DEPARTMENT OF
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ACTIVE PHARMACEUTICAL INGREDIENTS IN AQUEOUS MATRICES: AN INTEGRATED APPROACH FOR ASSESSING EFFECTS

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ABSTRACT

The occurrence of active pharmaceutical ingredients (APIs) in the environment, due to their incomplete removal at the sewage treatment plants (STPs), has been well-documented since the mid-1990s. The possible adverse effects to unintentionally exposed organisms preoccupy, nowadays, the scientific community. Since a broad range of APIs is used and consequently released into the environment and each API is subjected to continuous biotic and abiotic transformation, APIs are present in the environment as multi-component mixtures. This dissertation is among the first research efforts to investigate the effects of multi-component mixtures of APIs to biological systems.

In the framework of this study a battery assay was developed for the investigation of acute toxicity, chronic toxicity, genotoxicity, biodegradability and estrogenicity. Ten different bioassays using organisms from the trophic levels of producers, consumers and decomposers were evaluated. Namely, the bioassays were the *Pseudokirchneriella subcapitata* and *Lepidium sativum* chronic toxicity tests, the *Daphnia magna* and *Artemia salina* acute toxicity tests, the Cytokinesis-block micronucleus cytome genotoxicity test, the *Vibrio fischeri* acute and chronic toxicity tests, the *Pseudomonas putida* chronic toxicity test, the Closed Bottle biodegradability test and the yeast estrogen screen.

Three underlining objectives were set to fulfill the purpose of this dissertation. The first was to investigate the effects of wastewater to the battery assay. Physicochemical parameters were monitored in parallel. The weak correlations between the physicochemical parameters and the results obtained from the assessment of the effects indicate that the "traditional" parameters are not sufficient to explain the toxicity observed mainly during summer periods. A hazard classification identified freshwater microorganisms to be the most affected. A clustering approach demonstrated that the quality of the wastewater of each STP is different due to significant difference between the values of the physicochemical parameters investigated.

The second objective was to assess the effects of APIs. Eight APIs were investigated namely, atenolol, metoprolol, propranolol, diclofenac, ibuprofen, erythromycin, ofloxacin and sulfamethoxazole. These APIs were selected due to their occurrence at higher concentrations in wastewater of Cyprus. The APIs were assessed as single compounds and in various combinations using selected bioassays from the battery assay. A risk characterization approach identified propranolol and ofloxacin as having significant risk for algae and bacteria, respectively. The Chou-Talalay method was applied to quantify the predominantly antagonistic effects of mixtures. No acute effects were found from the exposure to the investigated mixtures of APIs. Wastewater was found to decrease the toxicity of the APIs, suggesting that in a multi-component mixture, both stimulating and inhibiting substances, co-exist and potentially interact.

The third objective was to increase our understanding on the effects of transformation products of APIs. The photolytic and photocatalytic treatment of photolabile APIs, like ofloxacin, may result to a reduction of the concentration of the parent compound and, at the same time, to the formation of oxidation by-products, with different characteristics. These transformation products were identified through advanced chromatographic analysis. During the treatment applied, the by-products formed led to a decrease of the chronic toxicity and an increase of the genotoxicity. The biodegradation of the by-products formed was very low and improved in the presence of sodium acetate, probably due to a co-metabolic process, which is well worth investigating in future studies.

To conclude, it is hoped that this research will provide assistance to governmental departments and other organizations dealing with pharmaceuticals in the environment.

ΠΕΡΙΛΗΨΗ

Η παρουσία ενεργών φαρμακευτικών συστατικών (ΕΦΣ) στο περιβάλλον είναι αποδεδειγμένη από τα μέσα της δεκαετίας του 90 και οφείλεται στη δυσκολία απομάκρυνσης τους στους βιολογικούς σταθμούς επεξεργασίας λυμάτων. Οι δυνητικές αρνητικές επιδράσεις λόγω της συμπτωματικής έκθεσης οργανισμών σε ΕΦΣ απασχολούν σήμερα την επιστημονική κοινότητα. Η πληθώρα ΕΦΣ που χρησιμοποιούνται ταυτοχρόνως και σε συνδυασμό με τη συνεχή βιοτική ή αβιοτική μετατροπή του κάθε ΕΦΣ στο περιβάλλον, οδηγεί στην παρουσία πολυσύνθετων μειγμάτων στο περιβάλλον. Η παρούσα διατριβή είναι από τις πρώτες προσπάθειες, μεταξύ άλλων, της διερεύνησης των επιπτώσεων των πολυσύνθετων μειγμάτων ΕΦΣ σε βιολογικά συστήματα.

Στα πλαίσια αυτής της εργασίας μια σειρά βιοδοκιμών αναπτύχθηκαν για τη διερεύνηση της οξείας τοξικότητας, της χρόνιας τοξικότητας, της γονοτοξικότητας, της βιοαποικοδοσιμότητας και της οιστρογονικότητας. Εξετάστηκαν δέκα διαφορετικές βιοδοκιμές χρησιμοποιώντας οργανισμούς από τα τροφικά επίπεδα των παραγωγών, των καταναλωτών και των αποικοδομητών. Ονομαστικά εξετάστηκαν τα εξής: βιοδοκιμές χρόνιας τοξικότητας με *Pseudokirchneriella subcapitata* και *Lepidium sativum,* βιοδοκιμές οξείας τοξικότητας με *Daphnia magna* και *Artemia salina* γονοτοξική βιοδοκιμή κυτταροχαλασίνης-Β εκτίμησης μικροπυρήνων βιοδοκιμές οξείας και χρόνιας τοξικότητας με *Vibrio fischeri,* βιοδοκιμές χρόνιας τοξικότητας με *Pseudomonas putida,* βιοδοκιμή βιοαποικοδόμησης τύπου κλειστού δοχείου και βιοδοκιμή εκτίμησης οιστρογονικότητας με γενετικά τροποποιημένο ζυμομύκητα.

Η παρούσα διατριβή έχει τρεις κύριους ερευνητικούς στόχους. Ο πρώτος στόχος είναι η διερεύνηση των επιδράσεων των λυμάτων στην πιο πάνω σειρά βιοδοκιμών με την παράλληλη παρακολούθηση των φυσικοχημικών παραμέτρων τους. Οι ασθενείς συσχετίσεις μεταξύ των φυσικοχημικών παραμέτρων και των αποτελεσμάτων από την εκτίμηση των επιπτώσεων υποδηλώνουν ότι η παρακολούθηση των «παραδοσιακών» φυσικοχημικών παραμέτρων δεν είναι ικανοποιητική για να επεξηγηθεί η αυξημένη τοξικότητα που εντοπίστηκε κυρίως κατά τους καλοκαιρινούς μήνες. Μια μέθοδος ομαδοποίησης (cluster analysis) οδήγησε στο συμπέρασμα ότι η ποιότητα των λυμάτων σε κάθε σταθμό που

ελέγχθηκε είναι διαφορετική, αναγνωρίζοντας επίσης τις κυριότερες χημικές παραμέτρους για την ποιοτική τους κατηγοριοποίηση.

Ο δεύτερος στόχος ήταν ο έλεγχος των επιπτώσεων των ΕΦΣ. Τα οκτώ ΕΦΣ που διερευνήθηκαν ήταν η ατενολόλη, μετοπρολόλη, προπρανολόλη, δικλοφενάκη, ιβουπροφένη, ερυθρομυκίνη, οφλοξακίνη και σουλφαμεθοξαζόλη. Αυτά τα ΕΦΣ επιλέγησαν λόγω των αυξημένων συγκέντρωσεών τους στο περιβάλλον. Η εκτίμηση των ΕΦΣ έγινε στο κάθε συστατικό ξεχωριστά καθώς και σε μείγματα αυτών. Η μέθοδος χαρακτηρισμού των κινδύνων που εφαρμόστηκε αναγνώρισε ότι η προπρανολόλη και η οφλοξακίνη εμπεριέχουν κινδύνους για τα φύκη και τα βακτήρια που μελετήθηκαν, αντίστοιχα. Η μέθοδος Chou-Talalay χρησιμοποιήθηκε για την ποσοτικοποίηση των ανταγωνιστικών κυρίως επιδράσεων των μειγμάτων που εξετάστηκαν. Η δράση αυτή ενισχύθηκε όταν τα μείγματα ΕΦΣ βρίσκονταν σε μήτρα επεξεργασμένων λυμάτων, υποδηλώνοντας ότι σε πραγματικές μήτρες, ενισχυτικές και κατασταλτικές ουσίες συνυπάρχουν και ενδεχομένως αλληλεπιδρούν. Δεν εμφανίστηκε οξεία τοξικότητα μετά από την έκθεση στα μείγματα των ΕΦΣ.

Ο τρίτος στόχος ήταν να διερευνηθούν οι επιδράσεις παραπροϊόντων οξείδωσης των ΕΦΣ. Η φωτολυτική και φωτοκαταλυτική επεξεργασία φωτοευαίσθητων ενεργών φαρμακευτικών ουσιών, όπως η οφλοξακίνη, μπορεί να οδηγήσει στη μείωση της πρότυπης ουσίας και στην παράλληλη δημιουργία παραπροϊόντων οξείδωσης με διαφορετικά χαρακτηριστικά. Στην συγκεκριμένη περίπτωση τα παραπροϊόντα οξείδωσης που αναγνωρίστηκαν μπορούν να οδηγήσουν σε μειωμένη χρόνια τοξικότητα και αυξημένη γονοτοξικότητα. Τέλος, η δυνατότητα βιοαποικοδόμησης των παραπροϊόντων, ενώ βρέθηκε να είναι πολύ χαμηλή, μπορεί να ενισχυθεί στην παρουσία οξικού νατρίου, η οποία αποδίδεται σε ένα είδος συμμεταβολισμού, το οποίο χρήζει περαιτέρω διερεύνησης στο μέλλον.

Εν κατακλείδι, η διατριβή προτείνει μια μεθοδολογία για την εκτίμηση των επιδράσεων πολύπλοκων πολυσύνθετων μειγμάτων, η οποία θα μπορούσε να εφαρμοστεί από αρμόδια κυβερνητικά τμήματα και άλλες οργανώσεις που δραστηριοποιούνται στον τομέα του ελέγχου των ΕΦΣ στο περιβάλλον.

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"When setting out upon your way to Ithaca, wish always that your course be long, full of adventure, full of lore…" from Ithaca of Kavafis. This excerpt could precisely describe this six-year-old journey. When difficulties arose one after another, the idea of reaching my *Ithaca* would always give me the strength to carry on. Today though, being so close to my *Ithaca*, I comprehend that the joy of the journey, the adventures, the achievements of small targets and the feeling of belonging to a group were so addictive that a part of me is already missing this journey. It is a pleasure to thank those who, in one way or another, contributed and offered their valuable assistance in the preparation and completion of this thesis.

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"...ὅτι δὲ οὐδὲ γίνεται οὐδὲ φθείρεταί τι τῶν ὁμοιομερῶν, ἀλλ' ἀεὶ τὰ αὐτά ἐστι, δηλοῖ λέγων· 'τούτων δὲ οὕτω διακεκριμένων γινώσκειν χρή, ὅτι πάντα οὐδὲν ἐλάσσω ἐστὶν οὐδὲ πλείω (οὐ γὰρ ἀνυστὸν πάντων πλείω εἶναι), ἀλλὰ πάντα ἴσα ἀεί' ταῦτα μὲν οὖν περὶ τοῦ μίγματος καὶ τῶν ὁμοιομερειῶν..."

Περί Φύσεως, Αναξαγόρας ο Κλαζομένιος

"Rien ne se perd, rien ne se crée, tout se transforme..."

Traité élémentaire de chimie, Antoine Lavoisier

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LIST OF ABBREVIATIONS AND SYMBOLS

AAS	Atomic Absorbance Spectrometry
ANOVA	ANalysis Of VAriance
API	Active Pharmaceutical Ingredient
BOD	Biological Oxygen Demand
CA	Concentration Addition
CAS	Conventional Activated Sludge
CBMN	Cytokinesis-block Micronucleus Cytome Assay
CBT	Closed Bottle Test
CFU	Colony Forming Units
CI	Combination Index
COD	Chemical Oxygen Demand
COX	CycloOXygenase
cytoB	CYTOchalasin B
d	day(s)
DNA	DeoxyriboNucleic Acid
DO	Dissolved Oxygen
DOC	Dissolved Organic Carbon
D_{ow}	Octanol/water distribution
DPD	N,N-Diethyl-P-phenyleneDiamine
EC	Effective Concentration
EREs	Estrogen Receptor Elements
EROD	EthoxyresoRufin-O-Deethylase
ESI	ElectroSpray Ionization
EU	European Union
FNU	Formazine Nephelometric Units
h	hour(s)
hERα	Human Estrogen Receptor
IA	Independent mode of Action
IN	Inlet
ISO	International Organization for Standardization

K_a	Dissociation Constant
K_d	Sorption Coefficient for Sludge
LC	Lethal Concentration
LOD	Limit of Detection
LOEC	Lowest-Observed-Effect Concentration
Log D_{lipw}	Liposome-Water Distribution Coefficient
Log D_{ow}	N-octanol/Water Distribution Coefficient
Log K_{ow}	N-octanol/Water Partition Coefficient
LOQ	Limit of Quantification
MEC	Measured Environmental Concentration
MIC	Minimum Inhibitory Concentration
min	minute(s)
MN	Micronucleus
MRM	Multiple Reaction Monitoring Mode
MS/MS	Tandem Mass Spectrometry
NADPH	Nicotinamide Adenine Dinucleotide PHosphate
NBUD	Nuclear BUDs
NDI	Nuclear Division Index
NOEC	No-Observed-Effect Concentration
NPB	NucleoPlasmic Bridges
NPOC	Non-Purgeable Organic Carbon
OD	Optical Density
OECD	Organization for Economic Cooperation and Development
OUT	Tertiary chlorinated treated effluent
P	Vapor Pressure
PC	Positive Control
PDA	PhotoDiode Array Detector
PEC	Predicted Environmental Concentration
pK_a	Acid Dissociation Constant
PTP	Photo-Transformation Product
RI	Relative Intensity
ROS	Reactive Oxygen Species
SC	Solvent Control

SPE	Solid-Phase Extraction
SSWC	Seawater Complete Medium Agar
STE	Secondary Treated Effluent
STP	Sewage Treatment Plant
ThOD	Theoretical Oxygen Demand
T_m	Melting point
TOC	Total Organic Carbon
TOF	Time-Of-Flight
TP	Transformation Product
TQD	Triple Quadrupole Mass Spectrometer
TS	Total Solids
TSS	Total Suspended Solids
TU	Toxic Units
UPLC	Ultra Performance Liquid Chromatography
USEPA	US Environmental Protection Agency
WET	Whole Effluent Toxicity

CHAPTER 1. THEORETICAL BACKGROUND

1.1 A growing dependency on chemicals: Focus on Active Pharmaceutical Ingredients

Modern-day society is dependent on the products and services that chemicals help to provide; however, without care and attention they may also pose a threat to human health and the environment. It has been estimated that environmental factors are responsible of roughly 80% of all cancers and 25-33% of the global burden of disease [1]. Chemicals are amongst the most important environmental factors identified to cause adverse effects, as these are produced and consumed daily in significant amounts.

The increase of the occurrence of chemicals in the environment has led to the creation of the term 'xenobiotic'. A xenobiotic is a chemical which is found in an organism although it is not normally produced or expected to be present in it. It can also include substances which are present in much higher concentrations than usual.

Specifically, active pharmaceutical ingredients (APIs) are xenobiotics for humans, because the human body does not produce them itself nor are they part of a normal diet. They are considered xenobiotics in the environment as their release is unintended. Approximately 2650 pharmaceutical products are available for human use in Cyprus [2], a comparable number with larger European countries e.g., United Kingdom 3000 [1] and more than 4 tonnes of pharmaceutical products are yearly prescribed [3]. They are usually classified according to their biological activity (e.g., antibiotics, analgesics, anti-neoplastics, anti-inflammatory substances, anti-histamines, X-Ray contrast media, etc.).

APIs belong to an extremely interesting class of xenobiotics, because they are produced in large quantities and are designed to be biologically active, so as to interfere with specific biochemical and physiological activities, and they are characterised by a great molecular diversity. They are considered as micropollutants since they are found at μg/L or ng/L levels in the environment.

The release of APIs into the environment is an issue of increasing concern which takes place for many years now. This happens mainly through the Sewage Treatment Plants (STPs) discharges of effluents or via direct agriculture and human disposal, as shown in Figure 1.1.

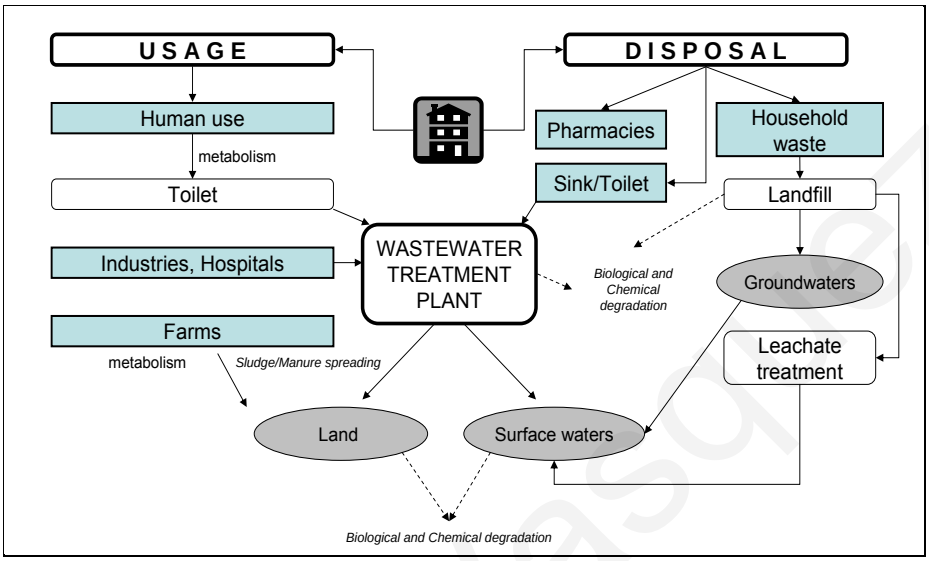


Figure 1.1 The release of active pharmaceutical ingredients and their possible routes into the environment [4]

The existence of APIs in environmental water was firstly reported in the 1970s by Tabak and Brunch [5] and Norpoth et al. [6] and in wastewater in the 1980s [7]. In fact at present, there is evidence of the occurrence of some 160 different APIs in STP effluent, surface water and groundwater [8]. APIs are a topical issue due to the extent to which they are present, not only in treated wastewater that is released in aquatic and terrestrial ecosystems, but also in drinking water. Of the 61 APIs listed in Richman and Castensson [9] as being present in environmental compartments, 15% were reported to exist in drinking water. Nineteen APIs have been recently detected in wastewater of Cyprus [10], whereas none in drinking water [11].

