone antibiotics was observed on *B. calyciflorus* (with the exception of norfloxacin) and *T. pyriformis* with the respective LC_{50} or IC_{50} values in the range of reported quinolone concentrations occurring in the environment, and thus a possible negative impact on the natural population of these organisms.

Abstrakt

Chinolonová antibiotika představují chemicky značně homogenní skupinu výhradně syntetických látek objevených v šedesátých letech minulého století. Dodnes si tato antibiotika zachovala významnou pozici v antimikrobiální terapii.

Chinolony (nebo přesněji fluorochinolony) jsou často používané v humánní terapii k léčbě jak běžných, tak i závažných onemocnění a navíc jsou považovány za důležitou součást boje proti bioterorismu (jako bioteroristická zbraň je hodnocený například antrax). Někteří zástupci fluorochinolonů rovněž patří mezi nejčastěji používaná veterinární antibiotika, a to včetně jejich velkoplošného použití v chovu ryb. Jenom v samotné České republice se roční spotřeba fluorochinolonových antibiotik pohybuje v řádech milionů DDD (definovaných denních dávek).

Fluorochinolony jako výlučně syntetické sloučeniny nemají v životním prostředí přirozený zdroj, proto je jejich výskyt v suchozemských i vodních ekosystémech považován za důsledek lidské činnosti. Nebudeme-li uvažovat o přímé aplikaci fluorochinolonů do vodního prostředí (jako prevenci nebo léčbu nemocí zvířat ve vodním hospodářství), představují vstupní bránu těchto látek do okolního prostředí odpadní vody. Četné vědecké práce z různých států poukázaly na fakt, že dokonce i po několikastupňovém čištění odpadních vod je úroveň fluorochinolonů na výtoku z čističky odpadních vod většinou v řádu desítek až stovek ng L^{-1}). Vliv fluorochinolonů na životní prostředí přitom nebyl dostatečně prozkoumán.

V této práci jsem se zaměřila na vypracování ekotoxikologického profilu třech v České republice nejčastěji používaných fluorochinolonových antibiotik (ciprofloxacinu, norfloxacinu a ofloxacinu). V toxikologických testech byly použité čtyři sladkovodní druhy, abych získala relevantní informace o akutní toxicitě testovaných antibiotik. Experimentálními organismy byly řasa *Pseudokirchneriella subcapitata*, prvok *Tetrahymena pyriformis*, vířník *Brachionus calyciflorus* a korýš *Thamnocephalus platyurus*. Všechny testy byly provedené jako testy na akutní toxicitu. Počáteční koncentrace fluorochinolonů byly 12 mg L^{-1}, 3 mg L^{-1}, resp. 0,3 mg L^{-1} a pro každý test bylo připraveno alespoň osm různých koncentrací použitých antibiotik.

Získané výsledky naznačují, že akutní toxicita jednotlivých chinolonů se liší v závislosti na testovaném organismu. Nízká akutní toxicita byla nalezena pro řasu *P. subcapitata* a korýše *T. platyurus*. Na druhé straně, značnou akutní toxicitu vykazovala použitá antibiotika vůči vířníku *B. calyciflorus* (s výjimkou norfloxacinu) a prvoku *T. pyriformis*. V těchto případech byly hodnoty LC_{50}, resp. IC_{50} srovnatelné s koncentracemi fluorochinolonů nalezenými v životním prostředí, což by mohlo negativně ovlivnit populace těchto organismů v jejich přirozeném prostředí.

1. Preface

Since the appearance of the first antimicrobial agents used in human and veterinary therapy (sulfonamides and penicillin), their importance increased gradually. In time, numerous chemical compounds, both naturally occurring and synthetic, were added to the nowadays large group of antibacterial chemotherapeutic agents. Antibiotics significantly decreased mortality on various diseases, shortened the period of healing, prevented from delayed effects of the disease-causing organisms. On the veterinary field antimicrobials allowed a considerably higher production with a decreased loss of the animals.

Due to the frequently use (and often overuse) of antibiotics in both human and veterinary therapy, several new problems have arisen in the past decades. Resistance of bacterial species to antibiotics definitively became an important issue in the antimicrobial chemotherapy. The well-known multi-resistant bacterial strains were one of the most severe and often lethal consequences of non-rational antibiotics usage. Because the resistance of microbes to antibiotics has direct impacts on human health, the research on this field was very intense and solved several vital questions. The results of this research have also been implemented in the praxis – the surveillance of antimicrobial agents consumption and the elaboration of rational antibiotic therapy guidelines may serve as good examples.

A completely different problem is the occurrence and fate of antimicrobial agents in the environment. Antibiotics in large quantities are directly applied to the aquatic environment in the aquaculture, and great amounts of various antibiotics are daily delivered via the wastewaters of both communal and hospital origin. The wastewater treatment plants do, in fact, eliminate a significant proportion of the whole amount of antimicrobials, but numerous chemotherapeutic agents can to various extent still be found in the final effluents of the wastewater treatment plants.

The fate of most antimicrobials in the environment is unknown. Do they remain in the aquatic ecosystem or are they adsorbed on the soil particles and enter the terrestrial ecosystem? Do they undergo any kind of degradation and lose their antimicrobial effect? Are they stored in a particular compartment or are they distributed evenly to the whole environment? And most importantly: how do they influence the living part of the ecosystems? What are their effects on non-target

organisms? And what the consequences of the potentially altered ecosystems will be? These questions need to be answered in order to perform a proper environmental risk assessment of the large group of antibacterial agents.

This study is focused on the ecotoxicological screening of three very frequently used fluorochinolone antibiotics on selected freshwater species and is intended to investigate the acute toxicity of tested fluoroquinolones. The tested compounds were chosen because they are commonly used in antibacterial therapy in the Czech Republic, are often found in the wastewaters in many countries and the knowledge about their fate in the environment and their impact on aquatic ecosystem is fairly poor. Published works dealing with the influence of fluoroquinolones on model plant and animal species are scarce and often focused on only one or two different species.

Present research is related to other studies supervised by Mgr. Jitka Vytlačilová, which were elaborated at the Department of Pharmaceutical Botany and Ecology, Faculty of Pharmacy in Hradec Králové, Charles Univeristy in Prague. Other works deal with selected pharmaceuticals (not restricted to antimicrobial agents) commonly used in the Czech Republic (e.g. paracetamol).

Aims of the Present Research

1. To determinate the acute toxicity of three fluoroquinolone antibiotics commonly used in the therapeutic praxis on selected freshwater experimental organisms.

Solved questions:

- What concentration range of the antibiotics should be used for the ecotoxicity tests?
- Do the selected fluoroquinolones have similar acute toxicity on the tested experimental organisms?

2. To estimate the environmental risk of three fluoroquinolone antibiotics with regard to their acute toxicity on selected freshwater experimental organisms.

Solved questions:

- Are the obtained acute toxicity data of the investigated chemotherapeutical agents in accordance with available published works?
- Based on the obtained results, are there any environmental risks associated with the quinolone antibiotics tested?

2. Introduction

At the beginning, I would like to explain the structure of the introduction presented. In this thesis, I deal with ecotoxicological screening of three selected antibiotic substances commonly used in infection treatment. For a more deep understanding of their effects on organisms tested, it is useful to have at least a fairly knowledge of the structure, chemical and biological properties of these antibiotic agents. Therefore, I will provide some basic information on the history and the therapeutic aspects of one particular group of antibiotics – namely quinolones – which compounds used in this research they belong to.

Further, I will try to encompass the most important facts regarding the effects of antibiotic agents on wastewaters and surface waters, and their consequences for aquatic environment with heavy emphasis on quinolones. And lastly, I will deal with organisms and biological tests used for the purpose of ecotoxicological screening.

2.1. Quinolones

In the present days, there are many substances with antibiotic activity. Some of them are used widely, some are restricted to treatment of particular diseases or biological groups and some are long forgotten or not explored yet. Due to the excessive amount of these compounds, the classification of antibiotics was established; dividing them into groups based on chemical structure and origin. Quinolones represent a relatively homogenous group of antibacterial agents, with their history reaching into early 1960s and extending up to the present days.

2.1.1. Characterization of Quinolones

2.1.1.1. Chemical Structure and the Structure-Activity Relations

The structure of quinolone antibiotics was derived originally from quinine. The first compound of this chemotherapeutical group to be discovered was

nalidixic acid in 1962. During the following years, some structural changes improving the pharmacokinetic and pharmacodynamic qualities were made.

The basic chemical traits common to quinolones involve the presence of a heterocyclic structure (namely quinoline, less often cinnoline or naphtyridine), a carboxylic acid in position 3 and a ketone in position 4 (see Figure 2.1.). Positions 3 and 4 are crucial for the antibiotic activity (Anderson and MacGowan, 2003; Hartl, 2006).

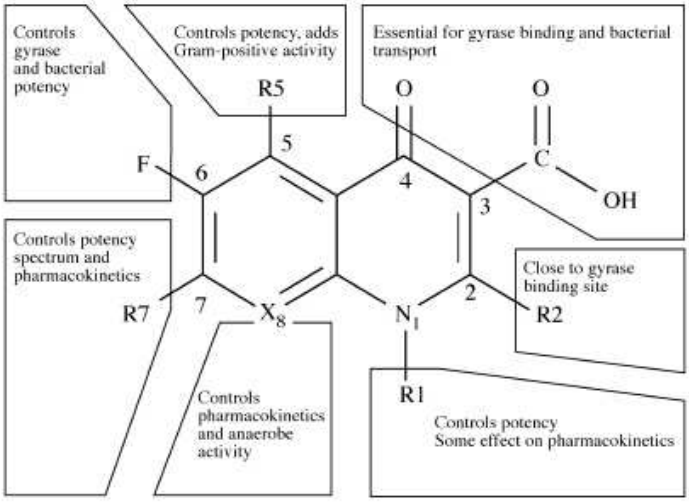

Figure 2.1. The general chemical structure of quinolone antibiotics with basic structure-activity assessment. From Anderson and MacGowan, 2003.

Modifications in some positions revealed to be undesirable, while other changes enhanced pharmacokinetic and pharmacodynamic features of the new compounds. Among the most important structural manipulations was the addition of fluorine atom to position 6, by which a significant increase in gyrase-blocking activity was achieved. Compounds with this particular structural trait were named **fluroquinolones** (Domagala, 1994; Anderson and MacGowan, 2003 and references therein)**.**

For the control of potency, several positions are of great importance. Aside from the essential gyrase-binding site (carbons 3 and 4), groups R1, R5 and R7 contribute to the resulting antibacterial activity (Andersson and MacGowan, 2003 and references therein).

All of three of quinolones used in this thesis are structurally similar, the main difference being a morpholine ring in ofloxacin and a cyclopropyl group in ciprofloxacin – both substitutions do not occur in the two other compounds (see

Figure 2.2.). They all contain a structural trait that allows increased biological availability of these compounds – namely the presence of piperazine molecule as the R7 substitute (Hartl, 2006).

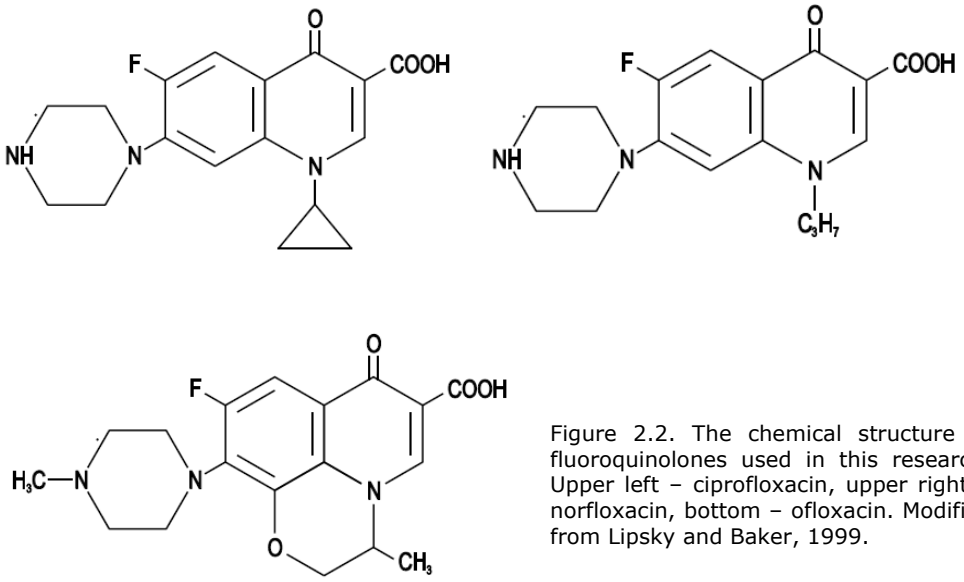

Figure 2.2. The chemical structure of fluoroquinolones used in this research. Upper left – ciprofloxacin, upper right – norfloxacin, bottom – ofloxacin. Modified from Lipsky and Baker, 1999.