A raising concern with regard to APIs is not only the parent compounds but also the plethora of by-products that can be formed from ingestion and all the way until release in the environment. When APIs enter the human or animal body, they can be metabolized in hepatic cells or biotransformed by the microbes present in the alimentary tract. When excreted to the environment as parent compounds, metabolites and/or transformation products, they are subjected to further biological and/or non-biological transformation and degradation (Figure

1.2). From an environmental perspective, each API should be regarded as a multi-component chemical mixture due to its possible transformation through biotic and abiotic processes.

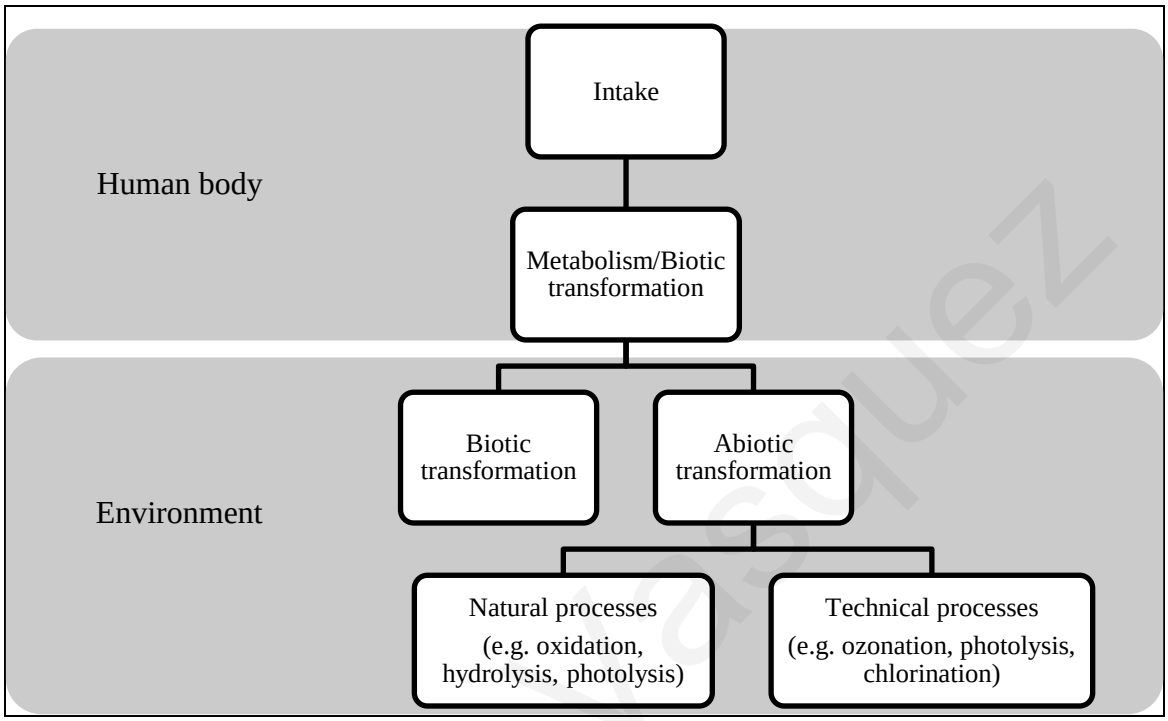


Figure 1.2 Physical and biological transformation of active pharmaceutical ingredients in the human body and the environment

The scientific concern regarding the presence of APIs in the environment has not yet been translated into legislative tools *i.e.*, limits, and there is still no specific legislation with an environmental approach regarding APIs. However, an environmental risk of pharmaceutical products planned for marketing within the European Union (EU) requires evaluation since 1993 according to EEC/2309/93 [12]. Moreover, a tool to control diffuse pollution from APIs to the aquatic environment is the Guideline on the environmental risk assessment of APIs for human use [13] that has been published by the European Medicines Agency in 2006. In September 2009, the U.S. Environmental Protection Agency (USEPA) published the final Contaminant Candidate List-3, which is a drinking water priority contaminant list for regulatory decision making and information collection, in which a typical API (erythromycin), an explosive which is also used as an API (nitroglycerin), and eight hormones (17α-ethynilestradiol, 17β-estradiol, equilenin, equilin, estriol, estrone, mestranol and norethindrone) were included [14]. In Europe, this issue may be addressed through the main environmental legislation, such as the Water Framework Directive (2000/60/EC), which is the

most important legislation regarding Europe's water policy and has given a central role to the ecological risk-based decision-making. In fact, the revision of the list of priority pollutants proposed the inclusion of an APIs (diclofenac) and two hormones (17α-ethynilestradiol, 17β-estradiol). A summary of the relevant chemical and environmental legislation applied nowadays in EU focusing on APIs is presented in Annex I.

1.2 Physicochemical characteristics of Active Pharmaceutical Ingredients

1.2.1 Introduction

Prior to any ecotoxicity testing, it is extremely important to collect all available information regarding the toxic substance and/or the matrix under study, and its route to the ecosystem. A number of aspects taken into account such as molecular structure, dissociation constant, solid state, solubility, etc. are presented below. This may help estimate whether or not an effect could be expected and select the most suitable bioassays to be performed.

Molecular structure

One key aspect of APIs that differentiates them from other xenobiotic chemical classes is that no homogeneity in molecular weights, chemical structures and functionalities is present [15]. Nowadays, the categorization of APIs is done by their action (e.g., anti-inflammatory APIs, antibiotics, etc.), their chemical structure (e.g., fluoroquinolones, sulfonamides, etc.) or biochemical target (e.g., β-blockers, serotonin reuptake inhibitors, etc.). Different classes of APIs were addressed by this thesis: Non-steroidal anti-inflammatory APIs, β-blockers and antibiotics belonging to the fluoroquinolones, sulfonamides and macrolides. The molecular weights ranged between 250-300 g/mol, with the exception of ibuprofen and erythromycin that have molecular weights of 206 and 734 g/mol, respectively.

Solid state chemistry and solubility

Many APIs exist as solid forms, often as salts with a tendency to form polymorphs which arise when molecules stack differently in the solid state. Pseudopolymorphs may form after hydration or solvation.

The solubility of an API in a specific solvent is its concentration in a saturated solution at equilibrium with the solid [15]. In the present thesis, the solubility of the APIs in water or in organic solvents, where applicable, was taken into account when performing the toxicity assaying since some of the APIs were relatively non-soluble e.g., propranolol, ibuprofen and erythromycin. The simplest salt form and the most thermodynamically stable polymorph were used in the present thesis.

The effects on solubility, dissolution rate, chemical and physical stability, melting point, colour, filterability, density and flow properties affect bioavailability; hence the toxicity of the substance [15].

Dissociation constant and zwitter ionization

The degree of ionization of an API at a specific pH is described by the dissociation constant (K_a). The negative logarithm of K_a, pK_a is the acid dissociation constant. The larger the value of pK_a, e.g., atenolol, metoprolol, propranolol, the smaller the extent of dissociation. Ionized molecules, e.g., diclofenac, usually have greater solubility and are less likely to partition to lipid-like substances. Ionization of an API molecule permits ion exchange in sludge and soil systems [15]. Some API molecules, also known as zwitterions, e.g., ofloxacin, have the ability to be charged both positively and negatively, even though the molecule as a whole is neutral [16,17]. This characteristic affects the sorption potential of APIs to soils [17].

Octanol-water distribution coefficient, liposome-water distribution coefficient and sludge-water distribution coefficient

The octanol-water distribution (D_{ow}) is defined as the ratio of the concentration of a diluted API (sum of the concentrations of both ionized and non-ionized) in two phases in equilibrium; n-

octanol -a substitute for lipids- and water. The n-octanol/water partition coefficient (K_{ow}) is the ratio of the concentration of the non-ionized molecules only, in two phases in equilibrium; n-octanol and water. In the case of non-ionizable APIs, the log D_{ow} equals with the log K_{ow}. To measure the log K_{ow} of ionizable APIs, the pH of the API solution should be adjusted such that the predominant form of the API is non-ionized.

A log D_{ow} <1 indicates that an API is unlikely to significantly bioconcentrate or sorb onto organic matter, e.g., atenolol, metoprolol, ofloxacin, sulfamethoxazole. A log D_{ow} ≥3 indicates a significant bioconcentration and sorption potential [15]. None of the APIs studied in the context of this thesis have a significant bioconcentration and sorption potential.

In the case of ionizable APIs, the non-ionized/ionized ratio in a matrix influences their absorption and disposition profiles [18]. Additional uptake mechanisms are possible for APIs, but the non-ionized form is more nonpolar and thus considered to passively cross membranes more readily than the ionized form of an API [19]. For APIs that can ionize, distribution into lipid is a function of the pH and the pK_a. However, the liposome-water distribution coefficient (log D_{lipw}) may be more useful than the log D_{ow} for predicting accumulation of ionizable APIs [20]. The log D_{lipw} is usually positively linearly correlated with the bioconcentration potential. Diclofenac and propranolol have the highest log D_{lipw} among the APIs studied in the framework of this thesis.

The sorption coefficient for sludge (K_d) is defined as the ratio of the API concentration associated with the solid to the API concentration in the surrounding aqueous solution when the system is at equilibrium. A $K_d \leq 0.5$ L/g suggests that physical removal by adsorption plays a limited role in the elimination of an API in wastewater treatment plants, e.g., atenolol, metoprolol, propranolol, erythromycin, etc. [21].

Exposure, bioavailability and lipophilicity

In order to be absorbed, a pollutant should get in contact with an organism. Exposure can be achieved through skin, respiratory and alimentary tracks. Regarding APIs in the environment, the alimentary tracks seem to be the most important way of exposure.

The bioavailability of an API, which is the concentration that directly influences an organism, as well as absorption, is closely connected to the lipophilicity. It is a crucial

parameter in toxicology, as it defines whether the API can actually be absorbed by an organism. Most of the toxic substances are lipid-soluble and persistent to metabolism or breakdown. The fact that an API is found in high concentrations does not necessarily mean that it has an impact on the environmental organisms. The bioavailability is affected by environmental conditions, such as the chemical and physical conditions of water, the geochemistry of the sediments, etc. Additionally it may have seasonal and spatial variation. APIs are usually designed to have high bioavailability, in order to have a low dosage and reduce undesired side effects. The bioavailability and lipophilicity of APIs for ecotoxicity testing is usually related to the K_{ow} or to the bioavailability calculated for humans. The bioavailability and lipophilicity usually increases as the K_{ow} increases. At very high K_{ow} though, the uptake might be hindered by the molecule size or the cell membrane. A K_{ow} around one indicates capacity for absorption, e.g., atenolol, metoprolol and sulfamethoxazole [22].

Metabolism

One of the main purposes of metabolism is to make toxic substances easier to excrete and not to bioaccumulate. This is achieved by creating more hydrophilic substances. As described by Lindman [23] the metabolism of toxic substances is commonly divided into phases. Phase I consists of various fundamental chemical reactions, such as oxidation, reduction, hydrolysis, etc., that end up in transforming the toxic substance into a slightly more hydrophilic molecule. Highly reactive intermediates and free radicals are usually formed by mainly a class of hepatic enzymes of the cytochrome P450 family. Other enzymes that participate in Phase I include esterases, reductases, etc. Phase II involves conjugation with highly water-soluble ligands, such as glucuronic and sulphuric acid, for excretion in urine and bile. This phase usually detoxifies the organisms. However in some cases, sulphuric acid conjugates may create mutagenic metabolites. These reactions are mainly catalyzed by transferases by adding an endogenic polar moiety. Metabolites are usually more polar, water-soluble and with reduced pharmacological activity than parent compounds [15]. APIs may undergo metabolism [24] or can leave the human body unchanged [25]. The metabolites may be a glucuronide or sulphate conjugate, a “major” metabolite or a complex mixture of many metabolites. There is evidence that conjugates of glucuronic and sulphuric acid are capable of re-transforming to the parent compound during sewage treatment [26]. Atenolol, ibuprofen and ofloxacin are examples of

APIs that are almost not metabolized in the human body, leaving the body in the form of the parent compound.

Biotransformation

Microbial metabolism is usually referred to as biotransformation or biodegradation. Biodegradability studies simulating sewage treatment plants are useful for understanding in which form the substances enter into the environment. Microbial metabolism can occur aerobically or anaerobically. Aerobic biotransformation of APIs may take place in the environment and in most sewage treatment plants' bioreactors in which oxygen is present [27-29]. Microbes can biodegrade organic compounds such as APIs, directly or indirectly. Direct metabolism is present when the carbon is oxidised and used as energy source, whereas indirect metabolism (co-metabolism) takes place when the oxidation does not produce any energy to the microbes and is enhanced by other compounds which are oxidised and used as energy source [30]. In a limited extent, biodegradation may take place in the sewer systems, whereas, it mainly occurs at the secondary stage of sewage treatment plants, where the density of microbes is higher. Atenolol, diclofenac and sulfamethoxazole are examples of APIs that can be biotransformed in various environmental compartments.

Bioaccumulation, biomagnification and mixture effects

If a toxic substance is effectively absorbed and slowly metabolized, it has the capability to accumulate in the biological organisms. Sometimes, after absorption, the substance can be accumulated in a specific tissue or protein molecule. This means that the substance concentration in the organism will increase during time. The hydrophobicity of the substance or its metabolized by-products will determine whether excretion will take place or not and if yes, to what extent. Some studies have been performed explaining how APIs can bioaccumulate in living organisms [31]. Even though some bioaccumulation has been reported in the literature for APIs, they are not considered as bioaccumulative based on the EU regulations [32].

If a toxic substance enters a lower trophic level and has the capability to bioaccumulate, then as the trophic level increases, there is also an increase of the concentration of the toxic

substance related to the body weight of organisms, in higher trophic levels. Fortunately, the lack of lipid solubility of most drugs and metabolites generally prevents biomagnification [33]. However, the study of biomagnification of APIs is still at a very early stage. One example of biomagnification has been reported for carbamazepine [34].

The exposure situation in reality consists of a panorama of different substances that interact with each other and also with the environment and organisms in various complex ways. Effects of synergism and antagonism are just some of the noticed effects of complex mixtures [35,36].

Following are presented the chemical groups and the APIs examined in the present thesis in a concise manner. A detailed presentation of the occurrence of the investigated APIs is given in Annex II.

1.2.2 β-blockers

β-adrenergic receptors are crucial for the normal functioning of the sympathetic branch of the vertebrate autonomous nervous system. In mammals, β-blockers compete antagonistically with the catecholamines such as, noradrenaline and adrenaline, at the β-adrenergic receptors, ultimately reducing the rate and force of contraction [37]. β-blockers are widely used for treating cardiovascular diseases such as, high blood pressure, ischemic heart disease and arrhythmia. Their metabolism varies and are amongst the most frequent APIs found in treated wastewaters, and consequently in the environment. β-adrenergic receptors have been found in a plethora of organisms, indicating that their presence in the environment may pose risks. Atenolol, metoprolol and propranolol were selected to be studied for this thesis.

1.2.2.1 Atenolol

Atenolol or 2-[4-[2-hydroxy-3-(propan-2-ylamino)propoxy]phenyl]acetamide (CAS No. 29122-68-7, C14H22N2O3, Figure 1.3) is a mixture of two enantiomers R- and S-atenolol. S-atenolol is the pharmacologically active substance. Its physicochemical characteristics are

summarized in Table 1.1. It has low lipophilicity, is readily soluble in water and has a low sorption affinity.

Atenolol is a selective β_1-blocker, poorly metabolized in the human body. Approximately 50% of the administered dose is excreted in the urine, of which 90% remains unchanged, and a small percentage of atenolol-glucuronide (0.8–4.4%) and hydroxyatenolol (1.1–4.4%, hydroxylation of the benzilic position) are produced [25].

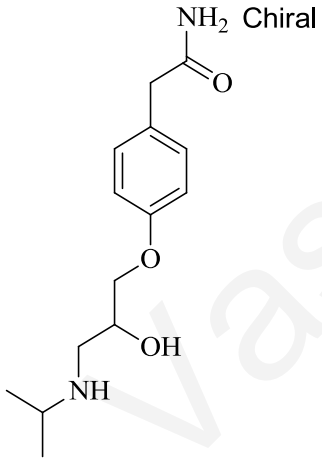


Figure 1.3 Chemical structure of atenolol

Table 1.1 Physicochemical characteristics of atenolol

Molecular mass	266.34 g/mol	[38-40]
Acid dissociation constant, pK_a	9.6	[38-40]
Charge at pH 7	Positive	[41]
Melting point, T_m	147 ºC	[38-40]
Vapor pressure, P	1.025×10^{-7} Pa	[42]
Henry's law constant	1.378×10^{-18} atm m^3/mol	[42]
Water solubility	13.3 g/L	[38-40]
Octanol-water partition coefficient, log K_{ow}	0.16, 0.50	[38-40]
Octanol-water distribution coefficient, log D_{ow}	-2.14	[41]
Liposome-water distribution ratio at pH 7, log D_{lipw}	0.51	[43]
Sorption coefficient for sludge, K_d	0.04 L/g_{COD}	[44]

As shown in Annex II, the range of concentrations of atenolol in the influent of sewage treatment plants (STPs) has been found to vary between 160-11239 ng/L, and in the effluent 30-15000 ng/L. In surface and ground water the concentrations of atenolol reported ranged between 3-1237 ng/L and 4-664 ng/L, respectively. Atenolol is not so persistent in sediments and has been found at the range of 0.49-36.4 ng/g.