The cyclopropyl substitute addition in ciprofloxacin resulted in further increased biological availability of the antibiotic agent. Due to the presence of abovementioned morpholine ring in ofloxacin, this substance is chiral and can be used as racemic mixture or enantiomer (named levofloxacin; Hartl, 2006).

2.1.1.2. Important Properties of Quinolones in Relation to Environmental Risk Assessment

According to available literal sources, **photochemical properties** of quinolones have been studied extensively because of the impact of the relatively low photostability of these antibacterial drugs on humans and animals (see Chapter 2.1.2.2.). On the other hand, as will be discussed in Chapter 2.2.2.2., photodegradation seems to be a very efficient process of the elimination of some quinolone antibiotics from the environment.

During the process of the photodegradation of quinolones in neutral aqueous solutions, several reactive species are generated – e.g. carbon centered

radical, hydroxyl radical and singlet oxygen were reported (Viola *et al.*, 2004 and references therein; Vargas *et al.*, 2009).

Fasani *et al.* (2004) studied the photochemistry of lomefloxacin. The authors reported that lomefloxacin and most likely other 8-halo-substituted fluroroquinolones endure fragmentation of C-F bond under various experimental conditions that results in the generation of a cationic structure, which is both chemotherapeutic inactive and aggressive (Fasani *et al.*, 2004)

Other structural part of quinolones most prone to degradation after light exposure is the piperazine ring in position 7 (occurring e.g. in ciprofloxacin, norfloxacin and ofloxacin; Córdoba-Díaz *et al.*, 1998 and references therein).

Quinolones, as shown in previous chapter, contain both acidic and basic groups, thus marking them as zwitterions. The dissociation constants (pK_a) for these compounds are environmentally relevant – e.g. norfloxacin and ciprofloxacin occur as zwitterions in very similar pH values (Lindberg *et al.*, 2006).

The **pH value** of environment has, to an extent, an influence on the distribution of quinolones (e.g. Belden *et al.*, 2006 in their study of ciprofloxacin sorption onto particulate organic matter under different pH values).

Nowara *et al.* (1997) reported that quinolones (e.g. enrofloxacin) appeared to be in the clay soil solutions (pH around 6.0) as anions and tended to interact with the exchangeable cations bound to soil surface.

However, pH value of raw sewage is higher (7.1 – 7.5; Lindberg *et al.*, 2006) and quinolones should exist as zwitterions in this pH range – which means that the sorption of quinolones to particulate matter may be influenced by other factors. Golet *et al.* (2003) and Lindberg *et al.* (2006) suggested hydrophobic and electrostatic interactions as factors impacting sorption of quinolones to particulate matter at higher pH values.

2.1.2. Therapeutic Use of Quinolones

Since the discovery of quinolones, these antibacterial agents have their place in treatment of various infection diseases. Not all of these substances are

used in the present human medicine – some of them being abandoned in favor of more effective agents and some restricted in the clinical use because of their adverse effects. For practical purposes, quinolones are divided into several generations in accordance to their structure and antibiotic activity.

2.1.2.1. Generations of Quinolones

Ball (2000) suggested that there are at least two different approaches of classification of quinolones: the first one being the chronological, based on development of new molecules, and the second one implying "natural selection", including for example the frequency of prescription of various quinolones. However, there are several more opinions of the classification of this chemotherapeutic group, distinguishing or not between quinolones, naphtyridones, fluoroquinolones and other sub-groups based on their minor chemical changes and placing particular antibacterial agents into 3 or 4 generations respectively, sometimes with the addition of several sub-generations.

First-generation quinolones are represented by the early substances discovered back in the 1960s – nalidixic acid (sometimes considered as a member of the naphtyridones), oxolinic acid and the very first of the fluoroquinolones – flumequin. These quinolones were used for treatment of the urinary tract infections, because of their pharmacokinetic properties (Ball, 2000). Flumequin is still used for veterinary purposes (e.g. in treatment of bacterial infection in fish – Lalumera *et al.*, 2004).

All of the three quinolones tested in this work (norfloxacin, ciprofloxacin and ofloxacin) belong into the 2. generation, which is characterized by relatively wide antibacterial activity and convenient pharmacokinetic properties when compared to 1. generation. All of them have enhanced activity, particularly against Gram-negative pathogens, but with restricted effect on Gram-positive bacteria. Among this group of antibacterial agents, ciprofloxacin is the most potent against *Pseudomonas aeruginosa* (Ball, 2000; Hartl, 2006).

Chemotherapeutic agents of the second generation have been available since the mid-1980s and have brought important changes in treatment of several infections. Second-generation quinolones are still widely used in practice. The

most common indications of these chemotherapeutic agents are: urinary tract infections, skin and soft tissues infections, acute exacerbations of chronic bronchitis, nosocomial pneumonia, chlamydial infections, gonorrhea, and infections caused by *P. aeruginosa* (Hooper, 1998; Ball, 2000; Lincová and Farghali, 2007). Furthermore, ciprofloxacin seems to be a very reliable agent against *Bacillus anthracis* (considered as a bioterrorist weapon) infection and in post-prophylactic treatment when administered twice daily (Deziel *et al*., 2005). On the other hand, there are some evidences that use of ciprofloxacin against Gram-positive bacteria (namely *Streptococcus pneumoniae*) is not appropriate and ciprofloxacin should be replaced by a more active chemotherapeutic agent, e.g. levofloxacin (Lister and Sanders, 1999).

Third-generation quinolones (sparfloxacin, grepafloxacin, tosufloxacin) are represented by substances with renewed strong antibacterial activity against Gram-positive bacteria (such as the genus *Streptococcus*), but with decreased activity against *P. aeruginosa*. This balanced broad spectrum of activity is used in pulmonary bacterial infection treatment. Furthermore, these agents have a very favorable pharmacokinetic profile, which allows a once-daily administration scheme (Ball, 2000).

As to fourth-generation quinolones (moxifloxacin, gatifloxacin, trovafloxacin), these substances are considered to be reserved antibiotics meant only for treatment of life-endangering infections, which cannot be eradicated by other chemotherapeutical agents. They have enhanced activity against Gram-positive bacteria and their pharmacokinetic profile allows their administration in only one daily dose (Hartl, 2006; Lincová and Farghali, 2007).

2.1.2.2. Restriction in Therapeutic Use of Quinolones

Quinolones, as every other group of pharmaceuticals, are not without adverse effects. Because of them, quinolones are not suitable in treatment of all human patients.

Generally, quinolones are well-tolerated antibiotics, with adverse effects in about 2 – 8% of patients. The most common adverse effects of quinolones are

(i) gastro-intestinal effects such as diarrhea, (ii) phototoxicity and (iii) arthropathy (Lincová and Farghali, 2007).

Phototoxicity of quinolones is more pronounced in compounds with 8-halo-substitution (e.g. Hartl, 2006). Tokura (1998) suggested that quinolones have the same photoantigenic epitope (recognizable by both T cells and imunoglobulins) resulting in fluoroquinolone photosensitivity and cross-reactivity.

Regarding arthropathy, patients should be warned beforehand to avoid physical activity during therapeutic treatment with quinolone antibiotics. Because of uncertainty of atrhropathic influence in children, quinolones are generally contraindicated in children patients, except for several indications (Lincová and Farghali, 2007). The newest data on quinolone-induced arthropathy, as well as clinical application of quinolone antibiotics in children patients, are explored by Sendzik *et al*. (2009).

2.1.2.3. Current Data on the Therapeutic Use of Quinolones in the Czech Republic

As of January 2010, not all quinolones are in therapeutic use in the Czech Republic. Following information is based on data displayed at the website of the State Institute for Drug Control – for additional information, more details and product names see www.sukl.cz.

According to the State Institute for Drug Control, in the Czech Republic there are no medicinal products with the first-generation quinolones as the pharmacologically active compound.

Second-generation quinolones represent the most frequently used group of quinolones in the Czech Republic. Of the quinolones presented in this study, norfloxacin is the most commonly prescribed. According to the State Institute for Drug Control, there are nearly 500 registered medicinal products in the ATC group J01MA (Fluoroquinolones). Of all these products, over 350 have ciprofloxacin as the pharmacologically active compound, 12 of them have ofloxacin and only 7 norfloxacin. Several of the medicinal products are in the form of an infusion solution, and therefore intended for hospital use only (for further details, product names and forms see www.sukl.cz).

There are currently no registered medicinal preparations with third- or fourth-generation quinolones in the Czech Republic.

Currently, available data on quinolones consumption regardless of administration route (i.e. all types of medicinal preparations) and patient (ambulant or hospitalized) in the Czech Republic are from the years 2007 and 2008 (based on the data provided by AISLP (Prague); see Table 2.1.). Norfloxacin represents the fluoroquinolone with the highest consumption in both years, while ofloxacin is the antibacterial agent with the lowest consumption in both years. As to financial value of the fluoroquinolone antibiotics use in the Czech Republic, in 2007 the costs for quinolones was around 166 millions CZK, in 2008 the costs for quinolones were higher, around 174 millions CZK (data obtained by AISLP, Prague).

Table 2.1. Quinolones consumption in the Czech Republic in years 2007 and 2008. Data obtained from AISLP, Prague. DDD = defined daily dose

	Thousands of DDD		% of All Quinolones DDD	
Quinolone	**2007**	**2008**	**2007**	**2008**
Ciprofloxacin	1 729	1 888	30,99	33,80
Norfloxacin	2 375	2 306	42,56	41,28
Ofloxacin	1 334	1 263	23,91	22,61
All Quinolones	5 580	5 586	100,00	100,00

Overall, according to ESAC (European Surveillance of Antimicrobial Consumption), the antibiotic consumption in ambulant patients (i.e. excluding hospitals) in Czech Republic in 2006 lies in the range from 14.4 to 18.69, expressed in defined daily doses (DDD) per 1000 inhabitants per day, which is the second-lowest level of use of antibiotics in the countries with antibiotic surveillance by ESAC (for further details see the internet pages of European Surveillance of Antimicrobial Consumption, http://app.esac.ua.ac.be/public/).

2.2. Environmental Exposure and Risk Assessment of Antibacterial Agents in Surface and Waste Waters

Chemotherapeutic agents were proved to be a powerful and irreplaceable means against various bacterial infections. However, the consumption of these

substances is, especially in the last decades, excessive. For example, Wise (2002) estimated the annual use of antibiotics to be about 100 000 to 200 000 tons globally. The use of antibiotics is not restricted to the area of human medicine; it includes veterinary, agri- and aquacultural purposes. During the last years, the concern about the effects of antibiotics use on environment significantly increased and several studies on this topic were conducted worldwide (e.g. studies of the last decade focused on quinolones: Halling-Sørensen *et al.*, 2000; Golet *et al.*, 2002; Cardoza *et al.*, 2005; Lindberg *et al.*, 2006; Xu *et al*., 2007).

The most imminent problem is the possible development of resistance to chemotherapeutic agents in bacteria that could result in impairment of infections treatment in human and animal patients. Secondly, there is the risk of affecting the natural inhabitants of aquatic and terrestrial environment and its following consequences could result in degradation of community structure and impaired function of the whole ecological system. And thirdly, there are also practical issues of exposing humans and/or animals to inadequate amounts of antibiotics due to the use of sewage treatment plant's solid products (e.g. their use in fertilizers).

2.2.1. Approaches on the Risk Assessment of Use of Antibiotics on the Environment

There are several ways to look at the risk assessment of antibiotics use on environment. As to administration point of view, different countries utilize different official procedures in order to evaluate the risk of use of chemically active entities – e.g. Bound and Voulvoulis (2004) provide a comparison of risk assessment strategies of pharmaceuticals in the aquatic environment.

From a "practical" point of view, there are several aspects of the flow of the antibiotic in the environment that can be evaluated. Antibiotics administered to ambulant or hospitalized patients undergo to some extent chemical changes in the human body – their metabolism is dependent on several factors including chemical structure and enzymatic apparatus of the organism – and are eliminated in the form of more or less active compounds into the sewage waters. From this point of view, the sewage treatment plants represent a good starting platform.

Based on their goals relevant to environmental risk assessment discussed in this thesis, in following chapters there are listed the basic three types of scientific projects which can be found in literal sources (i.e. analytical studies, mass flow studies, and ecotoxicological studies). In order to avoid abundance of information provided, all works mentioned in the following text are at least partially focused on quinolones.

2.2.1.1. Analytical Studies

A number of studies focused solely on determination of various pharmaceuticals in sewage waters or sludge, and on improving or simplifying of the analytical methods (frequently used were liquid chromatography methods) designed for these purposes (e.g. Golet *et al*., 2001; Ferdig *et al*., 2005; Bueno *et al.*, 2007; Xu *et al*., 2007). A comprehensive review on analytical methods for the determination of antimicrobial agents (including fluoroquinolones) in various types of surface waters is provided by Seifrtová *et al*. (2009).

The main contributions of these studies are: (i) determination of chemotherapeutic agents concentrations in different environmental samples (raw sewage, sludge, sewage treatment plant effluents, surface waters and soil samples) and (ii) gaining of efficient analytical methods that can be applied in common life use in various institutions. And while both aims are very reasonable and bring an insight on the topic, no actual risk assessment is provided by these studies.

2.2.1.2. Mass Flow Studies

Mass flow studies are works that try to pursuit the flow of pharmaceuticals in the environment. These studies include exploring the fate of antibiotics during the whole process of sewage water treatment or the distribution of the chemotherapeutical agents to aquatic or terrestrial environments. The works were carried out mostly as short-term case studies as shown on following examples of studies dealing with quinolone antibiotics.

Lindberg *et al.* (2006) conducted their study in a sewage treatment plant in Sweden, receiving water from an area inhabited with a population of

approximately 80 000 people. They used liquid and solid samples collected on three days on various stages of sewage treatment in order to determinate the degradation of different antibiotics, including ciprofloxacin and norfloxacin, and to estimate the efficiency of the mechanical, chemical and biological treatment of waste waters.

Slightly different circumstances were investigated by Golet *et al.* (2002) in their field work in Switzerland. The scientists tried to determinate the exposure of fluoroquinolones (10 substances) in waste and river water of the Glatt Valley Rivershed, located in a heavy populated area near Zurich (175 000 inhabitants, 260 km^2). The experiment was performed using samples obtained in two different seasons (one-week composite samples collected in winter and in summer).

An analogical study was conducted by Nakata *et al.* (2005) who researched the occurrence of a wide range of fluoroquinolones (9 substances) in effluents of a sewage water treatment plant and adjacent surface waters on the USA/Canada border. Only ciprofloxacin was found in final effluent of the sewage water treatment plant and probably due to dilution of the effluent, none of the tested antibiotics was detected in sampled natural waters.

Cardoza *et al.* (2005) researched the factors affecting disappearance rate of ciprofloxacin in surface waters, using both field and laboratory systems. The study focused on two main factors that influence the elimination of ciprofloxacin from aquatic environment – photodegradation and adsorption onto particulate organic carbon.

Mass flow studies often use the knowledge gained in analytical studies. Their significance is in verifying the concentrations of antibiotics in various environments as well as – at least to some degree – in trying to assess the risks of antibiotics use. However, in most cases, the risk assessment is reduced to comparing obtained data with literal sources and no ecotoxicological tests were actually performed.

2.2.1.3. Ecotoxicological Studies

As to the accuracy of environmental risk assessment, these are the studies that bring the most valuable data. The goal of these works is to elaborate the

reliable ecotoxicological profile of respective pharmaceuticals using battery of toxicity tests. In order to properly assess the toxicity of a substance, organisms of all three trophic levels (i.e. producer, consumer and decomposer) need to be tested – it is widely accepted that any effect on any of the trophic levels may have heavy impact on the other trophic levels and disturb the whole ecosystem.

Aside from tests providing data concerning acute toxicity, there is also the need for performing tests enlightening both sub-chronic and chronic toxicity of chemical substances. Because of these facts, ecotoxicological studies are largely time-consuming and financially demanding.

However, not every single ecotoxicological study does, in fact, cover all of the mentioned trophic levels. For example, Nie *et al.* (2009) focused on the effect of two chemically highly unrelated compounds (one of them being norfloxacin) on a freshwater algal species *Scenedesmus obliquus* – an autotrophic organism representing the producers. The reduction of tested organisms to one single species allowed a more in-depth research of the impacts of ciprofloxacin on the metabolism and growth of this specific microalga; not only the estimation of EC_{50}.

Similarly, Charoy (1995) aimed in the research for exploring the changes in swimming pattern of *Brachionus calyciflorus* (a freshwater rotifer) under toxic stress induced by four different toxicants rather than just determining basic toxicological parameters.

A study focused on effects of 25 pharmaceuticals most commonly occurring in aquatic environment on an aquatic higher plant species *Lemna gibba* was carried out by Brain *et al.* (2004). Among the five quinolone antibiotics tested (ciprofloxacin, norfloxacin, ofloxacin, levofloxacin and lomefloxacin), lomefloxacin showed the highest phytotoxicity. Overall, their toxicity demonstrated as bleaching of new fronds (due to damage of chloroplasts) and growth inhibition resulting in development of small new fronds and distinct anatomical changes.

Ecotoxicological aspects of two quinolones and their photodegradation intermediates were marginally studied by Sirtori *et al.* (2009a) in the form of measurement of the inhibition of bioluminescence emitted by the bacterium *Vibrio fischeri*.

A wide range of biological tests was performed by Halling-Sørensen *et al.* (2000) for ciprofloxacin and two other antibiotics. In this work, activated sludge bacteria, a green alga, a cyanobacterium, zooplankton and a fish species were used.

2.2.2. Quinolones in the Environment

According to available literal sources, quinolones are often found in the environment. And while sources of these chemotherapeutical agents are mostly known, it is the fate of the compounds that is not explored enough.

2.2.2.1. Occurrence of Quinolones in the Environment

Kümmerer (2009), in the first part of his two-part comprehensive review on antibiotics in aquatic environment, discussed also the **natural occurrence** of antibacterial agents in soils and surface waters. While some groups of chemotherapeutics can be found in trace concentrations naturally (e.g. β-lactams or streptomycins produced by soil bacteria), quinolones are, however, purely synthetic products and their presence in ecosystems is the result of human activity.

In concordance of the frequency of their therapeutic use, mostly second-generation quinolones are present in the environment. In the following text, an overview of both occurrence and reported concentrations of quinolone antibiotics is provided.

In **raw sewage**, the concentrations of quinolones were shown to occur in concentrations of the order of hundreds ng L^{-1} (from 200 to 300 ng L^{-1} in Lindberg *et al.*, 2006; from 270 to 600 ng L^{-1} in Golet *et al.*, 2003; from 300 to 600 ng L^{-1} in Golet *et al.*, 2002 and from 350 to 600 ng L^{-1} in Peng *et al.*, 2006), while in **final effluent** the concentrations of these antibiotics were significantly lower – reduced down to one fifth or less of the original content (i.e. 40 to 70 ng L^{-1} in Lindberg *et al.*, 2006), or to undetectable concentration (ofloxacin content in Peng *et al.*, 2006).

For several Canadian sewage treatment plants, Miao *et al.* (2004) analyzed only final effluent samples, however, their findings on three quinolones concentration (ciprofloxacin, norfloxacin and ofloxacin) are very similar – from 50 to 120 ng L^{-1} with the highest content observed for ciprofloxacin.

Ciprofloxacin concentration was studied also by Hartmann *et al.* (1999) in composite waste water samples collected from different waste water streams of German hospitals and it ranged from 0.7 to 125 ng L^{-1}.

Zorita *et al.* (2009) focused on determination of various pharmaceuticals (including ciprofloxacin, ofloxacin and norfloxacin) in different sewage waters in Sweden. Their results show that quinolone antibiotics can be detected also in household sewage in concentrations varying from 16 ng L^{-1} (ofloxacin) to nearly 4000 ng L^{-1} (ciprofloxacin). Concentrations of quinolone antibiotics in hospital sewage were as expected higher than those in household sewage.

Information on concentration of quinolones in **solid samples**, except for sludge samples of sewage treatment plants, is quite scarce (noted also by Hernando *et al.*, 2006). Concentration of quinolones in the sludge samples are generally in order of low mg kg^{-1} dry substance (e.g. Lindberg *et al.*, 2007) and because of these high contents, the sludge should be treated accordingly.

As for other solid samples tested on quinolone antibiotics content, Lalumera *et al.* (2004) determined content of flumequin in sediment samples of several Italian fish farms to be fairly low, mostly around the detection limits (0.1 μg kg^{-1} DW) with an extreme exception of nearly 600 μg kg^{-1} DW.

2.2.2.2. Factors Influencing the Elimination of Quinolones from Environment

Lindberg *et al.* (2006) suggest that quinolones undergo to a great extent the process of **sorption** onto sludge, as the reduction from aqueous phase was approximately 80% for both ciprofloxacin and norfloxacin. On the other hand, Halling-Sørensen *et al.* (2000) found no significant amount of ciprofloxacin to be sorbed onto sludge. The distribution between water and particulate matter may depend on water temperature as suggested by Lindberg *et al.* (2006) who compared relative sorption of quinolones onto sludge and temperature of water samples with data from a work of Golet *et al.* (2003). Cardoza *et al.* (2005)

observed that the sorption of ciprofloxacin onto particulate matter is higher in acidic environment and a similar observation was noted by Nowara *et al.* (1997).

Sorption on particulate matter seems to be one of the factors influencing the fate of quinolones in environment. Cardoza *et al.* (2005) conducted both field and laboratory experiments on ciprofloxacin and concluded that the proportion of particulate organic carbon affects the amount of ciprofloxacin in the environment: the not-sorbed fraction of the drug can undergo photodegradation (discussed in Chapter 2.1.1.2.), while the fate of ciprofloxacin sorbed onto solid particles remains fairly undisclosed. That sorption onto particulate material affects the proportion of ciprofloxacin, which can be involved in photodegradation, was confirmed by Belden *et al.* (2006): the photodegradation rate of ciprofloxacin was 2.3-fold slower in the presence of particulate organic material when compared to aqueous solution with no particulate matter.

During the mechanical and chemical treatment of quinolones in the sewage treatment plant, the **efficiency of removal** of quinolones (namely ciprofloxacin and norfloxacin) was around 55% and during the biological treatment only about 35% (Lindberg *et al.*, 2006). Also Halling-Sørensen *et al.* (2000) revealed that ciprofloxacin and structurally similar substances are to a great degree resistant against biodegradation in sludge.

As biodegradation appears not to be the most effective route of quinolones elimination, **photodegradation** became the central spot of scientific interest. Sirtori *et al.* (2009a) focused on the disappearance of flumequin and nalidixic acid of water solutions after two different solar photochemical treatments (the active compounds being TiO_2 and Fe^{2+} in combination with H_2O_2, respectively). The Photo-Fenton treatment (Fe^{2+}/H_2O_2) was confirmed as being the more effective – within 20 minutes of illumination, both pharmaceuticals with initial concentration 20 mg L^{-1} were totally eliminated. When using heterogeneous photocatalyst with TiO_2, the degradation of nalidixic acid was slower and not sufficient enough.

The process of photodegradation has a positive effect on the **mineralization** of quinolones. Under solar photochemical treatment, the proportion of mineralized pharmaceuticals was about 80%, while quinolones undergoing the standard sewage water treatment were mineralized to a much lesser extent (Sirtori *et al.*, 2009a).

Based on these findings, Sirtori *et al.* (2009b) suggested the treatment with Photo-Fenton as the final step in **treatment of sewage waters**. The addition of this procedure provides great time savings as well as very efficient degradation of pharmaceuticals even highly recalcitrant to biodegradation solely (such as nalidixic acid). Berto *et al*. (2009) also tested the Photo-Fenton as a possible step in sewage water treatment and their results confirm the effectiveness of this degradation process – within 30 minutes, mean chemical oxygen demand (COD) was reduced by over 40% and biological oxygen demand (BOD) by 50%.

Further, Sirtori *et al.* (2009a) revealed a significantly lower toxicity of photodegradation intermediates of two fluoroquinolones to the bacterial species *Vibrio fischeri.* This observation implies that photodegradation not only eliminates quinolones from environment, but also decreases the toxicity of these chemotherapeutical agents early in the process.