Atenolol has been found to be biotransformed via microbiologically initiated hydrolysis of the amide to carboxylic moiety to atenololic acid. Atenolol is transformed to a stable product and not mineralized [45].

1.2.2.2 Metoprolol

Metoprolol or 1-[4-(2-methoxyethyl)phenoxy]-3-(propan-2-ylamino)propan-2-ol, as atenolol (CAS No. 37350-58-6, $C_{15}H_{25}NO_3$, Figure 1.4) is a β_1-selective blocker. It is commercially available as tartrate and used as a racemic mixture in dosage forms (R-metoprolol/S-metoprolol: 50/50).

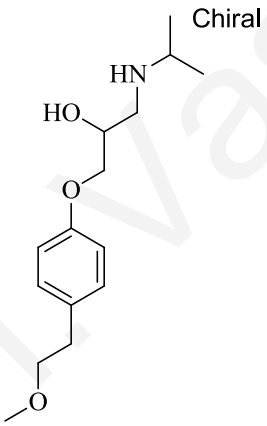


Figure 1.4 Chemical structure of metoprolol

Metoprolol undergoes oxidative metabolism in the liver principally in the CYP2D6 isoenzyme [46]. Metoprolol is metabolized via O-demethylation (65%), α-hydroxylation (10%) and N-alkylation (10%). Metabolites of metoprolol were found to be less toxic to mice than the parent compound [47].

About 10-15% of the applied metoprolol dose is excreted unchanged, and hence, it has been found present in influents of STPs at concentrations of 2-4900 ng/L [50], and in effluents at the range of 3-1700 ng/L. Metoprolol has been quantified in surface water in the range of 0.8 ng/L-738 ng/L, in soils at 0.32 ng g^{-1}, and in sediments in the range of 0.59-6.57 ng g^{-1}, which are provided in detail in Annex II. Table 1.2 shows its low sorption affinity.

Table 1.2 Physicochemical characteristics of metoprolol

Molecular mass	267.37 g/mol	[38-40]
Acid dissociation constant, pK_a	9.68	[38-40]
Charge at pH 7	Positive	[48]
Melting point, T_m	152.19 ºC	[38-40]
Vapor pressure, P	0.000305 Pa	[42]
Henry's law constant	1.40 x 10^{-13} atm m^3/mol	[42]
Water solubility	16.9 g/L	[38-40]
Octanol-water partition coefficient, log K_{ow}	1.88	[38-40]
Octanol-water distribution coefficient, log D_{ow}	-0.24	[49]
Liposome-water distribution ratio at pH 7, log D_{lipw}	1.43	[43]
Sorption coefficient for sludge, K_d	0.001 L/g_{cod}	[44]

1.2.2.3 Propranolol

Propranolol or 1-naphthalen-1-yloxy-3-(propan-2-ylamino)propan-2-ol (CAS No 525-66-6, $C_{16}H_{21}NO_2$, Figure 1.5, Table 1.3) is a non-specific β_1 and β_2-adrenergic antagonist and an effective serotonin (5-hydroxythriptamine; 5-HT) receptor antagonist, widely used cardiovascular API.

It has two enantiomers and it has been shown that the (S) enantiomer was more potent in humans than the corresponding antipodes. Propranolol is extensively metabolized in humans following three major oxidative pathways: side-chain glucuronidation (10-25%); naphthalene-ring hydroxylation (27-59%); and side chain N-desisopropylation (32-50%) [51,52].

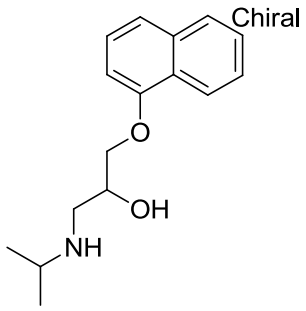


Figure 1.5 Chemical structure of propranolol

In humans, cytochrome P4501A2 (CYP1A2) catalyzes side chain N-desisopropylation; whereas 4- and 5-hydroxylation of propranolol is mainly catalyzed by CYP2D6 [51]. It may

be both a photosensitizing agent and light-unstable, similarly to other drugs that have chromophoric structures containing a naphthalene skeleton [53].

As presented in Annex II, propranolol has been identified in the influent of STPs at concentrations ranging from 10-1390 ng/L, and in the effluents at 10-510 ng/L. In the water bodies propranolol has been quantified in surface water between 0.1 and 178 ng/L, in ground water between 1.8 to 45 ng/L and in marine water between 93-2663 ng/L. Propranolol has been found in sediments at concentrations of 1.51 to 2.60 ng/g.

Table 1.3 Physicochemical characteristics of propranolol

Molecular mass	259.80 g/mol	[38-40]
Acid dissociation constant, pK_a	9.42	[38-40]
Charge at pH 7	Positive	[54]
Melting point, T_m	163-164ºC	[38-40]
Vapor pressure, P	6.13×10^{-5} Pa	[42]
Henry's law constant	7.98×10^{-13} atm m^3/mol	[42]
Water solubility	0.07 g/L	[38-40]
Octanol-water partition coefficient, log K_{ow}	3.48	[38-40]
Octanol-water distribution coefficient, log D_{ow}	1.34	[54]
Liposome-water distribution ratio at pH 7, log D_{lipw}	3.06	[43]
Sorption coefficient for sludge, K_d	0.32 L/g_{cod}	[44]

Sorption has been found to be the most significant removal mechanism for propranolol with a K_d of 207-320 L/kg [44,55,56]. Propranolol has been proved to photodegrade rapidly in river water with a half-life rate of 1.1 h and 4.4 h in air-saturated purified water [57,58]; whereas it may need up to 16 h during winter period [59]. No biodegradation was observed for propranolol [60].

1.2.3 Non-steroidal anti-inflammatory active pharmaceutical ingredients

Non-steroidal anti-inflammatory APIs act through modulation of eicosanoid metabolism. Eicosanoids are cyclooxygenase, lipoxygenase and cytochrome P450 products of C_{20} polyunsaturated fatty acids and include the subclass of prostanoids (prostaglandins, prostacyclines and thromboxanes) which play important physiological roles in practically every cell [61]. Non-steroidal anti-inflammatory APIs cause the inhibition of the enzymatic COX conversion of arachidonic acid to prostanoids. They are used worldwide for pain, fever,

osteo and rheumatic disorders. Non-steroidal anti-inflammatory APIs are known to cause nephrotoxicity due to the non-selective inhibition of COX [62]. Diclofenac and ibuprofen were examined to be studied in this thesis.

1.2.3.1 Diclofenac

Diclofenac or 2-(2,6-dichlorophenylamino)phenyl acetic acid (CAS No 15307-86-5, $C_{14}H_{11}Cl_2NO_2$, Figure 1.6) main physicochemical characteristics are summarized in Table 1.4. After intake, diclofenac is mainly metabolized in humans to its hydroxylated or methoxylated derivatives (3-hydroxydiclofenac (2%), 4-hydroxydiclofenac (16%), 5-hydroxydiclofenac (6%), 3-hydroxy-4-methoxydiclofenac, lactam-dehydrate of 4′-hydroxy diclofenac) and further conjugated, mostly to glucuronides (diclofenac acyl glucuronide) [63-65].

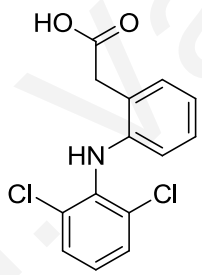


Figure 1.6 Chemical structure of diclofenac

Approximately 65% of the oral dosage of diclofenac is excreted via the urine [66]. Metabolism in fish bile has been recently studied and the main metabolites found were acyl glucuronides of hydroxylated diclofenac and an ether glucuronide of hydroxylated diclofenac [67].

Diclofenac, as presented in detail in Annex II, has been measured at concentrations from 0.4 up to 12370000 ng/L in influents and from 0.4 to 85000 ng/L in effluents. Regarding surface water, diclofenac concentrations ranged between 1.1-2200 ng/L. In groundwater diclofenac ranges between 9.7 and 590 ng/L. In sediments diclofenac ranged between 0.0085-144 ng/g, and in soils between 0.009 to 1.16 ng/g. Diclofenac was found to be readily biodegradable in agricultural soils [69], in river sediments [70] and in a membrane bioreactor

[71]. Another study though, demonstrated that diclofenac was not readily biodegradable, even in the presence of a carbon source [28].

Table 1.4 Physicochemical characteristics of diclofenac

Molecular mass	296.16 g/mol	[38-40]
Acid dissociation constant, pK_a	4.14	[38-40]
Charge at pH 7	Negative	[41]
Melting point, T_m	174.60 ºC	[38-40]
Vapor pressure, P	0.000291 Pa	[42]
Henry's law constant	4.73×10^{-12} atm m^3/mol	[42]
Water solubility	50 g/L	[38-40]
Octanol-water partition coefficient, log K_{ow}	4.51	[38-40]
Octanol-water distribution coefficient, log D_{ow}	1.37	[41]
Liposome-water distribution ratio at pH 7, log D_{lipw}	2.65	[43]
Sorption coefficient for sludge, K_d	65.5 L/kg	[68]

Photo-transformation has been identified as the main elimination process of diclofenac in water bodies [59,72]. However, photo-transformation (obtained after irradiation of a 50 mg/L diclofenac solution with natural sunlight) may be coupled by the formation of more toxic products as reported in previous studies using the green algae *Scenedesmus vacuolatus* [73,74].

1.2.3.2 Ibuprofen

Ibuprofen or 2-[4-(2-methylpropyl)phenyl]propanoic acid (CAS No. 15687-27-1, $C_{13}H_{18}O_2$, Figure 1.7) is a racemic mixture of R- and S-ibuprofen. The S-ibuprofen has been proved to be 160 times more potent than the R-antipode [75]. Its physicochemical characteristics are summarized in Table 1.5.

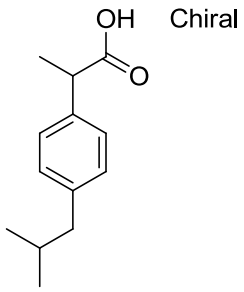


Figure 1.7 Chemical structure of ibuprofen

Ibuprofen competes with the arachinodic acid as a substrate for COX, acting as a reversible non-selective competitive inhibitor. A significant degree of ibuprofen (70−80% of the therapeutic dose) is excreted as the parent compound (free or conjugated) or in the form of metabolites [76,77].

Its physicochemical properties suggest a rather high mobility in the aquatic environment (Annex II). For instance, ibuprofen has been reported in influents up to 115 μg/L and in effluents up to 85 μg/L. In surface water, ibuprofen concentrations have been found to range between 1.2-14671 ng/L, in groundwater up to 3110 ng/L and in marine water up to 300 ng/L. In sediments it has been reported to range from 0.021 to 28 ng/g and in soils from 0.098 to 5.03 ng/g.

Table 1.5 Physicochemical characteristics of ibuprofen

Molecular mass	206.23 g/mol	[38-40]
Acid dissociation constant, pK_a	4.91	[38-40]
Charge at pH 7	Negative	[41]
Melting point, T_m	75-77 ºC	[38-40]
Vapor pressure, P	0.0756 Pa	[42]
Henry's law constant	1.52×10^{-7} atm m^3/mol	[42]
Water solubility	0.049 g/L	[38-40]
Octanol-water partition coefficient, log K_{ow}	3.97	[38-40]
Octanol-water distribution coefficient, log D_{ow}	1.71	[41]
Liposome-water distribution ratio at pH 7, log D_{lipw}	1.91	[43]
Sorption coefficient for sludge, K_d	37.8 L/kg	[68]

An oxidative transformation to corresponding hydroxy- and carboxy-metabolites of ibuprofen is considered to contribute to its high removal rate in STPs [78]. However, ibuprofen has been shown not to be easily phototransformed [57].

Ibuprofen has been shown to bioconcentrate in *Oncorhynchus mykiss* plasma under some conditions of treated wastewaters [79]; whereas when the bluntnose minnow *Pimephales notatus* was exposed to ibuprofen at concentrations up to 100 μg/L the potential for bioconcentration was low (BCF = 1.3), since the measured tissue concentrations were low too [80]. A study with fathead minnow *Pimephales promelas* and channel catfish *Ictalurus punctatus* also indicated that ibuprofen at 250 μg/L had a low bioconcentration capacity after a 14 d and 7 d exposure time, respectively [81].

1.2.4 Antibiotics

Antibiotics are considered natural or synthetic APIs that pose selective toxic action on bacteria and other single-celled microorganism [82]. They can be categorized according to the group of organisms to which they are active e.g., anti-bacterials or due to their chemical structure e.g., penicillins, tetracyclines, macrolides and sulfonamides. However, it should be mentioned that in some studies antibiotics are considered only the biologically active compounds that are of natural or semi-natural origin (e.g., β-lactams, tetracyclines); whereas the compounds from synthetic sources are categorized as antimicrobials (e.g., sulfonamides, quinolones) or antifungals (e.g., azoles) [83]. The antibiotics examined in the present thesis belong to the group of macrolides (erythromycin), fluoroquinolones (ofloxacin) and sulfonamides (sulfamethoxazole).

1.2.4.1 Erythromycin

Erythromycin or (3R,4S,5S,6R,7R,9R,11R,12R,13S,14R)-6-{[(2S,3R,4S,6R)-4-(dimethylamino)-3-hydroxy-6-methyloxan-2-yl]oxy}-14-ethyl-7,12,13-trihydroxy-4-{[(2R,4R,5S,6S)-5-hydroxy-4-methoxy-4,6-dimethyloxan-2-yl]oxy}-3,5,7,9,11,13-hexamethy/L-oxacyclotetradecane-2,10-dione (CAS No. 114-07-8, $C_{37}H_{67}NO_{13}$, Figure 1.8, Table 1.6) is a 14-member lactone macrolide to which two deoxy sugars are attached (L-cladinose and D-desosamine).

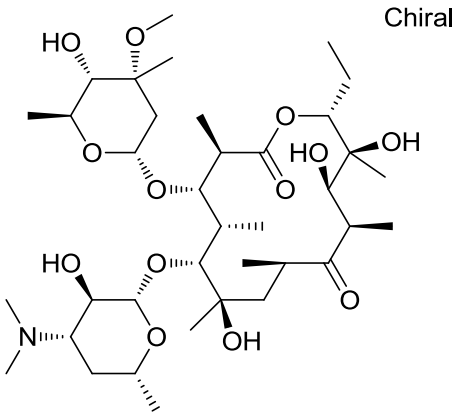


Figure 1.8 Chemical structure of erythromycin

Macrolides are usually bacteriostatic impeding the multiplication of bacteria rather than their death. They are used against respiratory tract infections e.g., pneumonia, chlamydia, diphtheria and tetanus [84]. They are used as an alternative in patients allergic to penicillin [85]. Erythromycin is produced by the actinomycete *Saccharopolyspora erythraea* and acts primarily via protein synthesis inhibition and in particular it inhibits the translocation of peptidyl-tRNA by binding to the 23S rRNA molecule in the 50S ribosomal subunit [86].

A 12-15% of the parent compound is reported to be excreted unchanged [84]. The most common transformation product of erythromycin in the environment is anhydro-erythromycin, which is less biologically active than the parent compound [83].

Table 1.6 Physicochemical characteristics of erythromycin

Molecular mass	733.93 g/mol	[38-40]
Acid dissociation constant, pK_a	8.8	[38-40]
Charge at pH 7	Positive	[87]
Melting point, T_m	191 ºC	[38-40]
Vapor pressure, P	2.83×10^{-23} Pa	[88]
Henry's law constant	n.d.	-
Water solubility	1.44 g/L	[38-40]
Octanol-water partition coefficient, log K_{ow}	3.06	[38-40]
Octanol-water distribution coefficient, log D_{ow}	1.14	[49]
Liposome-water distribution ratio at pH 7, log D_{lipw}	2.33	[89]
Sorption coefficient for sludge, K_d	0.165 L g_{SS}^{-1}	[86]

As shown in Annex II, it has been found in influents at concentrations up to 2700 ng/L and up to 6000 ng/L in effluents [90]. In surface water has been found to range between 1.4-1000 ng/L [91]. It has been shown to be quite stable during wastewater treatment, and in some cases higher concentrations were found in the treated effluents than in the inlet [92]. It has been recently detected in marine environments at concentrations close to the ones detected in surface water [93].

1.2.4.2 Ofloxacin

Ofloxacin or (RS)-7-fluoro-2-methyl-6-(4-methylpiperazin-1-yl)-10-oxo-4-oxa-1-azatricyclo[7.3.1.05,13]trideca-5(13),6,8,11-tetraene-11-carboxylic acid (CAS No 82419-36-1, $C_{18}H_{20}FN_3O_4$, Table 1.7) belongs to the fluoroquinolones therapeutic group which is a

broad spectrum synthetic antimicrobial. It has fluorine at C-6 and a carboxylic moiety at C-3 and is presented in Figure 1.9. Ofloxacin presents amphoteric characteristics [94] and is a racemic mixture of two optically active isomers with an asymmetric center at C-3 of the oxazine ring with l-ofloxacin (levofloxacin) being the more active of the two isomers [95].

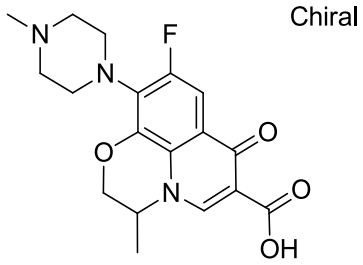


Figure 1.9 Chemical structure of ofloxacin

It is slightly metabolized with 70% of the parent compound leaving the human body unchanged [96]. Ofloxacin has been detected in sewage treatment plants influents and effluents, pharmaceuticals' production effluents, hospital effluents, groundwater, river and sea water, and drinking water [93,97].

As presented in Annex II, the concentrations in influents were found to be in the range of 115-31700 ng/L, in effluents 45 and 15000 ng/L, in surface water 33.1-306.1 ng/L [84], and in marine water 8.1-634 ng/L. Ofloxacin has been reported in sediments at concentrations ranging between 8.95-12.03 ng/g. It is amongst the most persistent fluoroquinolones with limited microbial biodegradation potential [98].

Table 1.7 Physicochemical characteristics of ofloxacin

Molecular mass	361.37 g/mol	[38-40]
Acid dissociation constant, pK_a	7.6	[38-40]
Charge at pH 7	Neutral	
Melting point, T_m	250-257ºC	[38-40]
Vapor pressure, P	4.8×10^{-8} Pa	[42]
Henry's law constant	1.16×10^{-21} atm m^3/mol	[42]
Water solubility	28.3 g/L	[38-40]
Octanol-water partition coefficient, log K_{ow}	-0.39	[38-40]
Octanol-water distribution coefficient, log D_{ow}	-0.34	[49]
Liposome-water distribution ratio at pH 7, log D_{lipw}	n.d.	
Sorption coefficient for sludge, K_d	n.d.	

Ofloxacin is photolabile and is subjected to direct and/or indirect photo-transformation [99,100]. In particular, ofloxacin is known to be sorbed onto soils and persist in sludge-treated soils in concentrations as high as few mg/kg [101] and has recently been characterized as a high hazard in the aquatic system due to the reuse of these sludge-treated soils on agricultural land [102].

1.2.4.3 Sulfamethoxazole

Sulfamethoxazole or 4-amino-N-(5-methylisoxazol-3-yl)-benzenesulfonamide (CAS 723-46-6, $C_{10}H_{11}N_3O_3S$, Figure 1.10, Table 1.8) is a bacteriostatic sulfonamide. Sulfonamides were the first clinically effective antimicrobials, used for preventing and curing bacterial infections in humans [103]. They have a broad range of activity and are used mainly against urinary tract infections. In many cases they are used in combination with trimethoprim for the treatment of otitis, bronchitis, sinusitis and pneumonia [104]. They are derivatives of para-aminobenzenesulfonamide (sulfanilamide) and have hydrophilic and amphoteric characteristics.

The active site for antibacterial action is the sulfur-benzene ring bond. A metabolic pathway, in which sulfonamides act as structural analogues of p-aminobenzoic acid inhibiting dihydropteroate synthase, for the production of dihydrofolic acid, needed for DNA synthesis, is targeted by sulfonamides [97,105,106].

It leaves the body unchanged only by 10-30% [107,108]. N^4-acetylsulfamethoxazole is one of its metabolites, which is inactive. However, cleavage of the N-acetyl group and retransformation to the API should not be overlooked, as it has been documented in previous studies [109]. Sulfamethoxazole-N_1-glucuronide is another main metabolite which is excreted by 9-15% [110].

As presented in Annex II, sulfamethoxazole has been found in influents at concentrations up to 3000 ng/L, in effluents at concentrations between 190-9460 ng/L [93,111], in river water at concentrations up to 1900 ng/L [50], in groundwater at concentrations up to 1110 ng/L, and in sea water at concentrations up to 47 ng/L [93].

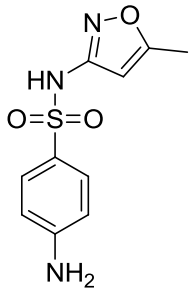


Figure 1.10 Chemical structure of sulfamethoxazole

Table 1.8 Physicochemical characteristics of sulfamethoxazole

Molecular mass	253.28 g/mol	[38-40]
Acid dissociation constant, pK_a	6.0	[38-40]
Charge at pH 7	Neutral-Negative	[41]
Melting point, T_m	167 ºC	[38-40]
Vapor pressure, P	0.000505 Pa	[42]
Henry's law constant	9.56×10^{-13} atm m^3/mol	[42]
Water solubility	610 g/L	[38-40]
Octanol-water partition coefficient, log K_{ow}	0.89	[38-40]
Octanol-water distribution coefficient, log D_{ow}	0.14	[41]
Liposome-water distribution ratio at pH 7, log D_{lipw}	1.32	[89]
Sorption coefficient for sludge, K_d	22.9 L/kg	[68]

Sulfamethoxazole is water soluble, with a half-life of 19 d under sunlight [105], hydrophilic and polar, able to be transferred to long distances and even deep aquifers. Sulfamethoxazole is usually negatively charged at pH close to 7, a fact that increases its transport velocity in porous media due to anion exclusion [112]. Sulfamethoxazole is subjected to direct photolysis [113].

Ambiguous information regarding the biodegradation potential of sulfamethoxazole is reported. For instance, it was found to be quite persistent when studied in an aquatic outdoor microcosm study [100,114]. In another study however, sulfamethoxazole was found to biodegrade at high concentrations of 383 mg/L during an activated sludge process using a sequencing batch reactor and its degradation could be performed more rapidly when acetate was used [115]. Sulfamethoxazole was also removed in a recent study during a 64-day exposure at concentrations of 20 μg/L by the river sediment [116].

1.3 Important aspects of environmental toxicology

The history of environmental toxicology is a quite short one since it was not until the mid-1900s that environmental effects of chemicals became a concern [117,118], mainly regarding effects of industrial wastes. Standardization and international acceptance of protocols for ecotoxicity testing has improved the quality of the data since the mid-1950s.

Organizations, such as the International Organization for Standardization (ISO) and the Organization for Economic Cooperation and Development (OECD) have contributed to this direction making ecotoxicity testing nowadays a very important part of environmental legislation.

A reason for this is that toxicity to living organisms is more comprehensible than the concentration of a chemical or an effluent for non-specialists and often is less expensive than chemical analysis. It can be used in a "weight of evidence" approach and as a complementary tool of analytical measurements.

Environmental safe levels take account of the toxicity of the substance, its persistence and ability to bioaccumulate and it can include mutagenicity, carcinogenicity and reproductive impairment [119].

In principle, environmental toxicity testing can be carried out at any biological level of organization. The endpoints to be studied in each organization level can be selected based on the objectives of the study. As a rule though, the majority of pollutants act initially at the molecular level following accumulation into the exposed organism, with effects then becoming apparent as physiological changes and effects on key individual parameters, such as growth, reproduction and survival [120]. Within an ecosystem, the existence and flow of energy and cycle of materials lead to the development of trophic structures, biotic diversity and nutrient cycles, as shown in Figure 1.11 [121].

A key corollary in ecotoxicity testing regarding the hierarchical levels of ecological organization is that detrimental effects at a given level of organization can propagate to higher

organization levels. However, effects at any organizational level may or may not propagate at higher levels. Similarly, neither an effect at a lower organizational level may be easier to detect, nor an effect at a higher organizational level may be easier to interpret. Additionally, experiments and observations at higher levels of biological organization generally are more effort and cost demanding [122].

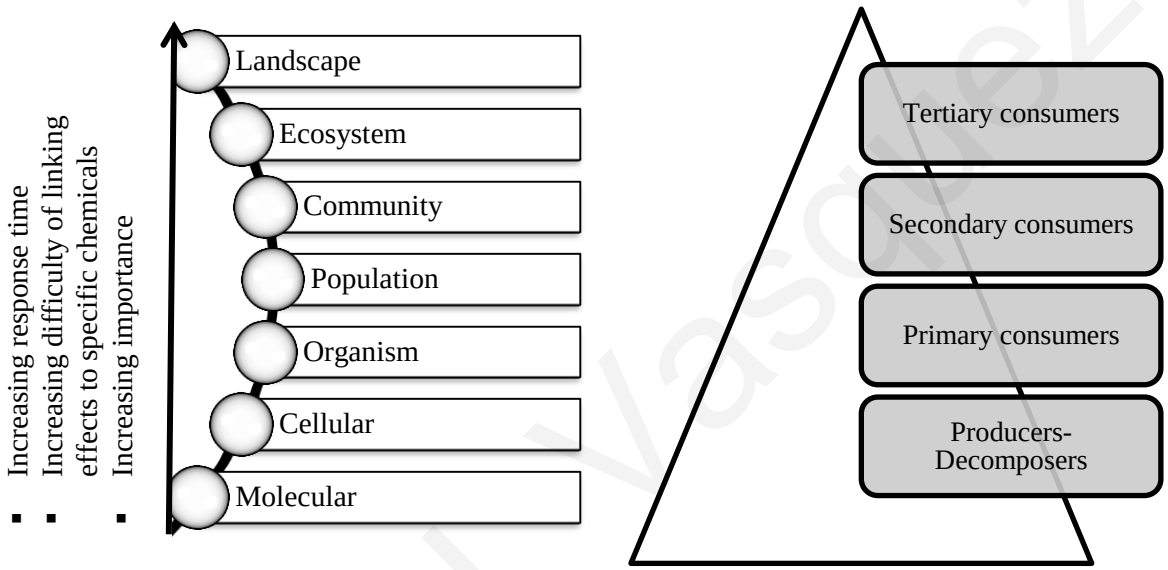


Figure 1.11 Hierarchical view of levels of biological organization

In practice, environmental toxicology still focuses on the organism level, relying on ecotoxicity data generated from single-species toxicity tests. Ecotoxicity testing may differ according to its (i) duration: short- to long-term; (ii) method of adding the test solutions: static, recirculation, removal, flow-through; (iii) type of the test: *in vitro*, *in vivo*; (iv) purpose of the study [123].

Organisms vary in sensitivity and the single-species approach has limitation in population and ecosystem extrapolation. As already mentioned in Section 1.1 though, the legislation focuses on achieving protection and preservation of the whole environmental entity and not that of single species. The need to evaluate effects at higher organizational levels has been acknowledged. However, the use of individual-based endpoints continues to dominate ecotoxicological assessments.

Furthermore, the USEPA using freshwater, estuarine and marine species has documented that toxicity of effluents correlates well with ecotoxicity measurements in the receiving water when effluent dilution is taken into account and the predictions of impacts from both effluent and receiving water toxicity tests compare favourably with ecological community responses in the receiving water [124].

The OECD has developed guidelines for the testing of chemicals. Tests developed include guidelines for assessment of effects to aquatic ecosystems (algae, water flea and fish), terrestrial ecosystems (terrestrial plants, earthworms, avian) and treatment processes (active sludge, respiration inhibition tests). For reference, the terms that are mostly used in this thesis and in the international scientific literature are briefly presented in Table 1.9.

Table 1.9 Terms widely used in environmental toxicology [125,126]

Term	Description
Acute toxicity	relatively short-term lethal or other effect, usually defined as occurring within 4 d for fish and macroinvertebrates and shorter times (2 d) for organisms with shorter life spans
Bioassay	is any controlled, reproducible test to quantitatively determine the presence, character, specificity or strength of a biological agent, upon a living organism, or other biological system
Chronic toxicity	toxicity involving a stimulus that lingers or continues for a relatively long period of time, often one-tenth of the life span or more. "Chronic" should be considered a relative term depending on the life span of an organism. A chronic toxic effect can be measured in terms of reduced growth, reduced reproduction, etc., in addition to lethality
Cumulative toxicity	effects on organisms caused by successive exposures
Dose	amount of toxicant that enters the organism. Dose and concentration are not interchangeable
Effective Concentration (EC)	toxicant concentration estimated to cause a specified effect in a designated proportion of test organisms. The effect is usually sublethal, such as a change in respiration rate or loss of equilibrium. The exposure time is specified; for example, the 96 h EC_{50} value for loss of equilibrium is the effective concentration for 50% of the test organisms in 96 h, for this kind of effect
Exposure time	time of exposure of test organism to test solution
Inhibiting Concentration	toxicant concentration estimated to cause a specified percentage inhibition or impairment in a qualitative biological function. For example, an IC_{25} could be the concentration estimated to cause a 25% reduction in growth of larval fish,

Term	Description
	relative to the control. This term may be used with any toxicological test that measures a change in rate, such as respiration, number of progeny, decrease in number of algal cells, etc.
In vitro	tests conducted in petri dishes or test tubes
In vivo	tests conducted using living organisms
Lethal Concentration (LC)	toxicant concentration estimated to produce death in a specified proportion of test organisms. Usually defined as median (50%) lethal concentration, LC_{50}, *i.e.*, concentration killing 50% of exposed organisms at a specific time of observation, for example, 96 h LC_{50}.
Lowest-Observed-Effect Concentration (LOEC)	in a full- or partial-life-cycle test, the lowest toxicant concentration in which the values for the measured response are statistically significantly different from those in the control
No-Observed-Effect Concentration (NOEC)	in a full- or partial-life-cycle test, the highest toxicant concentration in which the values for the measured response are not statistically significantly different from those in the control
Sublethal toxicity	exposure with detrimental effects but not causing death of the organism
Toxicity	potential for a constituent to cause adverse effects in a bioassay
Toxic unit (TU)	is the reciprocal of the test solution concentration that cause a specific effect, by the end of the exposure period

To summarize, prior to any ecotoxicity testing it is crucial to analyse the targets of the study to better select the biological organizational levels to be addressed. A differentiation among the assessment of effects strategies in toxicology and eco-toxicology of APIs is summarized in Table 1.10.

The assessment of adverse effects of effluents is a significant application of the environmental toxicity testing. The STPs usually receive high loads of effluents of variable qualitative characteristics due to industrial, hospital, touristic, and commercial human activities. An increasing number of xenobiotics that are suspected or already proved to be able to exhibit endocrine disrupting activity once released in the environment, such as cleaning chemicals and detergents, personal care products and APIs exist in the urban flows.

Additionally, in the case of combined sewage systems, like the ones present in Cyprus, the effluents are even more complex since these systems receive stormwater as well, which constitutes an important source for xenobiotics originating from diffuse sources of animal feedlots, atmospheric washout, traffic emissions, pesticides applications, etc. It should be noted that some of these xenobiotics not only pass through the treatment processes without being removed or only at a limited degree, but also, many biodegradation products such as nonylphenol, nonylphenol carboxylates [127], and transformation products [128,129] may as well be generated during the treatment.

Table 1.10 Characteristics of the assessment of the effects of active pharmaceutical ingredients in toxicology and ecotoxicology [130]

	Toxicology of APIs (humans)	**Eco-toxicology of APIs (environment)**
Number of compounds administered	One or only a few compounds at the same time	Unknown cocktail of different compounds
Desirable physicochemical properties	Stable	Ready (bio)degradable
Administration	Targeted, on demand, controlled	Diffuse
Wanted effects/side effects	Active, wanted effects, side effects	*Wanted effects* in target organism are most often "side effects" in the environment
Metabolism/biotransformation/affected organisms	One type of organism	Various type of organisms of different trophic levels

A remarkable progress has been accomplished during the last years in relation to analytical issues, mainly chromatographic techniques, the aim being the development of advanced technologies for the characterization of xenobiotics namely endocrine disrupting compounds, APIs and other micropollutants with emerging concern either individually or through multi-residue methods in matrices with complex mixture interactions like wastewater [131-137].

The importance of using both chemical analyses and toxicity tests for the quality characterization and control of STPs effluents in the framework of water quality programs is widely accepted nowadays [124]. The whole effluent toxicity (WET) approach entails various bioassays for acute and chronic toxicity determination and was formalized by the USEPA since 1985 [138]. The basic step is to test the effluents in their initial conditions without any treatment and dilution.

Among the bioassays cited in the literature in relation to the assessment of STPs effluents, the bioluminescent inhibition test using *Vibrio fischeri* is the most widely applied for wastewater monitoring [127,139,140].

Compared to chemical analysis alone, the WET programs have advantages in that they assess the potential biological effects of the chemicals present in wastewater, as shown in Table 1.11. The WET approach has led to the identification of detrimental effects in the environment of xenobiotics such as insecticides, surfactants and treatment polymers [141,142].

Table 1.11 Strengths and limitations of chemical-based and whole effluent toxicity approaches to toxicity assessment [143]

	Strengths	Limitations
Chemical-specific	More complete toxicology	Does not consider unknown/ uncharacterized substances
	Treatment systems designed for chemical-specific requirements	Bioavailability is not measured
	Fate can be modeled	Cannot account for interactions in a mixture
	Less expensive than biological measurements	Cannot directly assess biological impairment
	Can predict impacts	
Whole effluent toxicity	Accounts for un-known/ uncharacterized chemicals	Incomplete toxicology (few species tested)
	Evaluates aggregate toxicity (interactions)	Identification and treatment of toxicants difficult
	Toxicity is directly measured	Bioaccumulation and persistence not measured
	Bioavailability is assessed	Effects of ambient conditions on toxicity not considered
	Can predict impacts	

The WET approach has been included in the legislation of various countries as a tool for assessment of effects of real matrices and environmental protection. For instance, in 1988, Environment Canada undertook a 5-year study to quantify and regulate toxicity of industrial effluents discharged into the St. Lawrence River [144]. Yi et al. [145] pointed out that the Korean Ministry of Environment announced that a new standard protocol and legislation using *D. magna* acute toxicity tests would be gradually implemented from 2011 onwards to regulate wastewater effluent. For discharging effluents from STPs, the new legislation states that the TU of 24 h should be less than 1. However at European scale, there are no standard toxicity

tests yet or defined limits for the monitoring of STP effluents with the exception of Italy (DLgs152/2006, use of *D. magna*) [146], and Cyprus (Law 106 (I)/2002 use of *D. magna*, *P. subcapitata*, *V. fischeri*) [147].

1.4 Effects of the Active Pharmaceutical Ingredients investigated

A detailed review of the adverse effects of the selected APIs was performed. Specific information regarding the bioassays applied during the present thesis is presented in the Tables of each section [148-271].

A summary of the most common species used for WET approach and the assessment of effects of APIs is provided in Table 1.12.