2.3. Tests and Experimental Organisms Used in the Ecotoxicological Screening of Chemical Substances

2.3.1. Ecotoxicological Tests

As mentioned in Chapter 2.2.1.3., biological tests used in environmental risk assessment are designed to be either short-term, investigating acute toxicity of tested substance, or long-term, aiming for estimation of chronic (or sub-chronic, respectively) toxicity.

In general, the bioassays for ecotoxicological screening should be simple, preferably low-cost and accurate. The methods of endpoint measurement often involve spectral methods, e.g. colorimetry or measurement of optical density. The screening tests are optimized for each experimental organism.

When dealing with the ecotoxicological studies focused on antibiotics, some authors suggest (e.g. Kümmerer, 2009) that using short-term tests is not sufficient – as most mechanisms of resistance development in bacteria are functional in time span of several generations (i.e. order of days and not hours).

2.3.2. Experimental Organisms in Ecotoxicological Screening

The demands on experimental organisms are quite high: they (i) have to be representative for a larger group of species, (ii) should have well-known background data (biological properties, behavioral studies, ecology, metabolism and enzymatic apparatus), (iii) their growing should be easy to maintain and reproducible. Furthermore, the selected organisms should correspond with the environmental conditions of the particular research – e.g. using freshwater, sea and estuarial species in concordance with tested samples.

Not all plant and animal species meet these demands. However, the range of species used for experimental means is still fairly wide as new information is daily gained. In the following chapters, the most common species used for ecotoxicological screening purposes are listed. In addition, some basic characterization of the species and the most commonly evaluated biomarkers are provided.

2.3.2.1. Prokaryotic Organisms

Prokaryotic organisms represent a heterogeneous biological group with substantially different cell structure than those of plant or animal species. In ecotoxicological studies, they are valued for simple maintenance, rapid growth and short life-cycle turn.

The bacterial species probably most exposed to different toxicants are those of activated sludge in sewage treatment plants.

Of other bacterial species, Gram-negative bacteria *Vibrio fischeri* and the Gram-positive bacteria *Bacillus subtilis* are also often used as the indicators in ecotoxicological tests.

Botsford (2002) performed an extensive ecotoxicological testing with the bacterium *Sinorhizobium meliloti*. The research compared 23 other tests with the method using *Sinorhizobium* and concluded that bioassay with this bacterium represents a quick, simple and inexpensive method (Botsford, 2002).

2.3.2.2. Plant Species

Plants are considered as the first trophic level – the primary producers, although there are several exceptions – parasitic plants being the most blatant one. Plant material can be usually obtained at low cost and it is easy to work with. The knowledge gained from experimental work with plant species is quite extensive, but its extrapolation toward animals and, more specifically, humans is due to crucial differences between plants and animals very restricted. Lowe *et al.* (1995) suggested that the potency of using plant material instead of animal and/or human is high if an acceptable plant model is used.

In the numerous ecotoxicological studies, a large variety of plant species is used. Very common are tests conducted on **non-vascular plants**, esp. algae. According to available literal sources, algal genera *Chlorella, Cladophora, Pseudokirchneriella* (formerly *Selenastrum*) and *Scenedesmus* seem to be of the great interest, but there are also other species, frequently used for specific purposes. Using ecotoxicological test with *Chlorella vulgaris* was suggested as an appropriate treatment of hazardous waste waters of various origin by Silva *et al.* (2009). Nunes *et al.* (2008) explored the possibilities of the use of the marine microalga *Tetraselmis chuii* in ecotoxicology, pronouncing it as a suitable experimental organism for marine environment.

The use of algae in ecotoxicological testing is assessed in an in-depth review by Amparado and Persoone (1996).

As to **vascular plants**, ecotoxicological tests are performed on both terrestrial and aquatic species. The genus *Lemna* (and particularly species *Lemna gibba*, duckweed) is one of the most common plants used in testing of pharmaceuticals as well as non-pharmaceutical toxicants in aquatic environment. Terrestrial higher plants of the *Brassicaceae* family (e.g. *Sinapis alba*, *Letuca sativa*, *Raphanus sativus*) also often serve as experimental material.

Lowe *et al.* (1995) discussed the possibility of using **plant tissue cultures** in ecotoxicological screening. The main advantage of this experimental system is the close resemblance of protoplasts (naked plant cells that can be isolated enzymatically) to cultured animal cells.

Generally, growth inhibition is the most inspected endpoint in ecotoxicological studies performed on plants. It can be expressed as mass weight, number of cells, germination capacity or other appropriate surrogate. Also photosynthesis interference of tested plant species is very often the focus of ecotoxicological studies (e.g. Nie *et al.*, 2009 and Sánchez-Fortún *et al.*, 2009 for the freshwater microalga *Scenedesmus* or Brain and Cedergreen, 2009 – see text bellow).

A comprehensive review of **plant biomarkers** used in ecotoxicology is given by Brain and Cedergreen (2009). This work compiles information gained from over 150 experimental studies. Biomarkers are divided into 10 groups, including genes, enzymes, photosynthetic pigments and chlorophyll fluorescence measurement.

2.3.2.3. Invertebrate Animal Species

For ecotoxicological screening purposes mostly invertebrate animal species are used, mainly because of ethical and legislative issues. Rotifers and small crustaceans are proved to be suitable experimental organisms with favorable features concerning their keeping (e.g. a short life cycle, high reproductive rate and ease of culture).

As stated by Snell and Janssen (1995), the use of rotifers in ecotoxicological screening increased significantly since 1990s. In their review on the role of rotifers in ecotoxicology, the authors discuss the advantages of rotifers as experimental organisms from several aspects. According to Snell and Janssen (1995), especially the genus *Brachionus* is of the great interest, although others have been tested (such as *Philodina* in early 1970s or *Dicranophorus* in late 1970s).

The crustaceans represent another group of aquatic animals used in ecotoxicological studies. Among them, especially the genus *Artemia* (especially the marine species *Artemia salina*) is in the central point of scientific interest. The possibilities of this particular genus in ecotoxicology were explored by Nunes *et al*. (2006).

As for freshwater crustaceans, the cladoceran crustacean *Daphnia magna* is probably the most frequently used experimental organism. The variety of ecotoxicological assays that can be performed with this species is reviewed for example by Martins *et al.* (2007). An experimental organism with results comparable to *D. magna* was reported by Versteeg *et al.* (1997). The authors investigated the possibilities of the use of the genus *Ceriodaphnia dubia* in ecotoxicological studies and found that data obtained in studies with *C. dubia* are equivalent to those obtained with *D. magna* (Versteeg *et al.*, 1997). The suitability of *D. magna* utilization in ecotoxicology was, however, questioned by Koivisto (1995) who concluded that *D. magna* may be less sensitive to pollutants than other crustaceans because of its specific properties (such as size and habitat).

For terrestrial environmental pollution studies, annelids are of the great interest. Lowe and Butt (1997) evaluated the use of earthworms in chronic ecotoxicological studies and suggested the use of *Eisenia andrei* rather than the previously most used species *E. fetida* because of a better genetically homogenity.

The most frequent endpoints in ecotoxicological tests with invertebrate animal species are mortality, reproduction analysis and changes in behavior. Snell and Janssen (1995) provide an overview of biomarkers that can be determined in rotifers.

2.3.2.4. Experimental Organisms Used in the Present Research

In this thesis, only freshwater experimental organisms were used. For the ecotoxicological screening purposes following species were tested: the algal species *Pseudokirchneriella subcapitata*, the ciliate protozoan *Tetrahymena pyriformis*, the rotifer *Brachionus calyciflorus* and the crustacean *Thamnocephalus plytyurus*. For further information, see Chapter 3.2.1.

3. Materials and Methods

3.1. Materials

3.1.1. Tested Pharmaceuticals

For all toxicity tests conducted, three quinolone antibiotics were used: ciprofloxacin, norfloxacin and ofloxacin. All of them were of analytic grade quality and all three were purchased from Sigma Aldrich, Germany. Due to their chemical and physical properties, both solid chemicals and their solutions were stored at low temperature and wrapped with thin aluminum foil in order to protect them from any light sources. The solutions were utilized within a week of preparation, or new solutions were prepared.

Potassium dichromate (analytical grade quality, purchased from Fluka, Germany) was used for reference toxicity tests as a compound with a good-defined toxicity concentration for all tested organisms.

3.1.2. Plant and Animal Material

In order to determinate the acute toxicity of selected antibiotics on all three basic types of organisms based on their trophic levels (i.e. producer, consumer and reducer), several species were used. All selected experimental organisms are freshwater species frequently used in ecotoxicological screening of various pharmaceuticals.

Further details regarding experimental organisms included in this thesis are listed in sections describing the individual toxicity tests conducted.

3.2. Methods

3.2.1. Toxicity Tests

All of the selected toxicity tests were conducted according to their standard operational procedures, unless stated otherwise. The TOXKITs were purchased from MicroBio Tests Inc., Nazareth, Belgium. In this thesis, following toxicity tests were performed (listed in alphabetical order):

3.2.1.1. ALGALTOXKIT F™

This TOXKIT represents toxicity test with the freshwater algal species *Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum*; see Fig. 3.1.) – a typical producer. The used algal culture was de-immobilized and then algal inoculum was prepared according to standard operational procedure. Prior to testing, algal density of approximately 1 million cells mL^{-1} in the inoculum was determined. Dilution series of both investigated quinolone antibiotics and potassium dichromate dilution were prepared and then algal suspension was added. For each of the tested chemical compounds 8 concentrations of toxicant were prepared. The real concentrations started at 12 mg L^{-1} for the antibiotics and at 32 mg L^{-1} for potassium dichromate.

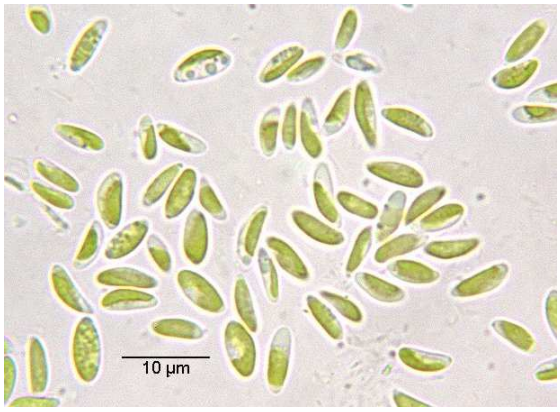

Figure 3.1. The freshwater microalga *Pseudokirchneriella subcapitata*.
From www.lifesciences.napier.ac.uk

Algae-toxicant dilutions and control samples were cultured in specific 10 cm long test vials. Test vials were placed into the holding tray in a random way

(with allowed air-exchange space) and put into an incubator at 25 °C with a constant and uniform illumination. Data were obtained after 0 and 72 hours of incubation.

The main difference from the ALGALTOXKIT F™ standard operational procedure was data collecting: the required spectrophotometer equipped with a holder for 10 cm cells was not available; therefore manual cell-number counting had to be accomplished. A light microscope at 400-fold magnification and a Bürker cell with ruled square measuring were utilized. The samples were observed in bright field and cells were counted according to a prior designed cell-counting system.

3.2.1.2. Modified PROTOXKIT F™

A toxicity test designed for acute toxicity determination using a freshwater ciliated protozan – *Tetrahymena pyriformis* (see Figure 3.2.). Slightly method modifications were implemented compared to standard operational procedure. Instead of 1 cm polystyrol spectrophotometric test cells polycarbonate multiwell test plates (with 96 test wells) were used. For a more detailed test schema see Figure 3.3.

Figure 3.2. The freshwater ciliated protozoan *Tetrahymena pyriformis*. From www.cadaster.eu.

For each of the tested chemical compounds 15 concentrations of the respective toxicant were prepared. The real concentrations started at 3 mg L^{-1} for the antibiotics and at 8 mg L^{-1} for potassium dichromate.

The whole process of test plates' preparation for measurement was conducted in a laminar flow box. Test plates were then placed into an incubator at 25 °C without illumination. Data were obtained after 0, 24 and 48 hours of incubation. The test plates were measured with the use of the anthos WinRead

software (Salzburg, Austria). Optical density of samples was measured at 562 nm directly in the test plates.