Table 1.12 Species used for ecotoxicity studies

Phylum (Class)	Species	Common name	Exposure time	Endpoint
Annelida	*Eisena fetida/andrei*	Redworm	14 d	Reproduction
Arthropoda (*Branchiopoda*)	*Artemia salina*	Brine shrimp	24 or 48 h	Immobilization
	Ceriodaphnia dubia	Water flea	48 h 6 d	Immobilization Reproduction
	Daphnia magna	Water flea	24 or 48 h 10 or 21 d	Immobilization Immobilization/ Reproduction
	Daphnia pulex	Water flea	24 h	Immobilization
	Moina macropora	-	7 d	Reproduction
	Streptocephalus proboscideus	-	24 h	Immobilization
	Thamnocephalus platyurus	Beaver-tail fairy shrimp	24 h	Mortality
Arthropoda (*Arachnida*)	*Hypoaspis aculeifer*	Mite	14 d	Reproduction
Arthropoda (*Collembola*)	*Folsomia candida*	Springtail	14 d	Reproduction
Arthropoda (*Malacostraca*)	*Gammarus pulex*	Freshwater shrimp	1.5 h	Activity
	Hyallela azteca	Lawn shrimp	14 d	Reproduction Biomarkers of oxidative stress Mortality
	Hydra vulgaris	Common brown hydra	96 h 7 d	Morphology and feeding behaviour
Bacillariophyta	*Cyclotella*	Diatom	96 h	Growth

Phylum (Class)	Species	Common name	Exposure time	Endpoint
	meneghiniana			
Bacteria	*Bacillus stearothermophilus*	-	3 h	Spore germination
	Blastomonas natatoria	-	24 h	Growth
	Legionella pneumophila	-	16 h	Growth
	Micrococcus luteus	-	24 h	Growth
	Pseudomonas aeruginosa	-	16 h	Growth
	Pseudomonas putida	-	16 h	Growth
	Staphylococcus aureus	-	24 h	Growth
	Vibrio fischeri	Luminescent bacteria	5, 15 or 30 min 24 h	Growth
Basidiomycota	*Ganoderma lucidum*	Bracket fungus	7 d	Biodegradation
	Irpex lacteus	Milk-white toothed polypore	7 d	Biodegradation
	Phanerochaete chrysosporium	-	7 d	Biodegradation
	Trametes versicolor	-	7 d	Biodegradation
Chlorophyta	*Chlorella vulgaris*	Green alga	48 h	Growth
	Denodesmus subspicatus	Pond scum, green weed	96 h 24 h	Growth Photosynthesis rate
	Dunaliella tertiolecta	Green alga	72 h	Growth
	Pseudokirchneriella subcapitata	-	72 or 96 h	Growth
Chordata (Actinopterygii)	*Danio rerio*	Zebrafish	48, 72 or 96 h	Egg and embryo mortality Hatching success Morphology Behaviour Development
	Oncorhynchus mykiss	Rainbow trout	28 d	Structural changes
	Oreochromis niloticus	Tilapia	48 h 10 d	Genotoxicity
	Oryzias latipes	Japanese medaka	14 d 28 d	Growth Reproduction
	Pimephales notatus	Bluntnose minnow	48 h	Biomarker
	Pimephales promelas	Fathead minnow	4 d	Hatching Survival
	Salmo salar	Atlantic salmon	5 d	Gene expression
	Salmo trutta	Brown trout	21 d	Histopathological alterations
Chordata (Amphibia)	*Xenopus laevis*	African clawed frog	96 h	Morphology
Cyanophycota	*Synechococcus leopoliensis*	-	96 h	Growth
	Synechocystis sp	-	72 h	Growth Biomarkers of

Phylum (Class)	Species	Common name	Exposure time	Endpoint
				photosynthesis
Mollusca	*Dreissena polymorpha*	Zebra mussel	96 h 7 d	Oxidative biomarkers Cytotoxicity Bioconcentration
	Mytilus edulis	Baltic blue mussel	21 d	Bioconcentration Growth Byssus strength Mortality
	Mytilus galloprovincialis	Mediterranean mussel	7 d	Cell signaling
	Planorbis carinatus	-	72 h 21 d	Mortality Growth Mortality Hatching success
	Potamopyrgus antipodarum	New Zealand mud snail	42 d	Growth Reproduction
Rotifera	*Brachionus calyciflorus*	Marine rotifer	24 h 48 h 7 d	Immobilization
Streptophyta	*Lactuca sativa*	Lettuce	14 d	Emergence Biomass
	Lemna gibba	Swollen duckweed	7 d	Weight Frond number chlorophyll a
	Lemna minor	Common duckweed	7 d	Reproduction of fronds
	Lepidium sativum	Garden cress	72 h	Emergence Growth
Tracheophyta	*Brassica napus*	Rape	14 d	Emergence Biomass
	Triticum aestivum	Spelt wheat	14 d	Emergence Biomass
	Vicia sativa	Vetch	14 d	Emergence Biomass

1.4.1 β-blockers

1.4.1.1 Atenolol

The assessment of effects of atenolol on a variety of organisms is shown in Table 1.13. It was found not toxic to microbial respiration at concentrations up to 100 mg/L and not genotoxic using the umuC test. When tested to the algae *D. subspicatus*, atenolol almost failed to register a toxic effect with an EC_{50} value of 620 mg/L [148].

No effects were found to terrestrial organisms at concentrations up to 1000 mg/kg. The plants *T. aestivum, B. napus* and *V. sativa* emergence and biomass after a 14 d exposure time and the reproduction of the springtail *F. candida*, the compost worm *E. fetida/andrei* and the predatory mite *H. aculeifer* were investigated. In the same study it was shown that atenolol was not toxic to the amphipod *H. azteca* and the snail *P. antipodarum* to the highest concentrations tested (approx. 10 mg/L) after a 14 d and 42 d exposure times, respectively [149].

At the same range with the terrestrial organisms, an effect was observed only with the *Daphnia* reproduction test, in which the mortality of the offsprings of the second generation (F_2) increased [149]. The cnidarian *H. vulgaris* showed similar effects at the same concentrations after a 7 d exposure time [150].

Atenolol at the highest concentration tested (100 mg/L) was not found to cause any effects when cytotoxicity on haemocytes, gill and digestive gland primary cell cultures of the zebra mussel *D. polymorpha* were investigated [151]. From a two weeks study, it was observed that exposure to 500 μg/L of propranolol was able to reduce growth rates of the Japanese medaka *O. latipes* [152]. The most sensible organism for atenolol was found to be *P. promelas* with a 4 d NOEC for hatching and survival of 10 mg/L and 28 d NOEC for growth of 3.2 mg/L [153]. Furthermore, it was found to produce differences in the expression of 480 candidate gene of the Atlantic salmon *S. salar* when exposed for 5 d at 11.08 ± 7.98 μg/L.

Table 1.13 Summary of ecotoxicity data for atenolol

Trophic level	Bioassay	EC_{50} (mg/L)	Exposure time	Reference
Producers	*P. subcapitata*	128.8 (NOEC)	72 h	[149]
	L. sativum	-		
Consumers	*D. magna*	200	48 h	[131]
		313	48 h	[148]
	A. salina	407	24 h	[155]
Decomposers	*Biodegradability*	6%	28 d	[149]
	V. fischeri	1304	30 min	[43]
	P. putida	>130	24 h	[156]
	Yeast estrogen screen	Non estrogenic	72 h	[149]

Atenolol was found to create toxic by-products to the dicotyledonous *L. sativa* when chlorinated in an aqueous solution [154]. The study tried to simulate common wastewater

disinfection procedures and although the tested concentrations used were higher than those usually present in effluents of sewage treatment plants and possible bioaccumulation caused by continual irrigations may have occurred, the dangers this may enclose should not be neglected.

Regarding effects for humans, atenolol has been found not to cause DNA damage (DNA strand brakes) at concentrations of 7990 μg/L; whereas it was found to cause long-term carcinogenic effects to both male and female rats when they were exposed at 500 mg/kg/d [157]. In a recent study, atenolol has been found to cause chromosome loss detected as micronuclei in the peripheral lymphocytes of treated patients with chromosomes 7, 11, 17 and X being preferentially present in the micronuclei [158].

1.4.1.2 Metoprolol

The assessment of effects of metoprolol on a variety of organisms is summarized in Table 1.14. Furthermore, *D. subspicatus* had an EC_{50} value of 7.9 mg/L and *L. minor* was not affected to concentrations up to 320 mg/L [148].

Table 1.14 Summary of ecotoxicity data for metoprolol

Trophic level	Bioassay	EC_{50} (mg/L)	Exposure time	Reference
Producers	*P. subcapitata*	-		
	L. sativum	-		
Consumers	*D. magna*	3.1 (LOEC heart rate)	30 min	[160]
		6 (LOEC fecundity)	9 d	[160]
		12	9 d	[160]
		63.9	24 h	[152]
		76.2	48 h	[161]
		200	48 h	[131]
		438	48 h	[148]
	A. salina	-		
Decomposers	*Biodegradability*	Non readily biodegradable	28 d	[162]
	V. fischeri	144	30 min	[43]
	P. putida	-		
	Yeast estrogen screen	No estrogenic	72 h	[163]

The acute toxicity of metoprolol after an exposure time of 48 h was found to be 8.8 mg/L for *C. dubia* and higher than 100 mg/L for both *H. azteca* and *O. latipes* [152]. The chronic effects after an exposure time of 28 d indicated that at concentration of 1 μg/L ultrastructural

changes occurred to the liver and kidney of the rainbow trout *O. mykiss* and even to gills, if exposed at 20 μg/L [159].

The effects and bioconcentration of metoprolol on the mussel *D. polymorpha* after an exposure time of 7 d were investigated at concentrations in the range of 0.534-534 μg/L. Gene expression in gills and digestive gland at higher concentrations was altered and a 20-fold bioconcentration at low concentrations was observed, even though metoprolol is water-soluble.

No mutagenicity was observed when the Ames test was applied to metoprolol [164]. Although metoprolol is considered as non-readily biodegradable [162] its biotransformation was accelerated under the light conditions, implying that photo-induced intermediates could be more easily biodegraded in river water [60].

Regarding human health impacts, the micronucleus test did not show significant genotoxic effects [164]. In a review by Brambilla [157], no effects on DNA strand breaks were observed. Moreover in the long-term carcinogenesis assay no effects were observed when performed on male mice, CD-1 mice and rats at concentrations up to 750 mg/kg/d; whereas when evaluated on female mice at the same concentrations lung adenomas were detected.

1.4.1.3 Propranolol

According to the published ecotoxicological studies available so far, propranolol exhibits higher acute toxicity than other β-blockers, as shown in Table 1.15. This could be partly due to its relatively higher log D_{OW} value and the fact that propranolol is a strong membrane stabilizer [165].

The blue-green alga *S. leopoliensis* was shown to have an EC_{50} value of 668 μg/L after a 96 h exposure time; whereas the most sensitive organism whithin the phytoplankton was the diatom *C. meneghiniana* with an EC_{50} value of 244 μg/L [166]. Propranolol was found to inhibit the growth of the green algae *D. subspicatus*, showing an EC_{50} value of 7.7 mg/L

[148]. However, a specific photosynthesis inhibition was observed after 24 h exposure time of *D. subspicatus* with an EC_{50} value of 0.65 mg/L [167].

The rotifer *B. calyciflorus* had an EC_{50} value of 2.59 mg/L after an acute 24 h exposure time and the crustacean *S. proboscideus* an EC_{50} value of 1.87 [168]. For the same exposure time *D. pulex* was found to have an EC_{50} value of 3.833 mg/L. Following a 48 h exposure time to propranolol, LC_{50} values of 29.8 and 0.8 mg/L^1 were obtained; whereas reproduction decreased with NOEC values of 1 and 125 μg/L for *H. azteca* and *C. dubia* respectively [152].

Propranolol was found to bioconcentrate in the Baltic Sea blue mussels *M. edulis* even at 1 μg/L when exposed for 3 weeks. Furthermore, a significantly lower scope for growth was observed when exposed to 1-10 mg/L, which indicated that the organisms had a smaller part of their energy available for normal metabolism, and secondly, they had lower byssus strength and lower abundance of byssus threads, resulting in reduced ability to attach to the underlying substrate. Higher mortality was observed at these concentrations; whereas lower concentrations (1–100 μg/L) tended to differ from the controls [169].

A subchronic test of 7 d with propranolol to the Mediterranean mussel *M. galloprovincialis* demonstrated that propranolol at concentrations of 0.3 ng/L was able to affect cell signalling and interacted with specific and evolutionally conserved biochemical pathways. It also induced a stress response and affected its physiology by interacting with the same molecular targets as in humans [170]. According to Solé et al. [171] an exposure time of 10 d to the same species provoked a decrease in the feeding rate with a NOEC of 11 μg/L and a LOEC of 147 μg/L. These concentrations caused a decrease of acetylcholinesterase activity and an increase of the carboxylesterase and glutathione-S-transferase activity in gills. An increase in the lipid peroxidation levels in gills and a decrease of the glutathione-S-transferase activity in the digestive gland were also observed. The LC_{50} of survival after a 24 h acute exposure time was 10.31 mg/L for *T. platyurus.*

A peculiarity has been reported regarding the toxicity of propranolol enantiomers on *D. magna* [172]. The immobilization percentages at 24 h of both enantiomers were similar with S-enantiomer being slightly more toxic than the R-enantiomer with EC_{50} values of 1.4 and 1.57 mg/L, respectively. When the enantiomers were examined for their chronic effects (21 d) the R-enantiomer was found to be more toxic than the S-enantiomer regarding immobilization.

Furthermore, with regard to the reproduction rate, an increase of the total number of neonates was observed for both enantiomers and a decrease on the number of neonates when exposed to 869 μg/L of R-enantiomer.

Measurement of Ethoxyresorufin-*O*-deethylase (EROD) activity as a biomarker for CYP1A activity was used to investigate propranolol effects on the rainbow trout *O. mykiss.* It was found to provoke an increase in EROD activity in the liver and gill at 200 μg/L, in both *in vivo* (albeit none significantly in the liver) and *in vitro*, thus supporting the use of the latter as a surrogate of the former [175]. The *in vitro* EROD induction was previously reported by Laville et al. at concentrations of 8 mg/L [176].

A 96 h exposure time of *O. latipes* had a LC_{50} of 11.4 mg/L [177] and a 48 h exposure time had a LC_{50} of 24.3 mg/L [152]. When the fish juvenile growth test was applied to *O. mykiss* the LOEC was 10 mg/L and the NOEC 1 mg/L [178]. Furthermore, *O. mykiss* is considered to have many additional β-receptor sub-types for different physiological functions, giving propranolol the potential to crossover into non-cardiovascular systems such as homeostasis, immunocompetence [37] and O_2 chemoreceptor activity [179].

Table 1.15 Summary of ecotoxicity data for propranolol

Trophic level	Bioassay	EC_{50} (mg/L)	Exposure time	Reference
Producers	*P. subcapitata*	0.77	72 h	[60]
		7.4	96 h	[166]
	L. sativum	-		
Consumers	*D. magna*	0.055 (LOEC heart rate)	30 min	[160]
		0.11 (LOEC reproduction rate)	9 d	[160]
		0.44 (LOEC dry body mass)		
		1.6	9 d	[160]
		2.7		
		2.75	48 h	[152]
		7.7	24 h	[173]
		15.6	48 h	[166]
			48 h	[148]
			24 h	[155]
	A. salina	407	24 h	[168]
Decomposers	*Biodegradability*	Non readily biodegradable	28 d	[174]
	V. fischeri	61	30 min	[166]
		81	30 min	[43]
		184	5 min	[168]
	P. putida	0.050	24 h	[156]
	Yeast estrogen screen	Non estrogenic	72 h	[167]

According to Huggett et al. [152], a two weeks exposure time to 500 μg/L reduced growth rates and a four weeks exposure time to 0.5 μg/L decreased fecundity of *O. latipes* with a decrease in the total number of eggs produced and the number of viable hatching eggs. Regarding *P. promelas*, a 3 d exposure time at 3.4 mg/L caused 100% mortality or severe toxic effects that required euthanasia. The most sensitive endpoints in the study though were the hatchability and the female gonadal somatic index with a LOEC of 0.1 mg/L. Furthermore, plasma concentrations of propranolol in male fish exposed to concentrations of 0.1 and 1.0 mg/L were 0.34 and 15.00 mg/L, respectively, which constitutes 436 and 1546% of measured water concentrations [180].

Experiments performed with *P. putida*, *P. aeruginosa*, *M. luteus* and *B. natatoria* at the higher concentrations tested (130 μg/L) growth was inhibited, and in most cases the death rate increased; associated changes were observed in the metabolic fingerprints [156].

Propranolol was found to cause DNA strand breaks to rat primary hepatocytes at concentrations of 7880 μg/L [157]. Its photo-transformation products did not exhibit any acute toxicity in mice or significant binding to β-adrenergic receptors using rat cerebellum cortex membranes and their binding to β-adrenergic receptors [53].

1.4.2 Non-steroidal anti-inflammatory drugs

1.4.2.1 Diclofenac

Diclofenac was found to inhibit the growth of the marine phytoplankton *D. tertiolecta* at concentrations of 25 mg/L and above [181]. It was shown to have an acute toxicity of 224.30 mg/L to *C. dubia* when exposed for 48 h; whereas sub-lethal effects as LOEC were observed at 25 mg/L for *B. calyciflorus* exposed for 48 h, 2 mg/L for *C. dubia* exposed for 7 d and 8 mg/L for *D. rerio* exposed for 10 d [96,182].

Biomarkers of oxidative stress in *H. azteca* such as lipid peroxidation, protein carbonyl content to evaluate oxidized protein content, and the activity of superoxide dismutase, catalase, and glutathione peroxidase were significantly altered by the exposure to 46.7 μg/kg

sediment; whereas the LC_{50} was 0.467 mg/kg [183]. In chronic toxicity tests of *D. magna* reproduction, the LOEC was found to be 0.2 mg/L [184]. Diclofenac inhibited the growth of the marine phytoplankton *D. tertiolecta* at concentrations of 25 mg/L and above [181].

No effects were observed in a study using eight biomarkers of the freshwater bivalve *D. polymorpha* when exposed for 96 h to concentrations up to 592 ng/L diclofenac [185]. Another biomarker, lipid peroxidation, was found to be affected at concentration of 1 μg/L in *D. polymorpha* exposed for 96 h [186]. The previous biomarker was sensitive for blue mussels *Mytilus spp* when exposed for 96 h at the same concentrations 1 μg/L [74].

Detrimental effects were observed for exposure time of 21 d at 1 μg/L to the rainbow trout *O. mykiss* to which induced tubular necrosis in the kidney, hyperplasia, and fusion of the villi in the intestine were observed. Furthermore the expression levels of COX in liver, gills, and kidney were significantly reduced and it was found to bioaccumulate in the bile by a factor of 509-657 [187]. A 28 d exposure time resulted in renal lesions and alterations of the gills at concentrations of 5 μg/L and bioconcentration of 12–2732 in the liver, 5–971 in the kidney, 3–763 in the gills, and 0.3–69 [184]. Furthermore, cytopathology effects in the liver, kidney and gills were observed at concentrations of 1 μg/L [159].