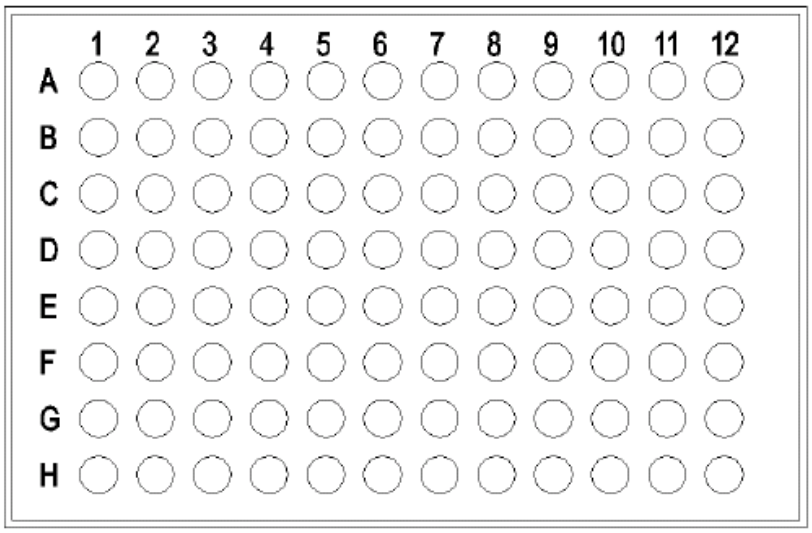

Figure 3.3. The multiwell test plate used in the modified acute toxicity test with *T. pyriformis*. Row B – peptone (200 µL), row C – peptone (150 µL) + tested chemical compound solution (50 µL), row D – peptone (150 µL) + *Tetrahymena* culture (50 µL), rows E – G – (three duplications) peptone (100 µL)+ tested chemical compound solution (50 µL) + *Tetrahymena* culture (50 µL). Columns 2 – 11 were used for different concentrations of tested chemical compounds. The model of test plate was constructed by Ing. Jan Dubánek.

3.2.1.3. ROTOXKIT F™ ACUTE

It is an acute toxicity test with a freshwater rotifer – *Brachionus calyciflorus* (Figure 3.4.). Following standard operational procedure, one day prior to the start of the toxicity test rotifer cysts were hatched in order to obtain test organisms of proper age (i.e. 0 to 2 hours old).

Figure 3.4. The freshwater rotifer *Brachionus calyciflorus*. From Turchin (2003).

The toxicity test was carried out using polycarbonate multiwell test plates (with 43 wells). Under a dissection microscope at 10-fold magnification, rotifers were transferred first into the rinsing trough and then into the test wells. For a more detailed test schema see Figure 3.5.

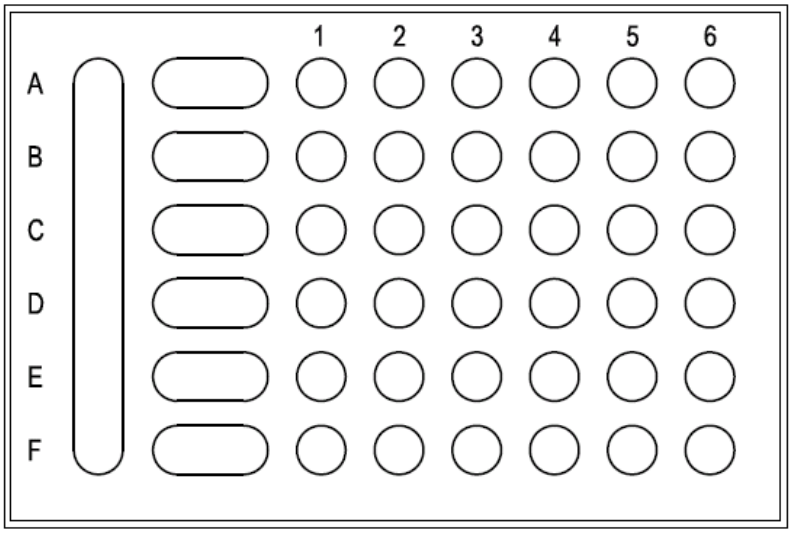

Figure 3.5. The multiwell test plate used in the acute toxicity test with *B. calyciflorus*. Rows A and B, C and D, and E and F, respectively – replications of the determination, each containing 300 µL of particular chemical compound solution and 5 experimental organisms. Columns 1 – 6 were used for different concentrations of tested chemical compounds. Unnumbered horizontal wells served as rinsing trough. The long transversal well was intended as hatching well. The model of test plate was constructed by Ing. Jan Dubánek.

For each of the tested chemical compounds 15 concentrations of the respective toxicant were prepared. The concentrations started at 12 mg L^{-1} for the antibiotics and at 32 mg L^{-1} for potassium dichromate.

Test plates were put in an incubator at 25 °C without illumination. Data (mortality of the rotifers) were collected after 24 hours of incubation.

3.2.1.4. THAMNOTOXKIT F™

This test is intended as an acute toxicity determining test with a freshwater crustacean – *Thamnocephalus platyurus* (Figure 3.6.).

As stated in the standard operational procedure, hatching of the cysts was performed 24 hours prior to the toxicity test. Polycarbonate multiwell test plates (with 24 test wells) were used for the toxicity test. After transferring from the hatching Petri dish into rinsing trough, the crustaceans were placed into the test

wells with effluent in the particular dilution. For a more detailed test schema see Figure 3.7.

Figure 3.6. The freshwater crustacean *Thamnocephalus platyurus*. From www.r-biopharm.com.

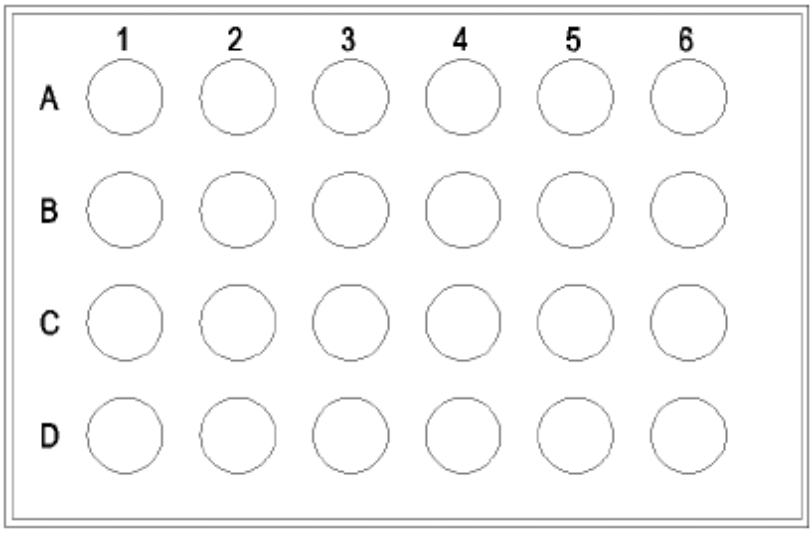

Figure 3.7. The multiwell test plate used in the acute toxicity test with *T. platyurus*. Rows A and B, and C and D, respectively – duplications of the determination, each containing 300 µL of particular chemical compound solution and 10 experimental organisms. Columns 2 – 6 were used for different concentrations of tested chemical compounds. Wells of the column 1 served as rinsing trough. The model of test plate was constructed by Ing. Jan Dubánek.

For each of the tested chemical compounds 9 concentrations of the respective toxicant were prepared. The concentrations started at 0.3 mg L^{-1} for the antibiotics and at 32 mg L^{-1} for potassium dichromate.

Test plates were put into an incubator at 25 °C without illumination. Data (mortality of the crustaceans) were collected after 24 hours of incubation.

3.2.2. Data Treatment

All data were obtained in accordance either with the standard operational procedures of each toxicity test performed, or in compliance with respective method modifications. The time schedule of data gaining was also following either the instructions or the altered test procedure.

The statistical processing of gained data (nonlinear regression) and the calculation of the commonly used ecotoxicological parameters for all toxicity tests conducted was performed by Mgr. Jitka Vytlačilová with the use of the statistical software GraphPad Prism (La Jolla, California, USA).

4. Results

4.1. Acute Toxicity of Tested Quinolone Antibiotics on Pseudokirchneriella subcapitata

Of all three fluoroquinolone antibiotics tested, ciprofloxacin and norfloxacin exhibited very similar acute toxicity on *P. subcapitata* (IC_{50} after 72 hours slightly below 8 mg L^{-1}), while ofloxacin was a little less toxic with the IC_{50} after 72 hours slightly above 9 mg L^{-1} (see Table 4.1.). The influence of ofloxacin on the growth rate rapidly decreased with increasing dilution of the tested antimicrobial agents (see Table 4.2. and Figure 4.1.).

Overall, the acute toxicity of the antibiotics was 20-fold lower than the acute toxicity of the standard toxicant – potassium dichromate (IC_{50} after 72 hours 0.40 mg L^{-1}).

Table 4.1. *Pseudokirchneriella subcapitata* acute toxicity test: IC_{50} values (mg L^{-1}) with confidence limits for each fluoroquinolone antibiotic.

	$IC_{50,72h}$
Ciprofloxacin	7.72 (7.17 – 8.317)
Norfloxacin	7.99 (7.57 – 8.43)
Ofloxacin	9.27 (8.81 – 9.77)

Table 4.2. *Pseudokirchneriella subcapitata* acute toxicity test: the used weight/volume and molar concentrations of fluoroquinolone antibiotics tested. w/v- weight/volume concentration of antibiotics (mg L^{-1}), all other concentrations are molar (µmol L^{-1}). CIP- ciprofloxacin, NOR- norfloxacin, OFL- ofloxacin.

	c1	c2	c3	c4	c5	c6	c7	c8
w/v	12.00	6.00	3.00	1.50	0.75	0.38	0.19	0.09
CIP	36.22	18.11	9.05	4.53	2.26	1.13	0.57	0.28
NOR	27.12	13.56	6.78	3.39	1.70	0.85	0.42	0.21
OFL	33.21	16.60	8.30	4.15	2.08	1.04	0.52	0.26

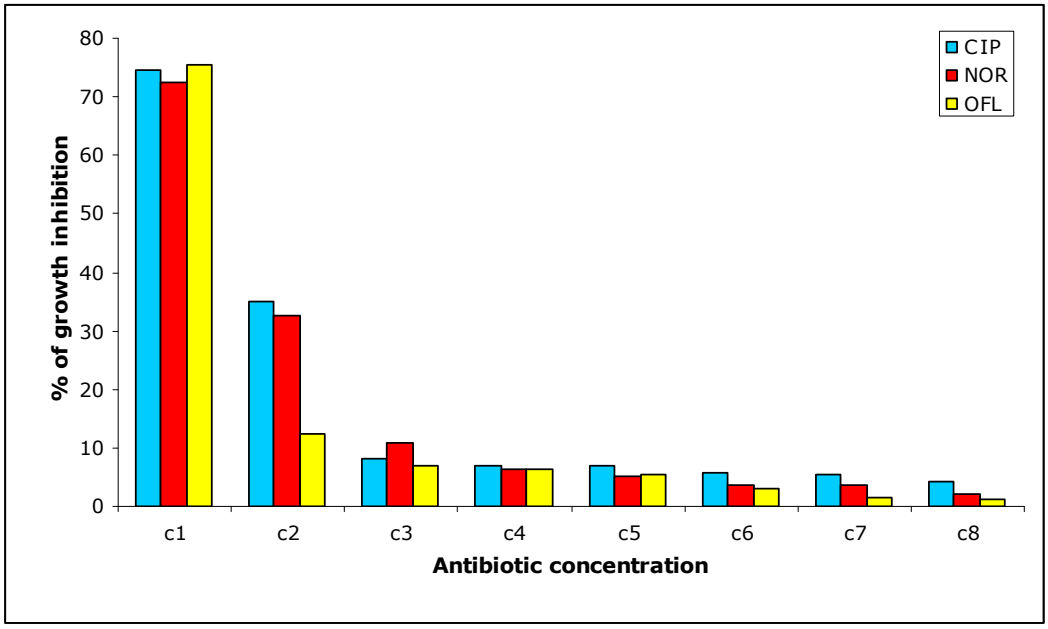

Figure 4.1. *Pseudokirchneriella subcapitata* acute toxicity test: the impact of antibiotics on the growth inhibition after 72 hours of treatment. The antibiotic concentrations decrease from c1 to c8. For the exact weight/volume and molar concentrations of the tested antibiotics see Table 4.2. CIP- ciprofloxacin, NOR- norfloxacin, OFL- ofloxacin.