Effects on the gene expression profile of *O. mykiss* were found at concentrations of 1.6 μg/L and the bioconcetration factor was found to be 4.02 ± 0.75 for the blood plasma and 2.54 ± 0.36 for the liver [188]. Laville et al. [176] demonstrated that diclofenac was cytotoxic to the PLHC-1 cell line with an EC_{50} value of 5.6 mg/L and estrogenic to a primary rainbow trout hepatocytes cell line with an EC_{50} value of 18.6 mg/L.

The effects to the brown trout *S. trutta* were observed at concentrations of 0.5 μg/L. At 7 and 14 d the haematocrit levels were affected; whereas after 21 d histopathological alterations were observed in the liver, gills and kidney. Moreover, diclofenac was able to hinder the stimulation of prostaglandin E_2 synthesis in head kidney macrophages *in vitro* [189]. Biomarkers of cellular toxicity (cytochrome P450 1A gene), p53-related genotoxicity (p53 gene) and estrogenicity (vitellogenin gene) were overexpressed in *O. latipes* after a 4 d exposure time to 1 μg/L [190]. Nanoinjection of diclofenac resulted in a decrease of the survival of injected embryos of *O. latipes* at hatching with an EC_{50} value of 6 ng/egg [191]. At

1 mg/L acute effects on the feeding behaviour (time to eat midge larvae) of *O. latipes* were monitored [192].

A decrease of reproduction was observed at 25 mg/L for *D. magna* after a 21 d exposure time and at 50 mg/L for *M. macrocopa* after a 7 d exposure time. Furthermore, a three-month exposure time of fish to 0.001‑10 mg/L of diclofenac caused a lower hatching success and a delay in hatch [193]. Diclofenac did not cause any effects to early-life stages of *D. rerio.* The parameters investigated were egg and embryo mortality, gastrulation, somite formation, movement and tail detachment, pigmentation, heartbeat, and hatching success after 48 to 96 h exposure times to up to 2000 μg/L [194]. In another study *D. rerio* specific effects were observed for hatching, yolk sac and tail deformation at concentrations above 1.5 mg/L when exposed for 72 h [195].

The nature of the bacterial community on a river biofilm was influenced by diclofenac at 10 and 100 μg/L [196]. Lotic biofilms (bacteria and algae) were found to be negatively affected when exposed to diclofenac at 100 μg/L for a 5 d exposure time [197]. Recently diclofenac has been qualitatively detected in the hair of Eurasian otters *Lutra lutra* indicating that wildlife is being exposed to this compound [198].

The most severe adverse effects of diclofenac though were found in three species of vultures the Indian white-rumped one (*Gyps bengalensis*), the Indian one (*Gyps indicus*) and the slender-billed one (*Gyps tenuirostris*) in India and Pakistan causing a population decline [199,200]. Renal failure and visceral gout were observed due to their scavenging behaviour feeding on carcasses of domestic cattle treated with diclofenac [201]. The LOEC causing renal failure was 0.007 mg/kg. Adverse effects on the same concentrations were found for the African vultures *Gyps coprotheres* [202].

The effects on four avian species: broiler chicks (*Gallus gallus*, 15 d old), pigeons (*Columba livia*, 3 months old), Japanese quail (*Coturnix japonica*, 4 weeks old) and mynah (*Acridotheres tristis*, independent young) when exposed to concentrations 0.25, 2.5, 10 and 20 mg/kg body weight for 7 d were depression, somnolence, decreased body weight and mortality. Serum creatinine levels were elevated and kidneys and liver were enlarged.

Histologically, the kidneys showed acute renal necrosis and the livers had fatty change and necrosis of hepatocytes. The kidneys and liver of broiler chicks and pigeons given 10 and 20 mg/kg diclofenac exhibited uric acid crystal aggregates (tophi) and associated lesions in the parenchyma [203]. When turkey vultures *Cathartes aura* were exposed to diclofenac no signs of toxicity, visceral gout, renal necrosis, or elevate plasma uric acid were observed at concentrations greater than 100 times the estimated median lethal dose reported for *Gyps* vultures, showing a different sensitivity among avian species [204].

Table 1.16 Summary of ecotoxicity data for diclofenac

Trophic level	Bioassay	EC_{50} (mg/L)	Exposure time	Reference
Producers	*P. subcapitata*	16.3	96 h	[166]
	L. sativum	-		
Consumers	*D. magna*	22	48 h	[96,166,182]
		40	48 h	[205]
		68	24 h/48 h	[35]
		80	48 h	[132]
	A. salina	-		
Decomposers	*Biodegradability*	Non readily biodegradable	28 d	[206]
	V. fischeri	11.45	30 min	[166]
		13.7	15 min	[207]
	P. putida	-		
	Yeast estrogen screen	Non estrogenic	72 h	[163]

Diclofenac was not found to cause bacterial mutation, cytogenotoxicity *in vitro* and *in vivo*, gene mutation in the mouse lymphoma cells, carcinogenicity on mouse at of concentrations 0.02-0.04× the high animal dose (mg/m^2/maximum recommended human dose) and on rats at concentrations up to 0.09×. Furthermore, no cell transformation and no effect on the dominant lethal assay were observed [208].

1.4.2.2 Ibuprofen

A summary of the ecotoxicity effects of ibuprofen is presented in Table 1.17. The effects of 10 μg/L ibuprofen to a riverine microbial community were monitored for 8 weeks indicating a toxic effect. Cyanobacteria were suppressed and bacterial biomass was reduced. The live-dead ratio was affected by the exposure [209]. Ibuprofen was not able to inhibit a number of endpoints monitored in *L. gibba* after a 7 d exposure time [210].

Ibuprofen was found to inhibit *D. subspicatus* algal growth with an EC_{50} value of 342 mg/L after an exposure time of 96 h [35]; whereas inhibited the duckweed *L. minor* growth causing an EC_{50} value of 22 mg/L after an exposure time of 7 d [211]. *L. minor* in a different study was found to have an EC_{50} value of 4 mg/L [212].

The behaviour of the amphipod *G. pulex* was found to be affected by quantifying its movements using a multispecies freshwater biomonitor in a test chamber. In particular, exposure to low concentrations (10–100 ng/L) resulted in a significant decrease in activity, whereas the activity of *G. pulex* at higher concentrations (1 μg/L – 1 mg/L) was similar to the control [213].

The cnidarian *H. vulgaris* was found to have an EC_{50} value of 22.36 mg/L when exposed for 96 h to ibuprofen; whereas its morphology and feeding behaviour was affected when exposed at 1.65 and 3.85 mg/L, respectively [214]. The most sensitive of the cnidarians was found to be *H. vulgaris* with a LOEC of 10 μg/L on feeding behaviour when exposed for 7 d [150]. The cladoceran *M. macrocopa* had an EC_{50} value of 72.60 mg/L when its immobilization was monitored for 48 h and an NOEC of 25 mg/L when the reproduction was assessed after a 7 d exposure time [215].

The mollusc *P. carinatus* had a LC_{50} of 17.1 mg/L when its survival was monitored for 72 h and a NOEC of 1.02, 2.43 and 5.36 mg/L when exposed for 21 d when the wet weight, the hatching success and the survival were monitored, respectively [216]. Exposure of *M. edulis* to 10 mg/L resulted in lower byssus strengths and byssus thread abundance compared to the control treatment when exposed for 3 and 2 weeks, respectively. The scope of growth of this organism was influenced at lower concentrations when exposed to 1 mg/L for two weeks [169].

The effects of ibuprofen at 0.2-8 μg/L were addressed to the bivalve *D. polymorpha* exposed for 96 h, which demonstrated a slight cyto-genotoxicity on the mussel hemocytes at the lowest concentration tested; whereas higher concentrations tested were able to significantly increase both genetic and cellular damage [217]. In addition, ibuprofen was suggested to have a considerable effect on the activities of antioxidant and detoxifying enzymes due to the notable oxidative status imbalances of the exposed specimens.

When *D. magna* was exposed to 40 and 80 mg/L for 10 d the number of offsprings reduced significantly. Interestingly, when a recovery period of 10 d followed the exposure period, ibuprofen-stressed daphnids produced offsprings faster and by the end of the experiment the average growth was comparable with control populations. This suggested that daphnids were susceptible during egg maturation [218].

T. platyurus was reported to have a LC_{50} of 19.59 mg/L after an acute 24 h exposure time and *O. latipes* had a LC_{50} of > 100 mg/L after a 96 h exposure time [177]. Ibuprofen had no effect on the oxidation rate of nicotinamide adenine dinucleotide phosphate (NADPH) and lipid peroxidation when *O. mykiss* hepatocytes were exposed for 60 min at concentration of 100 μM [219]. *O. latipes* demonstrated an alteration of the spawning behaviour when exposed to 0.1 μg/L for 42 d indicating that a different reproduction pattern was developed when exposed to ibuprofen [220]. A delay in egg hatching was observed when *O. latipes* was exposed for 120 d to concentrations of 0.1 μg/L [215]. *O. mykiss* fry were exposed to ibuprofen solutions for 4 d. Even at 1 μg/L the heat shock protein70 was induced in the trout liver [221].

At 1 mg/L, ibuprofen was shown to disturb the seawater-induced elevation in plasma osmolality and concentrations of Cl^- and K^+. This was accompanied by enhanced gill glycolytic capacity and reduced liver glycogen content suggesting enhanced metabolic demand to fuel ion pumps induced elevation in gill Na^+/K^+-ATPase activity [222]. After a 48 h exposure time of *P. notatus* to 50 μg/L and 100 μg/L a significant reduction of gill tissue of the prostaglantin (PGE_2) concentration by approximately 30% and 80% respectively was observed [80].

The results from daily observations of *D. rerio* for a total period of 7 d indicated that developing embryos tolerated lower (1 and 5 μg/L) doses of the ibuprofen readily but, exposure to higher doses (>10 μg/L) caused retarded development, decreased hatching rate and growth, cardiac anomalies, spinal curvature, pectoral fin malformation and behavioural alterations resulting in higher mortality of experimental embryos [223].

Ibuprofen was found to increase the frequency of micronuclei to the *O. niloticus* fish (Tilapia) at 300 ng/L in both acute (48 h) and sub-chronic (10 d) exposure times; hence

induced genotoxicity potential [224]. *X. laevis* was investigated and an EC_{10} of 30.7 mg/L was calculated for a 96 h exposure time when deformity was investigated as an endpoint [225].

Table 1.17 Summary of ecotoxicity data for ibuprofen

Trophic level	Bioassay	EC_{50} (mg/L)	Exposure time	Reference
Producers	*P. subcapitata*	2.3	96 h	[226]
	L. sativum	-		
Consumers	*D. magna*	13.4	14 d	[227]
		20	21 d	[132]
		51.44	48 h	[215]
		101.2	24 h/48 h	[35]
		108	48 h	[211]
	A. salina	-		
Decomposers	*Biodegradability*	Readily biodegradable	28 d	[228]
	V. fischeri	19.1	15 min	[207]
	P. putida	-		
	Yeast estrogen screen	Non estrogenic	72 h	[167]

An older study indicated that ibuprofen had antibacterial activity suppressing the growth of *S. aureus* when exposed to 150 mg/L [229]. Ibuprofen at 10 mg/L was able to be biodegraded by the rot-white fungi *T. versicolor, I. lacteus, G. lucidum* and *P. chrysosporium* after an exposure time of 7 d [230].

Ibuprofen was reported to be non-mutagenic using the Ames mutagenicity assay (in strains TA97a, TA100 and TA102) and weak genotoxic when using the *in vivo* genotoxicity test of sister-chromatid exchange in bone marrow cells of mice [231].

1.4.3 Antibiotics

1.4.3.1 Erythromycin

A summary of ecotoxicity data for erythromycin is presented in Table 1.18. Erythromycin has been found to inhibit the growth of the cyanobacteria *Synechocystis* sp. by 70% when exposed for 5 d to 1 mg/L; *L. minor* was found to be inhibited by 20% when exposed for 7 d at the same concentrations [212]. *L. gibba* was not inhibited using a 7 d static renewal test at concentrations up to 1 mg/L [210].

The effects of erythromycin to *P. subcapitata* have been recently been studied using a biomarker battery that included photosynthetic rate, chlorophyll fluorescence, Hill reaction activity, photophosphorylation activity, and ribulose-1.5-bisphosphate carboxylase activity and was found to cause acute effects (96 h) at concentrations of 0.6 mg/L [232].

Erythromycin was found to immobilize *B. calyciflorus* and *T. platyurus* exposed for 24 h and *C. dubia* exposed for 48 h with EC_{50} values of 27.53, 17.68 and 10.23 mg/L, respectively [107]. *D. rerio* was not killed when exposed for 96 h to concentrations up to 1000 mg/L [107]. No effects on the immobilization and morphology of adults and neonates, adult length, resting egg production, brood size (fecundity), and the proportion of male broods produced (sex ratio) when *D. magna* was exposed to 6 and 30 d at concentrations of 1-100 μg/L [233]. The growth of *B. calyciflorus* at 48 h and the number of female rotifers of *C. dubia* at 7 d were affected with an EC_{50} value of 0.94 and 0.22 mg/L [107]. It has been found that erythromycin may affect the microbiological population in aquaculture by changing the bacterial composition, rather than the numbers of total viable aerobic bacteria or erythromycin-resistant bacteria at 25 mg/L [234].

Erythromycin was reported to cause membrane lysis of Gram negative bacteria *L. pneumophila* with a Minimum Inhibitory Concentration (MIC) of 0.5 mg/L when exposed for 16 h [235]. At sub-inhibitory doses of 1.5 mg/L repression of lectin production in *P. aeruginosa* [236] and modification of the cell surface structure and hydrophobicity were observed [237]. Erythromycin was found to inhibit ammonification, nitritation and nitratation at concentrations higher than 20 mg/L. It also affected heterotrophs, particularly filamentous bacteria by causing floc disintegration and breakage of filaments. Cell lysis was observed [86]. Adverse effects such as inhibition of the specific evolution rate of COD and $N\text{-}NH_4^+$ and destruction of flocs were observed in activated sludge when exposed at 10 mg/L erythromycin for 24 h [238].

Erythromycin was found to cause mortality to *A. salina* when exposed to 10 mg/L for 120 h [244]. The respiration inhibition test OECD 209 was applied to erythromycin for an exposure time of 20 h and the IC_{50} was greater than 100 mg/L [245]. Only part of the bacterial population of activated sludge was found to be affected by erythromycin with an EC_{50} value ranging between 39-43 mg/L [241].

Table 1.18 Summary of ecotoxicity data for erythromycin

Trophic level	Bioassay	EC_{50} (mg/L)	Exposure time	Reference
Producers	*P. subcapitata*	0.02	72 h	[107]
		0.0366	96 h	[239]
		0.0366	144 h	[240]
		0.13	48 h	[241]
	L. sativum	-		
Consumers	*D. magna*	22.45	24 h	[107]
		210.57	24 h	[242]
		387.7	48 h	[242]
	A. salina	-		
Decomposers	*Biodegradability*	Non readily biodegradable	28 d	[243]
	V. fischeri	>100	30 min	[107]
		560	15 min	[241]
	P. putida	-		
	Yeast estrogen screen	-		

No mutagenic effect was observed when the AMES test, to both the TA98 evaluating frameshift mutations and the TA100 monitoring base pair substitutions for erythromycin [107]. Erythromycin at 1 and 100 mg/L did not affect the methanogens of an anaerobic batch reactor and the biogas production, meaning that a substantial percentage of the population was resistant to erythromycin. The conversion of butyric acid though was inhibited when erythromycin was present, indicating that specific substrate degradation pathways can be affected [246].

Erythromycin was not able to produce an increase in the frequency of biomarkers as sister chromatid exchanges or chromosomal aberrations in either the presence or absence of metabolic activation to Chinese hamster ovaries [247].

1.4.3.2 Ofloxacin

Ofloxacin functions by inhibiting the enzyme complex DNA gyrase, a type II topoisomerase, and topoisomerase IV, thereby inhibiting cell division [248]. A summary of ecotoxicity data is presented in Table 1.19. Ofloxacin was found to be phytotoxic to *L. gibba* at μg/L when exposed for 7 d with an EC_{50} value of 532-1374 μg/L, depending on the endpoint assessed [210]. *L. minor* when exposed to ofloxacin had an inhibition on the reproduction of fronds after a 7 d exposure time with an EC_{50} value of 126 μg/L.

Levofloxacin was found to inhibit the O_2 evolution and the photosystem II (PSII) activity of the *Synechocystis* sp. at concentrations of 0.1-10 mg/L after 12 h exposure time [249]. The spore germination of *B. stearothermophilus* was inhibited by a 3 h exposure time with a LOEC of 23 μg/L [250].

Ofloxacin was found to immobilize *B. calyciflorus* and *T. platyurus* after an exposure time of 24 h and *C. dubia* after an exposure time of 48 h with an EC_{50} value of 29.88, 33.98 and 17.41 mg/L respectively [107]. Lethality of *D. rerio* at the maximum concentration tested (1000 mg/L) did not increase markedly after an exposure time of 96 h [107]. Chronic exposure time of 48 h for *B. calyciflorus* in which growth was evaluated and 7 d for *C. dubia* in which the number of females were counted had an EC_{50} value of 0.53 and 3.13 mg/L respectively. Ofloxacin was found to have genotoxic properties at concentrations of 1-2 μg/L present in hospital effluents [251]. It was reported to display high activity not only against bacterial topoisomerases [252], but also against eukaryotic topoisomerases [253]. According to Li et al. [254] it could also induce oxidative stress, lipid peroxidation and DNA oxidative damage to chondrocytes.