4.2. Acute Toxicity of Tested Quinolone Antibiotics on Tetrahymnena pyriformis

After 24 hours of treatment, the most toxic antimicrobial agent on *T. pyriformis* was ciprofloxacin (IC_{50} 5.82 10^{-3} mg L^{-1}), although both remaining fluoroquinolones exhibited high acute toxicity on the experimental organism (IC_{50} slightly above 2 mg L^{-1} for norfloxacin and slightly above 1 mg L^{-1} for ofloxacin; see Table 4.3. and Figure 4.2.A). The $IC_{50,24h}$ value of the standard toxicant, potassium dichromate was 20.82 mg L^{-1} (17.27 – 25.11 mg L^{-1}).

After 48 hours of treatment, the toxicity of tested antibiotics surprisingly changed, with norfloxacin as the most active in growth inhibition of *T. pyriformis*. The IC_{50} values of all three fluoroquinolones decreased at least one order of magnitude (to 10^{-4} mg L^{-1} for ciprofloxacin and ofloxacin, and to 10^{-5} mg L^{-1} for norfloxacin, respectively; see Table 4.3. and Figure 4.2.B).

This particular trend (i.e. the increase of growth inhibition after prolonged exposition to antibiotics and the highest toxicity switch between ciprofloxacin and norfloxacin) is noticeable throughout the whole concentration range of tested antibiotics (see Figure 4.2., Figure 4.3. and Table 4.4.).

Table 4.3. *Tetrahymena pyriformis* acute toxicity test: IC_{50} values after 24 and 48 hours of treatment (mg L^{-1}) with confidence limits for each fluoroquinolone antibiotic.

	$IC_{50,24h}$	$IC_{50,48h}$
Ciprofloxacin	$5.82\ 10^{-3}$ ($4.94\ 10^{-3}$ – $6.85\ 10^{-3}$)	$1.15\ 10^{-4}$ ($7.22\ 10^{-5}$ - $1.85\ 10^{-4}$)
Norfloxacin	2.19 (1.82 – 2.64)	$3.80\ 10^{-5}$ ($1.61\ 10^{-5}$ - $8.99\ 10^{-5}$)
Ofloxacin	1.08 (0.58 – 2.03)	$2.35\ 10^{-4}$ ($1,91\ 10^{-4}$ - $2.89\ 10^{-4}$)

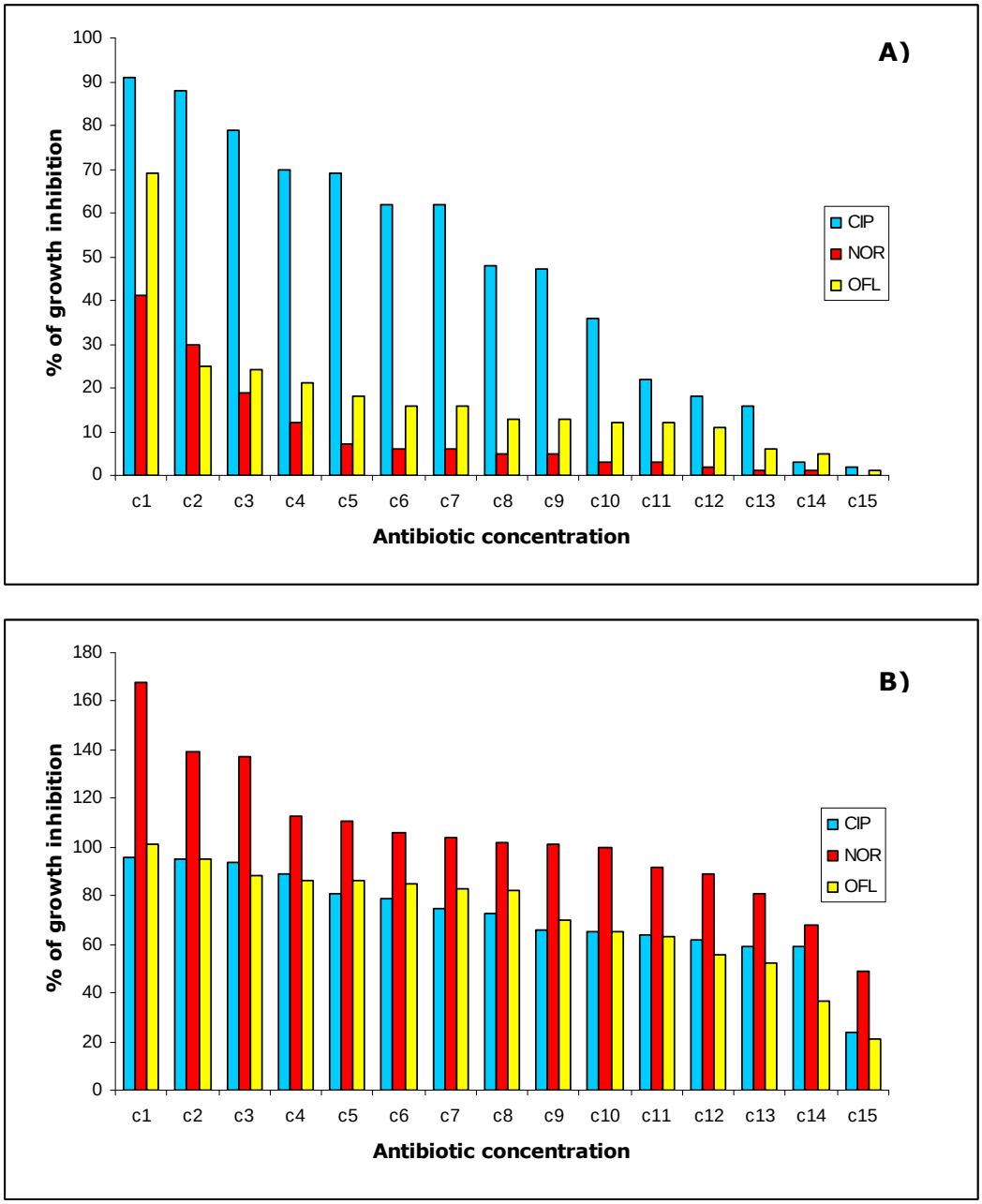

Figure 4.2. *Tetrahymena pyriformis* acute toxicity test: the impact of antibiotics on the growth inhibition after **A)** 24 and **B)** 48 hours of treatment. The antibiotic concentrations decrease from c1 to c15. For the exact weight/volume and molar concentrations of the tested antibiotics see Table 4.4. CIP- ciprofloxacin, NOR- norfloxacin, OFL- ofloxacin.

Table 4.4. *Tetrahymena pyriformis* acute toxicity test: the used weight/volume and molar concentrations of fluoroquinolone antibiotics tested. w/v- weight/volume concentration of antibiotics (mg L^{-1}), all other concentrations are molar (nmol L^{-1}). CIP- ciprofloxacin, NOR- norfloxacin, OFL- ofloxacin.

	c1	c2	c3	c4	c5	c6	c7	c8
w/v	3.0000	1.5000	0.7500	0.3750	0.1875	0.0938	0.0469	0.0234
CIP	9054	4527	2264	1132	566	283	141	71
NOR	6780	3390	1695	848	424	212	106	53
OFL	8302	4151	2075	1038	519	259	130	65

	c9	c10	c11	c12	c13	c14	c15
w/v	0.0117	0.0059	0.0029	0.0015	0.0007	0.0004	0.0002
CIP	35.37	17.68	8.84	4.42	2.21	1.11	0.55
NOR	26.49	13.24	6.62	3.31	1.66	0.83	0.41
OFL	32.43	16.21	8.11	4.05	2.03	1.01	0.51

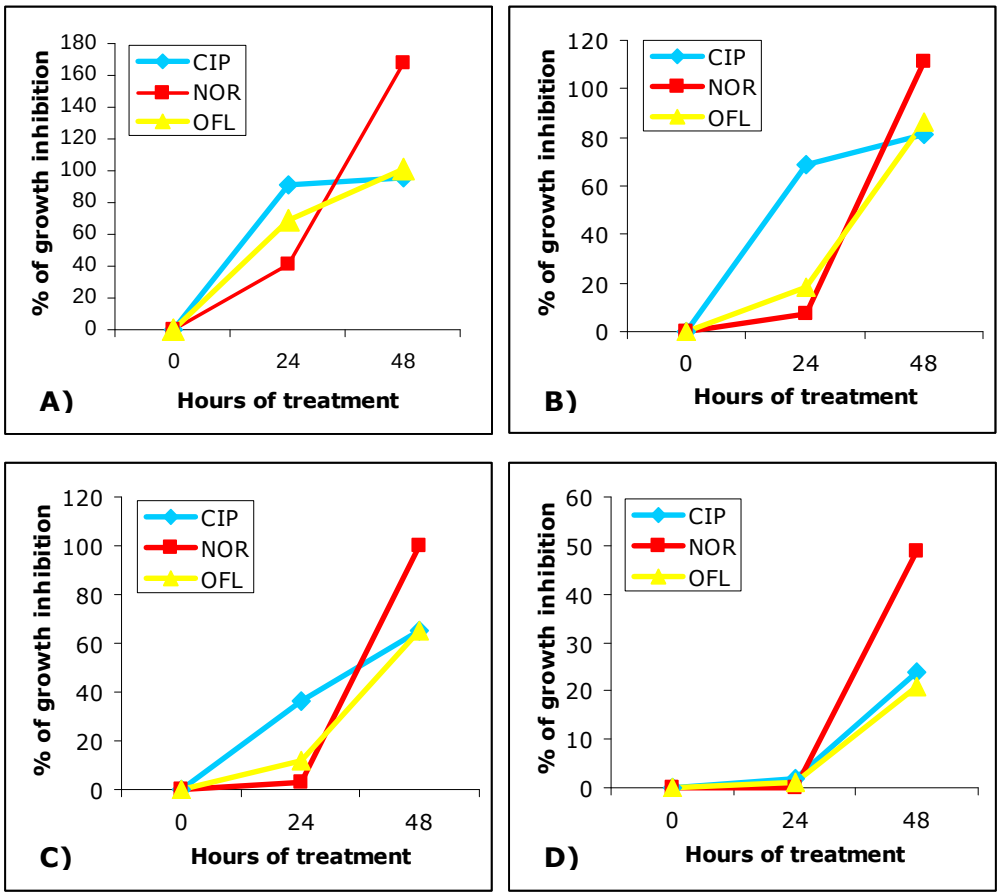

Figure 4.3. *Tetrahymena pyriformis* acute toxicity test: the impact of selected antibiotic concentrations on the growth inhibition. Concentrations: **A)** c1 **B)** c5 **C)** c10 **D)** c15. For the exact weight/volume and molar concentrations of the tested antibiotics see Table 4.4. CIP- ciprofloxacin, NOR- norfloxacin, OFL- ofloxacin.

4.3. Acute Toxicity of Tested Quinolone Antibiotics on *Brachionus calyciflorus*

The acute toxicity of the tested antibiotics on *B. calyciflorus* after 24 hours treatment varied considerably: the highest acute toxicity was found for ofloxacin (LC_{50} was 6.05 10^{-4} mg L^{-1}), while norfloxacin was significantly less toxic (LC_{50} was slightly above 144 mg L^{-1}) and the LC_{50} value for ciprofloxacin was somewhere in between (LC_{50} 0.057 mg L^{-1}; see Table 4.5.). In other words, norfloxacin slightly affected the mortality of the experimental organisms only when applied in the three highest concentrations, ciprofloxacin influenced the mortality in two thirds of the concentration range (i.e. c1 – c9), and ofloxacin impacted mortality of the crustaceans in the whole tested concentration range (see Figure 4.4. and Table 4.6.).

The LD_{50} value of the standard toxicant, potassium dichromate, after 24 hours of treatment was 13.70 mg L^{-1} (9.60 – 17.80 mg L^{-1}).

Table 4.5. *Brachionus calyciflorus* acute toxicity test: LC_{50} values after 24 hours of treatment (mg L^{-1}) with confidence limits for each fluoroquinolone antibiotic.

	$LC_{50,24h}$
Ciprofloxacin	5.74 10^{-2} (4.79 10^{-2} – 6.87 10^{-2})
Norfloxacin	144.2 (78.04 – 266.6)
Ofloxacin	6.05 10^{-4} (5.72 10^{-4} – 6.40 10^{-4})

Table 4.6. *Brachionus calyciflorus* acute toxicity test: the used weight/volume and molar concentrations of fluoroquinolone antibiotics tested. w/v- weight/volume concentration of antibiotics (mg L^{-1}), all other concentrations are molar (nmol L^{-1}). CIP- ciprofloxacin, NOR- norfloxacin, OFL- ofloxacin.

	c1	c2	c3	c4	c5	c6	c7	c8
w/v	12.000	6.000	3.000	1.500	0.750	0.375	0.188	0.094
CIP	36217	18108	9054	4527	2264	1132	566	283
NOR	27122	13561	6780	3390	1695	848	424	212
OFL	33207	16603	8302	4151	2075	1038	519	259

	c9	c10	c11	c12	c13	c14	c15
w/v	0.0469	0.0234	0.0117	0.0059	0.0029	0.0015	0.0007
CIP	141.47	70.74	35.37	17.68	8.84	4.42	2.21
NOR	105.94	52.97	26.49	13.24	6.62	3.31	1.66
OFL	129.71	64.86	32.43	16.21	8.11	4.05	2.03

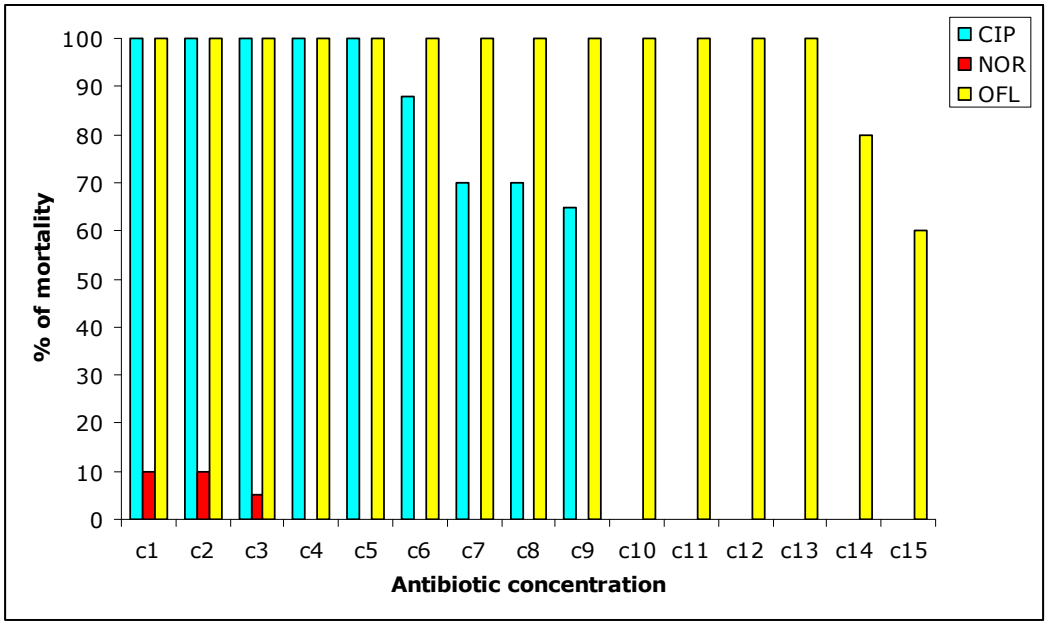

Figure 4.4. *Brachionus calyciflorus* acute toxicity test: the impact of antibiotics on the mortality of experimental organisms after 24 hours of treatment. The antibiotic concentrations decrease from c1 to c15. For the exact weight/volume and molar concentrations of the tested antibiotics see Table 4.6. CIP- ciprofloxacin, NOR- norfloxacin, OFL- ofloxacin.

4.4. Acute Toxicity of Tested Quinolone Antibiotics on Thamnocephalus platyurus

In the concentration range of antibiotics used for the toxicity tests (see Table 4.7.), none of the three tested fluoroquinolones exhibited acute toxicity on *T. platyurus*. The mortality of experimental organisms was 10% or less (see Figure 4.5.), which is not sufficient to provide reliable results for statistical analysis and calculation of appropriate ecotoxicological parameters.

Table 4.7. *Thamnocephalus platyurus* acute toxicity test: the used weight/volume and molar concentrations of fluoroquinolone antibiotics tested. w/v- weight/volume concentration of antibiotics (µg L^{-1}), all other concentrations are molar (nmol L^{-1}). CIP- ciprofloxacin, NOR- norfloxacin, OFL- ofloxacin.

	c1	c2	c3	c4	c5	c6	c7	c8	c9
w/v	300.00	150.00	75.00	37.50	18.75	9.38	4.69	2.34	1.17
CIP	905.41	452.71	22.35	113.18	56.59	28.29	14.15	7.07	3.54
NOR	678.04	339.02	169.51	84.76	42.38	21.19	10.59	5.30	2.65
OFL	830.17	415.09	207.54	103.77	51.89	25.94	12.97	6.49	3.24

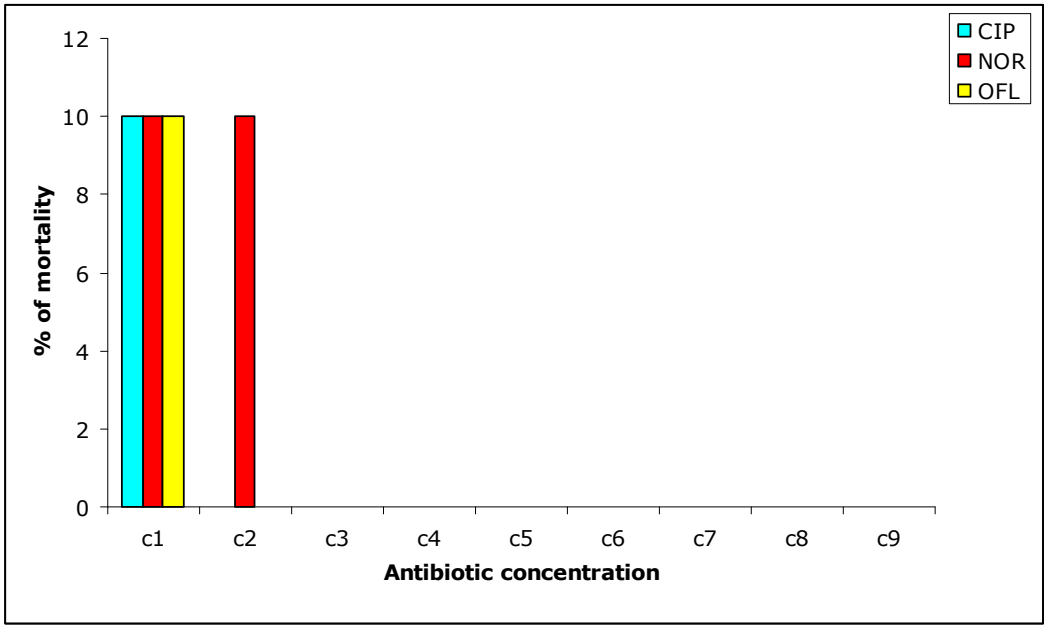

Figure 4.5. *Thamnocephalus platyurus* acute toxicity test: the impact of antibiotics on the mortality of experimental organisms after 24 hours of treatment. The antibiotic concentrations decrease from c1 to c9. For the exact weight/volume and molar concentrations of the tested antibiotics see Table 4.7. CIP- ciprofloxacin, NOR- norfloxacin, OFL- ofloxacin.

5. Discussion

5.1. Discussion of Methods Used

During the presented research have arisen several issues with the optimal methods. Some of them are of great influence on the reliability and consistence of obtained results, and therefore there is large space for discussion on a deeper level.

5.1.1. Changes in Standard Operational Procedure of ALGALTOXKIT^{TM}

Data collected in this test was performed with the use of a light microscope and Bürker cell for manual cell counting (for details see Chapter 3.2.1.1.). This change of standard operational procedure was necessary due to the unavailability of the required equipment (a spectophotometer for 10 cm cells) and caused several inconveniences.

First of all, the time frame of the test was heavily altered: while a spectrophotometric sample measurement takes about two minutes, the manual cell counting is more time-consuming (approximately 15 minutes for a single sample). For 10 samples, the difference is over two hours and for all 56 samples, the time schedule is shifted considerably (well over eight hours).

Secondly, the abovementioned altered schedule could lead with high probability to unreliable results. To minimize this threat, control samples were measured in regular intervals and the cell numbers of samples treated with toxicants were corrected accordingly.

However, the control samples were measured in two-hour intervals; that means that there is still possibility for the results to be underestimated and thus, the toxicity of the tested antibiotics may appear higher (i.e. the calculated IC_{50} values will be lower). The best option how to eradicate this particular error is to perform the test with the spectrophotometric measurement.

5.1.2. Quinolone Antibiotics Concentrations in the Modified PROTOXKIT™

In the modified protozoan toxicity test the concentration of quinolones starts at 3 mg L^{-1}. This inconvenience creating a slight discrepancy in the presented results was caused by an unpredictable behavior of the used experimental organism, *Tetrahymena pyriformis*.

As of November 2009, there was an inadequate growth response of *T. pyriformis* in all conducted toxicity tests. Various modifications of the experimental conditions were unsuccessful and different batches of experimental organisms also failed to provide accurate results.

Therefore, a new experimental organism, *T. thermophila*, is currently being tested, and the optimal experimental conditions are being adjusted. Thus, results published in this work represent the early conducted toxicological tests from before November 2009.

5.2. The Impact of Quinolone Antibiotics on Selected Experimental Organisms

In this chapter, the influence of the three quinolone antibiotics on the tested organisms will be discussed. I tried to include only studies focused on quinolones and their effects on the same experimental organisms that were used in presented research, but due to lack of publications fulfilling these criteria, there are some works with different antibacterial agent and/or experimental organism included. The lack of ecotoxicological studies concerning (not exclusively) quinolones was marked for example by Christensen *et al*. (2009).

5.2.1. The Effect of Quinolone Antibiotics on Freshwater Green Algae with the Emphasis on *Pseudokirchneriella supcapitata*

Based of the collected data (see Chapter 4.1.), the algal species *Pseudokirchneriella subcapitata* seems to have similar tolerance to all three tested quinolones. The growth rate of the algae was inhibited only by antibiotic

concentrations around 8 mg L^{-1} (ciprofloxacin, norfloxacin) and over 9 mg L^{-1} (ofloxacin; details in Chapter 4.1.).

That norfloxacin is influencing the growth rate and photosynthetic parameters in green algae prevalently in higher concentrations, has been observed also by Eguchi *et al*. (2004). The authors found that norfloxacin concentrations under 4 mg L^{-1} did not affect algal cultures of two freshwater algae, *P. subcapitata* and *Chlorella vulgaris*. Only concentrations of norfloxacin exceeding 10 mg L^{-1} (for *Pseudokirchneriella*) and 14 mg L^{-1} (for *Chlorella*), respectively lead to a significant growth and photosynthetic rate decrease (Eguchi *et al.*, 2004).

Even higher tolerance of green algae (*P. subcapitata* and *Scenedesmus obliquus*) to quinolone antibiotic was reported by Robinson *et al.* (2005) and Nie *et al.* (2009). Robinson *et al.* (2005) provided research focused on the toxicity of seven fluoroquinolone antibiotics (including ciprofloxacin and ofloxacin) on selected aquatic organisms. The authors reported that to *P. subcapitata*, both ciprofloxacin ($EC_{50,24h}$ 18.7 mg L^{-1}) and ofloxacin ($EC_{50,24h}$ 12.1 mg L^{-1}) were the least toxic of all fluoroquinolones investigated (Robinson *et al.*, 2005). Similar results were reported by Nie *et al.* (2009) who studied the influence of norfloxacin (in concentrations ranging from 3.75 to 60 mg L^{-1}) on *S. obliquus*. A decrease in growth rate and the content of chlorophyll *a* were observed only when the algal culture was exposed to norfloxacin concentration exceeding 15 mg L^{-1} (Nie *et al.*, 2009).

The finding, that ciprofloxacin seems to have similar toxicity levels as norfloxacin on green algae is supported by Nie *et al.* (2008) who observed growth rate inhibition of the green alga *C. vulgaris* only at ciprofloxacin concentrations higher than 12 mg L^{-1}.

The documented concentrations of quinolones in the environment are several orders of magnitude lower than the concentrations shown to cause growth inhibition – they vary in the rank of tens to hundreds of ng L^{-1} (see Chapter 2.2.2.1.) – which suggests that even the raw sewage with the highest amount of quinolones present should not affect the algal population in surface waters.