Table 1.19 Summary of ecotoxicity data for ofloxacin

Trophic level	Bioassay	EC_{50} (mg/L)	Exposure time	Reference
Producers	*P. subcapitata*	1.44	72 h	[107]
		4.74	96 h	[166]
		12.1	96 h	[255]
	L. sativum	-		
Consumers	*D. magna*	31.75	24 h	[107]
		76.58	48 h	[166]
	A. salina	-		
Decomposers	*Biodegradability*	Non readily biodegradable	28 d	[243]
	V. fischeri	0.0135	24 h	[256]
		>100	30 min	[107]
		90	30 min	[166]
	P. putida	0.01	16 h	[251]
	Yeast estrogen screen	-		

Although ofloxacin is toxic to mammalian cells in culture, its mechanism of action is still not completely understood. A reason may be that quinolones bind cooperatively to DNA, perhaps as a consequence of π-π stacking of planar quinolone rings [257]. It should be mentioned that since the dosing period of ofloxacin is usually short, carcinogenicity studies are not always compulsory for its governmental approval.

1.4.3.3 Sulfamethoxazole

A review of ecotoxicity data on sulfamethoxazole is presented in Table 1.20. Sulfamethoxazole could inhibit *L. gibba* after a 7 d exposure time to seven endpoints evaluated wet weight, frond number, chlorophyll a, chlorophyll b and carotenoids with EC_{50} values ranging from 0.79-81 μg/L [210]. The concentration of para-aminobenzoic acid was found to increase when *L. gibba* was exposed to sulfamethoxazole suggesting a specific mode of action at concentrations of 100-1000 μg/L during the 7 d exposure time [258].

The growth of *C. meneghiniana* and *S. leopoliensis* were found to be inhibited when exposed for 96 h to sulfamethoxazole with an EC_{50} value of 2.4 and 0.026 mg/L, respectively [166]. Sulfamethoxazole was toxic to *C. vulgaris* when exposed for 48 h with an EC_{50} value of 6.2×10^{-3} mM [259].

Immobilization of *B. calyciflorus* and *T. platyurus* after 24 h and *C. dubia* after 48 h was observed with an EC_{50} value of 26.27, 35.36 and 15.51 mg/L, respectively. *D. rerio* was not affected when mortality was monitored after an exposure time of 96 h to 1000 mg/L. Chronic exposure times of 48 h of *B. calyciflorus* and 7 d of *C. dubia* had an EC_{50} value of 9.63 and 0.21 mg/L [107]. *The* morphology feeding response, hydranth number and attachment of *H. vulgaris* were not found to be affected when exposed at 96 h at concentrations up to 100 mg/L [214].

B. stearothermophilus was inhibited to sulfamethoxazole when exposed for 3 h with a LOEC of 132.5 μg/L to its spore germination [250]. Sulfamethoxazole was found to mutagenic using the AMES test at high concentrations 6.25 and 50 mg/L with the TA98 and TA100 respectively [107]. It was found to be unstable in anaerobic mesophilic digesters [265,266]. Furthermore, it could inhibit the soil bacteria as means of leucine incorporation, and endpoint for estimating pollution-induced community tolerance when exposed to 20 and 500 mg/kg for 30 d. An increase in the fungal and a decrease in the bacterial phospholipid fatty acids were observed [267].

Sulfamethoxazole was found to be hepatotoxic and cause systemic hypersensitivity reactions [268]. However, the frequency of chromosomal aberrations in peripheral

lymphocytes [269] and in bone-marrow [270] did not increase. An increase of the number of micronuclei was observed in the bone marrow [270]. More recently sulfamethoxazole was found to be genotoxic in lymphocytes at 500 mg/L [271].

Table 1.20 Summary of ecotoxicity data for sulfamethoxazole

Trophic level	Bioassay	EC_{50} (mg/L)	Exposure time	Reference
Producers	*P. subcapitata*	0.146	96 h	[166]
		0.52	72 h	[107]
		1.9	72 h	[260]
		1.53	96 h	[239]
	L. sativum	-		
Consumers	*D. magna*	25.20	24 h	[107]
		123.1	48 h	[261]
		177.3	96 h	[262]
		205.2	48 h	[263]
	A. salina	-		
Decomposers	*Biodegradability*	Non readily biodegradable	28 d	[243]
	V. fischeri	23.3	30 min	[107]
		74.2	5 min	[262]
		78.1	15 min	[262]
		>84	30 min	[166]
	P. putida	0.256	16 h	[264]
	Yeast estrogen screen	-		

To summarize a presentation of the ecotoxicity data was performed by identifying the most toxic compounds for each of the bioassays evaluated in this thesis. The main findings are presented in Table 1.21.

Table 1.21 Summary of the most toxic Active Pharmaceutical Ingredients

Trophic level	Bioassay	API
Producers	*P. subcapitata*	Erythromycin
	L. sativum	-
Consumers	*D. magna*	Propranolol
	A. salina	-
Decomposers	*Biodegradability*	Ibuprofen was the only readily biodegradable
	V. fischeri	Diclofenac
	P. putida	Ofloxacin
	Yeast estrogen screen	No estrogenicity reported

CHAPTER 2. THESIS OBJECTIVES

Improvements in analytical techniques have led to an increased awareness of the occurrence of APIs in the environment. APIs as environmental pollutants were addressed during the late 1980s [272]. When introduced into the environment, APIs may affect the same pathways in animals, having identical or similar targets, or they may cause unexpected effects when having dissimilar modes of actions. The population decline of vultures due to indirectly exposure to diclofenac [199] and the impairment of reproduction of fish due to exposure to 17α-ethinylestradiol at environmentally realistic concentrations are only some examples of the unexpected effects of exposure of organisms to APIs [273].

Being exposed over their whole life at sites that receive effluents with APIs, aquatic organisms are particularly important targets, and the current lack of knowledge on chronic and mixture effects hampers ecotoxicological assessment. Concern is now raised on the potential adverse effects these compounds may have on non-target organisms, particularly under conditions of chronic exposure, as well as their effects as multi-component mixtures (parent compound and transformation products).

It should be mentioned that some transformation products, e.g., products of biotransformation, can be even more persistent than the parent compounds. The toxicity potency may vary and they may exhibit a higher potential for accumulation compared to the parent compound. As an example, a photo-transformation product of naproxen has been found to show higher toxicity than the parent compound [274].

As stated by Sanderson et al. [275], measured ecotoxicity data is available for less than 1% of APIs being used. Standard acute ecotoxicity data exist for a number of APIs; however such data alone are not sufficient for a comprehensive hazard and risk assessment. Muñoz et al. [276] have performed a Life Cycle Analysis to compare different scenarios involving wastewater reuse, with special focus on toxicity-related impact categories. The results highlighted the importance of including wastewater pollutants in Life Cycle Analysis of wastewater systems assessing toxicity, since the contribution of wastewater pollutants to the overall toxicity scores in the study was found to be above 90%. It is important to know that the

study considered as key pollutants not only the "traditional" ones like heavy metals, but also included non-regulated ones as well.

Based on the information provided above, the present thesis had the following scientific objectives, presented in Figure 2.1:

- Develop a flexible battery assay using bioassays capable of evaluating the biological potency of xenobiotics, such as APIs, and also of environmental samples, such as wastewater samples. The objective was to develop a battery assay that would take into account multiple endpoints of acute and chronic toxicity. The battery assay should include all trophic levels and adverse effects that are relatively less studied, such as genotoxicity and estrogenicity.
- Investigate whether environmental samples, such as wastewater, pose risks to the environment by implementing the battery assay developed.
- Select the APIs expected to be present in the environment (Cyprus was taken as the case study of the thesis) and assess their effects as single compounds using acute and chronic assays, in order to increase knowledge about their possible adverse effects. A prioritisation of substances and risk assessment was the objective.
- Evaluate the effects of a selection of APIs in mixtures by monitoring the effects. The objective was to assess the effects of APIs with similar and/or dissimilar modes of action.
- Increase the understanding on the effects of APIs as mixtures in true environmental samples, such as treated wastewater.
- Provoke transformation of APIs by abiotic and biotic processes and investigate its effects regarding the acute and chronic toxicity
- Monitor the environmental biopersistence of APIs and their biotransformation products after the application of abiotic processes.
- Investigate the genotoxicity of APIs after the application of abiotic processes with the use of a bioassay able to predict effects on humans.
- Identify transformation products of selected APIs after the application of abiotic processes and to propose possible transformation pathways.

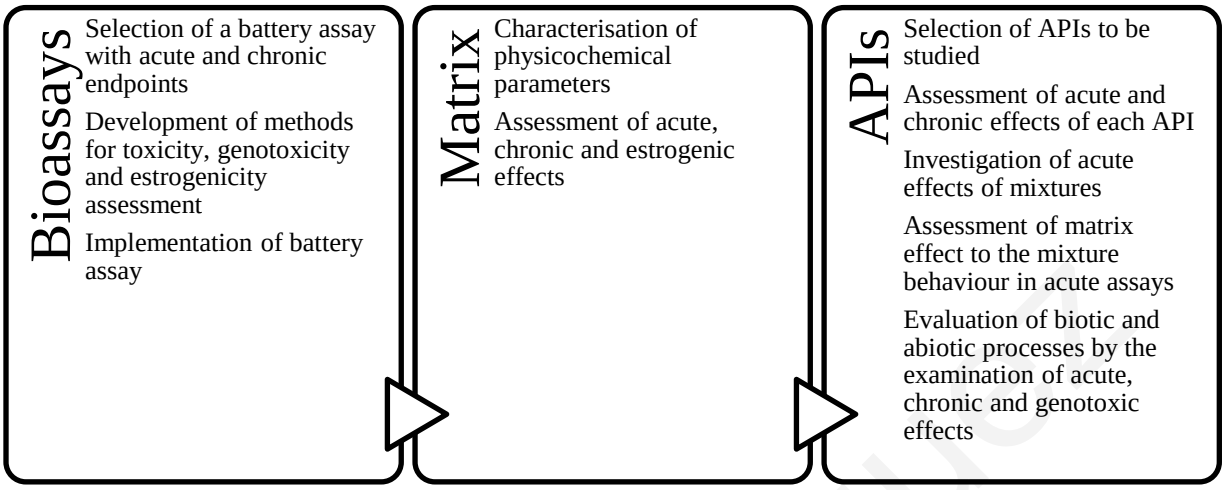


Figure 2.1 Scientific objectives of the present thesis

In the present thesis the use of a multi-species approach and a simplified ecosystem, as suggested by the OECD, were considered in the present thesis. The advantages of this methodology are the reduction of logistics, space, time and money, the easiness of interpretation and the increase of accuracy. A great disadvantage is that the simplification of an ecosystem decreases the ability to extrapolate results to the environment. The interactions between species, between species and the environment and between the stressor and the environment were addressed in a lesser extent.

The originality of this thesis relies in the fact that the battery assay developed and implemented includes organisms from all trophic levels, in which environmental and human endpoints were both addressed. The selection of endpoints that focus on chronic toxicity, estrogenicity and genotoxicity assessment were more sensible permitting testing in environmentally relevant concentrations, giving a more accurate envisage of the real environment. The latter was also enhanced by the investigation of APIs not only as single compounds, but also in multi-component mixtures, in which transformation products were also taken into account.

An integrated approach was considered, as shown in Figure 2.2. The first part of the thesis focused on assessing the environmental effects of real matrices (in this case wastewater) to a

battery assay. For this purpose a variety of methods was developed and physicochemical and ecotoxicological assessments were performed. Three sewage treatment plants were selected for sampling. The criteria for their selection were size, location, origin/sources of wastewater, treatment processes and wastewater reuse/discharge potential. On-site visits and employees' interviewing were performed at least once per year during 2006-2011 and information is provided in Chapter 4. The qualitative and quantitative characteristics of wastewater were determined. The parameters monitored such as, pH, conductivity, ammonia, Biological Oxygen Demand (BOD_5), Chemical Oxygen Demand (COD), heavy metals, provided information on the physicochemical characteristics of the wastewater; whereas the application of bioassays allowed assessing any adverse acute, chronic and estrogenic effects to organisms from different trophic levels.

This approach may be considered as an integrated one since a multi-species battery assay was developed. It is generally accepted that using a battery of test species provides a better insight concerning multiple toxic substances and additional information based on differing species sensitivity. Especially when evaluating environmental samples, e.g., wastewater, any changes in the proportional relationship of toxicity between species over time may indicate that the toxic substances present in the sample may vary [124].

The first part of the thesis was performed in the framework of the research project PHAREM (ΑΕΙΦΟ/0506/16) "Development and application of innovative advanced oxidation processes for the removal of active organic compounds in urban wastewaters and monitoring of toxicity", 2007-2009 which was co-financed by the Cyprus Research Promotion Foundation and the University of Cyprus. Study visits for the development of the battery assay took place during this period. In particular, during 2006-2007, a visit at the Institute for the Environment, University of Brunel took place for obtaining training on the yeast estrogen screen under the guidance of Dr. Alice Baynes, Dr. Edwin Routledge and Prof. John Sumpter. A visit at the Environmental Hygiene Division, Faculty of Sciences, University of Naples Federico II during 2007 also took place, for obtaining training on the implementation of acute and chronic assays on algae, plants and crustaceans under the guidance of Prof. Süreyya Meriç and Prof. Marco Guida.

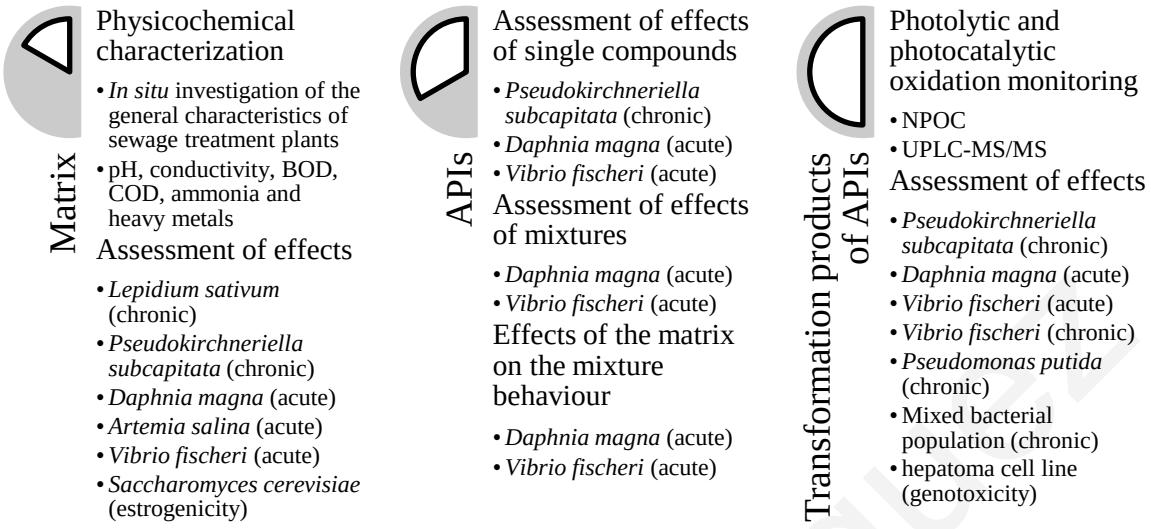


Figure 2.2 Schematic presentation of the methodology applied in the present thesis

The second part of the thesis focused on increasing the understanding on the assessment of effects of APIs as single compounds and in mixtures. Regarding APIs, a selection of eight substances was made mainly due to their occurrence in significant concentrations in treated wastewater and the environment as analysed in detail in Chapter 1. Furthermore, the long-term use forecast, the sale levels, the biodegradation and metabolism rate were some of the criteria taken into account for the selection of the APIs. The APIs studied were three β-blockers (atenolol, metoprolol, propranolol), two anti-inflammatory APIs (diclofenac, ibuprofen) and three antibiotics (erythromycin, ofloxacin, sulfamethoxazole).

The mode of action of the selected APIs already presented in Chapter 1 is quite different, as they belong to different pharmaceutical groups. Briefly, it can be noted that β-blockers act by competitively inhibiting β-adrenergic receptors. Propranolol acts to all β-receptors and has the ability to cause cell membrane stabilization, whereas atenolol and metoprolol act on the β_1-receptors and have no membrane stabilizing activity. Diclofenac and ibuprofen act by inhibiting either reversibly or irreversibly both of the two isoforms of the cyclooxygenase enzyme (COX-1 and COX-2), which catalyzes the synthesis of different prostaglandins [99,277]. Erythromycin is a macrolide and acts by penetrating the bacterial cell membrane and reversibly binding to the ribosomes so that binding of the transfer RNA is blocked and

subsequent protein synthesis is inhibited. Ofloxacin is a synthetic fluoroquinolone that inhibits the supercoiling activity of bacterial DNA gyrase, halting DNA replication. Sulfamethoxazole is a sulfonamide drug that interferes with folic acid synthesis in susceptible bacteria. The effects of the matrix (treated wastewater) on the behaviour of the APIs were investigated.

The second part of the thesis was implemented in the framework of TOMIXX (PENEK/0609/24) "Development of novel methods for the toxicity assessment of multi-component chemical mixtures to humans and the ecosystem", 2010-2012, funded by the Cyprus Research Promotion Foundation through Desmi 2009-2010, which is co-financed by the Republic of Cyprus and the European Regional Development Fund of the European Union.

In the third part of the thesis, the focus was placed on the transformation products of APIs. Atenolol and ofloxacin were chosen as model APIs due to their photosensitivity characteristics, as described in Chapter 1. Photo-transformation of ofloxacin was provoked and the assessment of effects of the transformation products formed during the photo(cata)lytic treatment was investigated, using chronic tests on bacteria and a genotoxicity test on human cells. The methodology applied can be used to assess the photo(cata)lytic treatment efficiency, not only by the removal of the parent compound but also by investigating whether the effects of the transformation products formed pose any greater risks.

The last part was performed in the framework of IX-AQUA (UPGRADING/DURABLE/0308/07) "Fate, effect and removal potential of xenobiotics present in aqueous matrices", 2009-2013, funded by the Cyprus Research Promotion Foundation through Desmi 2008, which is co-funded by the Republic of Cyprus and the European Regional Development Fund of the European Union. A four-month visit at the Department of Environmental Health Sciences, University Medical Center Freiburg to implement chronic toxicity assays with bacteria, biodegradability tests and a cytotoxicity-genotoxicity test on human cells under the guidance of Prof. Klaus Kümmerer and his research group, took place in 2010.