Furthermore, some of the algal genera (including *Pseudokirchneriella* and *Scenedesmus*) seem to have the ability of (at least partially) evolving into antibiotic-resistant species as shown by Sánchez-Fortún *et al.* (2009). The authors

tested the impact of one of the most used antibiotic agents utilized in aquaculture (namely chloramphenicol) on the growth rate and photosynthetic performance in *Scenedesmus intermedius*. After 72 hours of exposure chloramphenicol caused growth inhibition and decrease of photosynthesis; however, in the time span of 60 days the algal culture restored both the growth and photosynthetic rate because of spontaneous mutation resulting in the evolution of chloramphenicol-resistant cells (Sánchez-Fortún *et al.*, 2009).

Similarly, Nie *et al.* (2009) also observed cell adaptability of *S. obliquus* culture to norfloxacin exposure in a longer time span in both growth rate and chlorophyll *a* content. And Eguchi *et al.* (2004) reported that norfloxacin strongly inhibited the growth rate of *P. subcapicata* early in the study (the first day), while later (the third day), the growth rate of the norfloxacin-treated culture even exceeded the growth rate of control population. None of other antimicrobial agents tested in their research (erythromycin, oxytetracycline, several sulfonamides, and selected β-lactam antimicrobials) had shown similar properties (Eguchi *et al.*, 2004).

To extrapolate the results of three studies (one with a chemically different compound, even) on the presented research would be inappropriate. On the other hand, at least it has been suggested that the algal genera *Pseudokirchneriella* and *Scenedesmus* might potentially gain adaptive mechanisms to antibiotic agents and therefore would probably not be endangered by low quinolone concentrations present in the environment.

However, this may not be true for all algal species present in freshwater ecosystems (esp. the blue green algae; e.g. Robinson *et al*., 2005). Sanderson *et al.* (2004) provided a large research focused on the environmental risk assessment of several pharmacological groups of active substances to surface waters. In this study, the authors found that while antibacterial agents do not have a high potential to accumulate in biologic materials, they represent a significant threat to algal populations. There seems to be, however, only a restricted number of antibacterial agents with a high-level hazard quotient – about 10% of the whole amount of the therapeutically used antibacterial active compounds (Sanderson *et al.*, 2004).

5.2.2. The Effects of Quinolone Antibiotics on the Ciliated Protozoan *Tetrahymena pyriformis*

The genus *Tetrahymena*, particularly species *T. pyriformis* as well as *T. thermophila* have been proved to be useful in ecotoxicological studies (a large review of the use and the studies conducted on *T. pyriformis* was provided by Suavant *et* a.l, 1999).

According to available literary sources (Web of Knowledge) up to April 2010 there are, however, no published works dealing at least marginally with the toxicity of quinolone antibiotics on this particular genus. Although Suavant *et al.* (1999) in their review remarked that "Many substances have been tested with *Tetrahymena pyriformis*. Most of them are antibiotics…" the authors listed exactly three works focusing on the antibiotic agents: Krawczynska (1990) studying the effect of polymyxin B and two aminoglycosidic antibiotics on the phagocytic activity of *T. pyriformis*, Nilsson (1989) dealing partially with chloramphenicol and Wu *et al.* (1996) characterizing membrane action of chloramphenicol with the use of *T. pyriformis* motility inhibition. As antibiotics represent a large, non-homogenous group with compounds of substantially different chemical structure and mechanism of action, it is probably not appropriate to equate the effect of the mentioned antibiotics to the impact of quinolones on the *Tetrahymena* genus.

Further pursuit provided that such research was neither done with another frequently used protozoan genus, *Paramecium*. Most of the available studies focused on the effects of various heavy metals and herbicides on the both abovementioned protozoan genera (e.g. Miyoshi *et al.*, 2003 focused on *P. caudatum* and *P. trichium*; Sauvant *et al.*, 1999 dealing with *T. pyriformis*).

For these reasons, there is actually not a single work I could discuss results obtained in the presented research. The actual results suggest that *T. pyriformis* reacts diversely when exposed to different fluoroquinolones (ciprofloxacin being the most toxic) and that the length of treatment may increase the sensitivity of the test organism to investigated antibiotics (considerably increased toxicity of norfloxacin after 48 hours of treatment; see Chapter 4.2.). The $IC_{50,48h}$ values for all investigated quinolones are furthermore in the range of the reported fluoroquinolone antibiotics concentrations in the environment (see

Chapter 2.2.2.1.), and thus they may have negative influence on the naturally occurring populations of various *Tetrahymena* species.

5.2.3. The Effects of Quinolone Antibiotics on the Freshwater Rotifer *Brachionus calyciflorus*

In ecotoxicological screenings, the species *Brachionus plicatilis* is probably more used experimental organism than *Brachionus calyciflorus*, which caused some problems, especially regarding the availability of published works focused on *B. calyciflorus*. As *B. plicatilis* is a brakish-water inhabitant, while *B. calyciflorus* represents a freshwater species, their natural ecosystems are substantially different. Furthermore, there are works that show a great variability in response of two different *Brachionus* species (or even different strains of one species) to exposure of antimicrobial agents (Araujo and McNair, 2007 and references therein).

Overall, there are not many studies focused on toxicity tests on *B. calyciflorus* using quinolone antibiotics as toxicants (in fact, the only published research I managed to obtain was that of Isidori *et al*., 2005; see text bellow).

The present results show that the acute toxicity of the quinolone antibiotics on the experimental organism is considerably different for each of the three fluoroquinolones (the LC_{50} values vary in the range of several orders of magnitude – from 10^{-4} to 10^{2} mg L^{-1}; see Chapter 4.3.) with ofloxacin being the most toxic, which is in contradiction with published studies. For example, the mentioned work of Isidori *et al.*, (2005) reported $LC_{50,24h}$ for ofloxacin for *B. calyciflorus* slightly bellow 30 mg L^{-1}, although the chronic toxicity of ofloxacin was higher (the value of $LC_{50,48h}$ was 0.53 mg L^{-1}).

The present results suggest that ofloxacin may have a negative impact on the naturally occurring populations of *B. calyciflorus* as the LC_{50} value for this fluoroquinolone antibiotic is in the range of quinolone concentrations reported in the environment (see Chapter 2.2.2.1.).

Apart from the work of Isidori *et al.* (2005), there were, however, no studies I could discuss the obtained results with, as the only other research dealing

with the effect of the quinolone antibiotic ofloxacin focused on chronic toxicity rather than acute toxicity of ofloxacin on *B. calyciflorus* (Ferrari *et al.*, 2003).

5.2.4. The Effects of Quinolone Antibiotics on Freshwater Crustaceans *Thamnocephalus platyurus* and *Daphnia magna*

According to available literal sources (Web of Knowledge) as of April 2010, there are only two published works dealing with the effects of antimicrobial agents on the freshwater anostracan crustacean *Thamnocephalus platyurus* (Isidori *et al.*, 2005, and Kim *et al.*, 2009; see text bellow). There are, however, more studies acknowledging *T. platyurus* as a valuable experimental organism in the field of ecotoxicology and proving that results obtained in toxicity tests with this particular crustacean species are reliable and demonstrative in evaluating the toxicity of tested chemicals (e.g. Centeno *et al.*, 1995; Manusadzianas *et al.*, 2003; Torokne, 2004).

T. platyurus was used as an experimental organism in a research by Kim *et al.* (2009) focused on a large number of chemically unrelated pharmaceuticals (e.g. several NSAIDs, β-blockers, antihistaminic and antipsychotic drugs, and among all, selected antimicrobial agents: macrolide antibiotics – clarithromycin and erythromycin, and quinolone antibiotic levofloxacin). While clarithromycin was found to be toxic at least in high concentrations (the 24-hour medial lethal concentration was slightly above 90 mg L^{-1}), both erythromycin and levofloxacin did not exhibit any acute toxicity toward *T. platyurus* (Kim *et al.*, 2009).

In the present research, the whole range of antibiotic concentrations tested caused less than 10% mortality of experimental organisms (see Chapter 4.4.), suggesting that *T. platyurus* is not sensitive to the low concentrations of fluoroquinolones reported in the environment (see Chapter 2.2.2.1.) This finding is in accordance with the research of Isidori *et al*. (2005) who tested acute toxicity of six antibiotic agents (including ofloxacin) on *T. platyurus*. The authors reported that the $LC_{50,24h}$ concentration of ofloxacin on *T. platyurus* was slightly above 30 mg L^{-1} (Isidori *et al.*, 2005), which is a concentration 100-fold higher than the highest ofloxacin concentration used in the present research (see Chapter 3.2.1.4.)

and several orders of magnitude higher than the values of concentrations of quinolones reported in the environment (see Chapter 2.2.2.1.).

Persoone *et al.* (1994) compared the sensitivity of *T. platyurus* with the more used cladoceran crustacean species *Daphnia magna* on different types of environmental samples (e.g. effluents, river sediments, and sludge) and pure chemicals. A similar research was performed ten years later by Torokne (2004) who compared the sensitivity of *T. platyurus* with *D. magna* on selected samples contaminated with different chemicals (e.g. insecticide phosdrin, acaricide chlordimeform, and heavy metals). The authors concluded that both experimental organisms show similar sensitivity to exposed toxicants (Persoone *et al.*, 1994 and Torokne, 2004) and thus, it can be assumed that the sensitivity of the two species will be similar when exposed to antimicrobial agents.

Park and Choi (2008) included a quinolone antibiotic, enrofloxacin, in their study on the effects of commonly used antibiotics on aquatic systems. The authors showed enrofloxacin to be toxic to *D. magna* in concentrations exceeding 130 mg L^{-1} in the first 24 hours, however, the toxicity of enrofloxacin increased significantly in the next 24 hours (EC_{50} for 48 hours was 56.7 mg L^{-1}).

Similar results were provided by Kim *et al.* (2010), who in addition studied also the influence of pH values of the toxicants solutions, water temperature and UV B irradiance on the toxicity of tested antibiotics. The authors found that enrofloxacin had higher toxicity to *D. magna* at lower water pH values, but the absolute EC_{50} values at all three examined pH values were conformable to those of Park and Choi (2008) – EC_{50} for 24 hours was around 100 mg L^{-1} and EC_{50} for 48 hours was around 50 mg L^{-1} (Kim *et al.*, 2010).

These results suggest that while acute toxicity of enrofloxacin may be low (EC_{50} for the first 24 hours is several orders of magnitude higher than concentrations of quinolones reported in the environment; see Chapter 2.2.2.1.), there is the risk of chronic toxicity that may significantly influence crustacean populations in surface waters. Similar findings were reported by Yamashita *et al*. (2006) for levofloxacin. The authors observed no acute toxicity of levofloxacin on *D. magna*, although levofloxacin did exhibit chronic toxicity on this crustacean species (Yamashita *et al.*, 2006).

On the other hand, the toxicity of enrofloxacin significantly decreased when the antibiotic was exposed to UV B radiance (Kim *et al*., 2010), which confirms the accuracy of a photodegradation step in treatment of (especially hospital) wastewaters containing quinolones (Berto *et al.*, 2009; Sirtori *et al.*, 2009a, b).

Ciprofloxacin seems to be problematic regarding its toxic properties on *D. magna*: while Kim *et al.* (2010) reported the ciprofloxacin EC_{50} for 48 hours slightly above 1 mg L^{-1} (which suggests high acute toxicity), Robinson *et al.* (2005) in their study of seven quinolone antibiotics concluded that none of them (ciprofloxacin included) exhibits acute toxicity on *D. magna*. The results of present research (see Chapter 4.4.) can, however, not fully confirm the findings of either Robinson *et al.* (2005) or those of Kim *et al*. (2010) as the investigated antibiotic concentrations were lower than those used in both mentioned studies.

Conclusions

- In order to obtain relevant acute toxicity data, the concentrations of investigated fluoroquinolone antibiotics started at values several orders of magnitude higher (mg L^{-1}) than the documented values of fluoroquinolone antibiotics in the environment (ng L^{-1}).
- The acute toxicity of investigated fluoroquinolone antibiotics varied among the test species. Low acute toxicity of quinolone antibiotics was found for *P. subcapitata* and *T. platyurus*. High acute toxicity of the antimicrobials was reported for *B. calyciflorus* (with the exception of norfloxacin) and *T. pyriformis*.
- The obtained acute toxicity data of the investigated fluoroquinolones are mainly in accordance with available literal sources. There is, however, a lack of published studies dealing with the ecotoxicological screening of fluoroquinolones.
- Based on the obtained data and with regard to reported values of fluoroquinolone concentrations in the environment, all three investigated quinolones may have a negative impact on the population of *T. pyriformis* and ofloxacin may negatively influence the population of *B. calyciflorus*.

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2. http://app.esac.ua.ac.be/public/ (European Surveillance of Antimicrobial Consumption)
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