In summary, the selection of the major test endpoints used in this thesis was based on the legislation requirements (national and European) and on specific research objectives related to currently unanswered scientific questions, as discussed in the following chapters. For the acute exposures, survival and growth were used as endpoints. Survival was selected as it is easy to measure and a quite large toxicological database already exists, permitting comparison of the results. However, it should be mentioned that the major disadvantage of survival as an endpoint is that it usually lacks sensitivity. For the chronic exposures, growth was used as an endpoint. Growth is a more sensitive endpoint than survival, but is often characterized by greater variability due to its influence by test conditions that are less controllable than those of the survival tests. An endpoint for estrogenicity was investigated using a recombinant yeast assay, for increasing the understanding on the adverse effects that real matrices, such as treated wastewater may pose. The biodegradability potential of APIs was addressed. Finally, an endpoint for genotoxicity was studied, that enlightened less studied aspects of the biological potency of APIs.

CHAPTER 3. METHODOLOGY

3.1 Physicochemical assessment of the aqueous matrices used

Prior to the assessment of the various effects, the main physicochemical characteristics of the wastewater or of the solution treated by photo(cata)lysis, pH, temperature, salinity, dissolved oxygen and total free chlorine, were measured and adjusted according to the needs of the test to be performed. The parameters evaluated using Standard Methods [125] were conductivity, pH, temperature, dissolved oxygen (DO), biological oxygen demand (BOD_5), chemical oxygen demand (COD), total solids (TS), total suspended solids (TSS), ammonia and ammonium ions concentration, free chlorine and the concentration of selected heavy metals (Cd, Zn, Ni and Pb). Each experiment for the determination of these parameters was performed in triplicate.

3.1.1 Sample collection, handling and conservation

In order to examine wastewater samples, a specific strategy for the sampling procedure was designed, in which regulatory procedures for sampling were taken into account. A specific protocol for sampling and sample handling was prepared. Representative composite samples were collected from two treatment steps of three sewage treatment plants. The samples were taken in appropriately labelled amber glass bottles using an automatic sampler that collected a grab sample every 15 min for 24 h. Transportation to the laboratory was performed in ice cubes within two hours, and stored in darkness at 4 °C. The samples were tested within 72 h after collection. Collection and storage of samples was performed in such a way as to ensure that the toxicity of the samples obtained at source did not change markedly before the tests were conducted. Samples for toxicity testing were taken at least seasonally for the period 2007-2010 (four samplings/year) from the three sewage treatment plants. Spring samples were taken during March-May, summer samples during June-August, autumn samples during September-November and winter samples during December-February.

3.1.2 Chemicals used and solutions preparation

All chemicals used were at least of analytical grade. All the APIs used in the present thesis were provided by Sigma-Aldrich: Atenolol (A7655) ≥98% (TLC), (±)-metoprolol (+)-tartrate salt (M5361) ≥99% (TLC), D,L-propranolol hydrochloride (P-0884) ≥99% (TLC), diclofenac sodium salt (D6899), ibuprofen (14883) ≥98% (GC), erythromycin (E-7904), ofloxacin (O8757), sulfamethoxazole (S7507). A detailed description of each chemical used is included in the following subsections. All measurements were performed on an analytical balance (Shimadzu, AUW320). All solutions were performed using ultrapure water produced by Milli-Q & Elix water purification system. If solubility was not achieved using deionised water, an organic solvent was used (e.g., methanol, ethanol, dimethyl sulfoxide).

3.1.3 Parameters examined

3.1.3.1 Conductivity

Conductivity is a measure of the ability of an aqueous solution to carry an electric current. It depends on the presence of the total concentration, mobility, and valence of ions, and on the temperature of measurement. Solutions of most inorganic compounds are relatively good conductors. Conversely, molecules of organic compounds that do not dissociate in aqueous solution conduct very poorly current, if at all. The method implemented was 2510 B with the use of a Multi Parameter Equipment, measuring dissolved oxygen, conductivity, temperature and pH (WTW InoLab Multilevel 3).

3.1.3.2 pH

The pH or hydrogen ion activity describes the acidic or basic character of a solution, at a given temperature. pH is the -log [H^+], an "intensity" factor of acidity. The electrometric pH measurement was used (Method 4500-H^+ B) on a Multi Parameter Equipment, measuring dissolved oxygen, conductivity, temperature and pH (WTW InoLab Multilevel 3). A pH meter consisting of a potentiometer, a glass electrode, a reference electrode, and a temperature-

compensating device was used for the measurement. The electromotive force produced in the glass electrode system varies linearly with pH. This linear relationship is described by plotting the measured electromotive force against the pH of different buffers. Sample pH is determined by extrapolation. The electrode system was calibrated against two or three standard buffer solutions of known pH, rinsed and dried with a soft tissue prior pH measurement of the samples.

3.1.3.3 Temperature

The temperature has an impact on most of the physicochemical characteristics and was calculated in parallel with most parameters. The method 2550 was applied using a Multi Parameter Equipment, measuring dissolved oxygen, conductivity, temperature and pH (WTW InoLab Multilevel 3).

3.1.3.4 Dissolved oxygen

This method requires an instrument with a variable length of "gas permeable" tubing, connected to a pressure-measuring device. Dimethyl silicone rubber tubing often is used because it is highly permeable to dissolved gases, including water vapor. At steady state, the gauge pressure inside the tubing is equal to the difference in gas pressure (ΔP) between the total dissolved gas pressure and the ambient barometric pressure. When the water is in equilibrium with the atmosphere, ΔP equals zero. A dissolved oxygen meter was used and the method 2810 was applied using a Multi Parameter Equipment, measuring dissolved oxygen, conductivity, temperature and pH (WTW InoLab Multilevel 3).

3.1.3.5 Biological oxygen demand

The biochemical oxygen demand (BOD_5) determination is an empirical test in which standardized laboratory procedures are used to determine the relative oxygen requirements of wastewater, effluents, and polluted water. The test measures the molecular oxygen utilized during a specified incubation period for the biochemical degradation of organic material

(carbonaceous demand) and the oxygen used to oxidize inorganic material such as sulfides and ferrous iron. It may also measure the amount of oxygen used to oxidize reduced forms of nitrogen (nitrogenous demand) unless their oxidation is prevented by an inhibitor. Measurements of oxygen consumed in a 5 d test period were assessed.

The method 5210 B was applied which consists of filling with sample to overflowing, an airtight bottle of a specified volume and incubating it at 20 °C for 5 d. A BOD Measurement System (444406 OxiDirect) was used. Dissolved oxygen was measured initially and after incubation, and the BOD was computed from the difference between initial and final DO.

3.1.3.6 Chemical oxygen demand

Chemical oxygen demand (COD) is defined as the amount of a specified oxidant that reacts with the sample under controlled conditions. The quantity of oxidant consumed is expressed in terms of its oxygen equivalence. According to 5220 B method, a sample is refluxed in strongly acid solution with a known excess of potassium dichromate ($K_2Cr_2O_7$). A 2 h reflux time was used. When a sample is digested, the dichromate ion oxidized the COD material in the sample. This resulted in the change of chromium from the hexavalent (VI) state to the trivalent (III) state. Both of these chromium species are colored and absorb in the visible region of the spectrum. The dichromate ion ($Cr_2O_7^{2-}$) absorbs strongly in the 400-nm region, where the chromic ion (Cr^{3+}) absorption is much less. The chromic ion absorbs strongly in the 600-nm region, where the dichromate has nearly zero absorption.

3.1.3.7 Total solids

A well-mixed sample of 20 mL was evaporated in a weighed dish and dried to constant weight in an oven at 103 to 105 °C for 1 h, according to the method 2540. The increase in weight over that of the empty dish represents the total solids.

The calculation was performed using Equation 3.1.

$$mg\ total\ dissolved\ solid\ L^{-1} = \frac{(A - B) \times 100}{sample\ volume, mL}$$

$$A = weight\ of\ dried\ residue + dish, mg$$

$$B = weight\ of\ dish, mg$$

Equation 3.1 Calculation of total solids

3.1.3.8 Total suspended solids

The method 2540 was applied. A homogenized sample of 20 mL is filtered through a weighed standard glass-fibre filter and the residue retained on the filter is dried for minimum 1 h to a constant weight at 103 to 105 °C. The increase in weight of the filter represents the total suspended solids (TSS). The calculation is performed using the Equation 3.2.

$$mg\ total\ dissolved\ solid\ L^{-1} = \frac{(A - B) \times 100}{sample\ volume, mL}$$

$$A = weight\ of\ filter + dried\ residue, mg$$

$$B = weight\ of\ filter, mg$$

Equation 3.2 Calculation of total suspended solids

3.1.3.9 Ammonia and ammonium ions concentration

The Nessler method was used since it is a classic water quality measurement for more than a century. A distillation step using $MgCO_3$ and pumice stone in a heating mantle (BIBBY) was taken prior adding the Nessler solution. A tintometer (Nessleriser 2150) was used to compare the colour of the sample with a reference colour disk.

The following equations were used:

$$[NH_4{}^+]\ in\ mg\ L^{-1} = number\ of\ colour\ disk \times 0.004 \times dilution$$

$$[NH_3]\ in\ mg\ L^{-1} = number\ of\ colour\ disk \times 0.0033 \times dilution$$

Equation 3.3 Calculation of ammonia and ammonium ions concentration

3.1.3.10 Free chlorine

The chlorination of wastewater is used to destroy or deactivate pathogenic microorganisms and to improve its quality from the reaction of chlorine with ammonia, iron, manganese, sulfide, and other organic substances. However, it may produce adverse effects. Potentially carcinogenic chloro-organic compounds such as chloroform may be formed. Combined chlorine formed on chlorination of ammonia- or amine-bearing wastewater may adversely affect aquatic life.

Free chlorine includes aqueous molecular chlorine, hypochlorous acid, and hypochlorite ion. At the pH of wastewater, hypochlorous acid and hypochlorite ion usually predominate.

Free chlorine as hydrochloric and hypochlorous acids were measured by a colorimetric method. The reaction was buffered at approximately 6.3 pH; by oxidizing N,N-diethyl-p-phenylenediamine (DPD) with the amount of free chlorine and producing a reddish colour. The colour intensity of the solution determines the free chlorine concentration.

3.1.3.11 Heavy metals

A selection of four heavy metals was made. Cadmium (Cd), lead (Pb), nickel (Ni) and zinc (Zn) were monitored due to their high toxicity and legislation requirements. An Atomic Absorption Spectrometer (AAS) (Perkin Elmer - Aanalyst 200) was used and the method 3111B was implemented.

In flame atomic absorption spectrometry, a sample is aspirated into a flame and atomized. A light beam is directed through the flame, into a monochromator, and onto a detector that measures the amount of light absorbed by the atomized element in the flame. Because each metal had its own characteristic absorption wavelength, a source lamp composed of that element was used. Furthermore, the amount of energy at the characteristic wavelength absorbed in the flame is proportional to the concentration of the element in the sample over a limited concentration range. The AAS consists of a light source emitting the line spectrum of the elements studied, a flame for vaporizing the sample, a means of isolating an absorption

line and a photoelectric detector with its associated electronic amplifying and measuring equipment.

All glassware on the previous day of the experiment was washed with 10% HNO_3 prior use and left to dry. Ten mL 70% HNO_3 were added to 100 mL of samples and heated for 1 h at 90-95 °C. The samples were then filtered and deionised water was used to reach 100 mL. Stock solutions of 100 mg/L were used for the preparation of calibration curves.

3.1.3.12 Non-purgeable organic carbon

The organic carbon in solutions is usually composed of a variety of organic compounds in various oxidation states. The non-purgeable organic carbon, which is the fraction of Total Organic Carbon (TOC) not removed by gas stripping, was measured in the present thesis since the substances under study are not volatile.

A stock solution of 1000 mg/L TOC was prepared by dissolving 0.2125 g of oven-dried (110 °C for 1 h) anhydrous potassium hydrogen phthalate (KHP) in 100 mL Milli-Q water. A calibration curve was prepared using dilutions of the stock solution depending on the range of the TOC. Solutions of 7.5, 15 and 22.5 mg/L TOC were used as standards during monitoring of NPOC in samples.

The sample was acidified with HCl prior to being set in the equipment. The sample was sparged with high purity air, eliminating the inorganic content component. The sample was then injected into the column and combusted to CO_2. The CO_2 was detected by a non-dispersive infrared gas analyzer producing a detection signal generating a peak. The area of the peak is proportional to the carbon component of the sample and its concentration is calculated by comparing to that of the standard solutions.

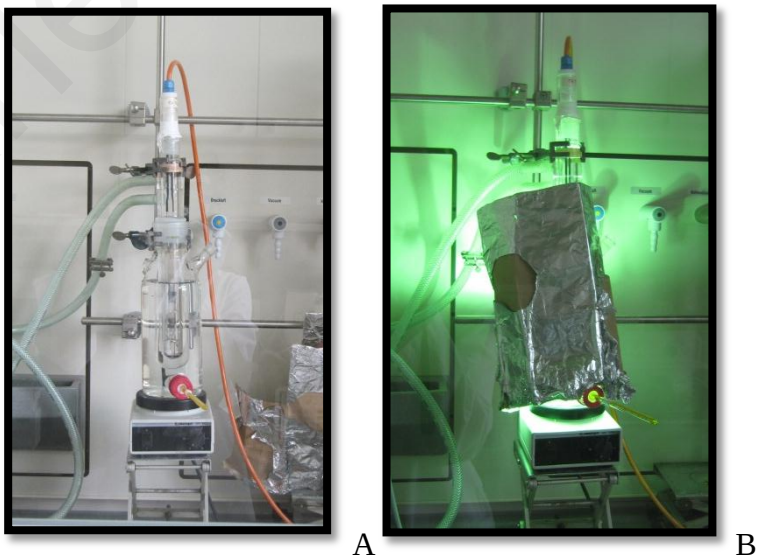
Samples were taken at various times (0, 2, 4, 8, 16, 32, 64 and 128 min) during the irradiation process (photo(cata)lytic) and passed through a 0.22 μm Milli-pore filter for measuring the Dissolved Organic Carbon (DOC). The concentration of DOC was assessed as

Non-Purgeable Organic Carbon (NPOC) with the use of a TOC- analyzer (Shimadzu, TOC-5000).

3.2 Photo-transformation experiments

Photo-transformation was achieved during photo(cata)lytic treatment. Photo-transformation was performed in a 850 mL Pyrex glass cylindrical immersion-type photoreactor containing 750 mL of ofloxacin solution (20 mg/L in water), shown in Picture 3.1. Photolytic irradiation experiments were performed using a medium pressure mercury vapour lamp 150 W (Heraeus TQ150, Hanau, Germany). The lamp emits polychromatic radiation in the range of 200-436 nm, with peak intensities at 254, 265, 302, 313 and 366 nm.

Photocatalytic experiments were performed in the same way as the photolytic experiments with the additional input of TiO_2 in a concentration of 1 g/L according to Hapeshi et al. [278], which is considered as the optimum catalyst dose for ofloxacin at the specific concentration levels. Prior to irradiation, the suspension was magnetically stirred at 700 rpm for 30 min in the dark, to ensure complete equilibration of adsorption/desorption of the substrate on the catalyst surface. Overnight dark experiments did not show higher adsorption of the substrate on the catalyst surface.



A B

Picture 3.1 Photoreactor (A, B) in which photo-transformation experiments were performed

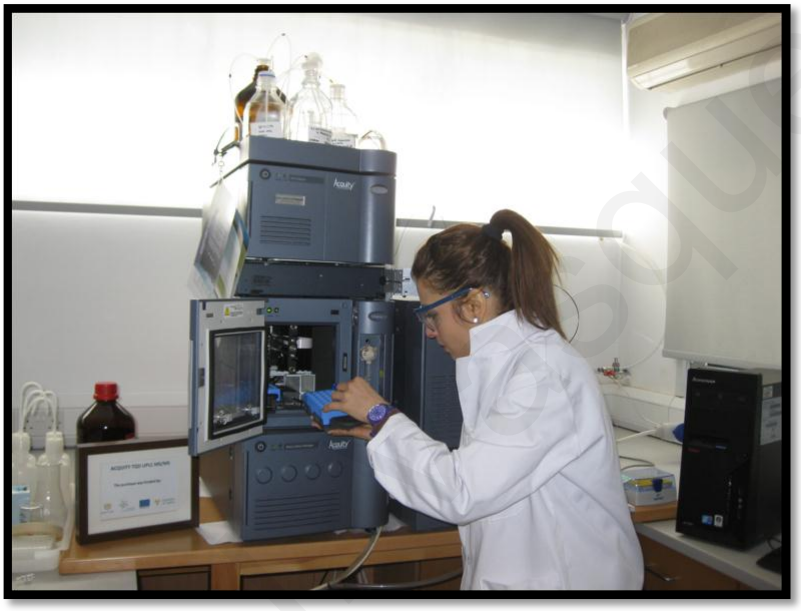
Photo(cata)lytic treatment at various times (0, 2, 4, 8, 16, 32 and 64 min) was examined, during which the solution was magnetically stirred, the temperature was kept constant at 20±1 °C through a cooling circuit and the pH of the solutions was neutral (inherent solution pH). All samples taken were sterile-filtered through a 0.22 μm filter (Milli-pore) and used as follows: (i) 15 mL for DOC monitoring, assessed on the same day of the experiment, (ii) 2 mL for UPLC-MS/MS analysis, stored at -80 ºC for maximum one month, (iii) 50 mL for chronic ecotoxicity experiment, left overnight before testing to reduce negative effects due to potential free radicals and other Reactive Oxygen Species (ROS) formed during the treatment processes applied and (iv) 5 mL for cytokinesis-block micronucleus cytome assay, purged with N_2O again to reduce O_2, potential free radicals and ROS formed and aliquoted in 500 μL and stored at -20 ºC for maximum one month.

3.3 Liquid chromatrography mass spectromentry analysis

The chromatographic analysis was performed with an ACQUITY TQD UPLC-MS/MS system (Water, UK) equipped with a sample organiser and sample, binary-solvent and column manager. A triple quadrupole mass spectrometer coupled with electrospray ionization (ESI) source was used for sample analysis. The equipment is shown in Picture 3.2. Data acquisition was performed with MassLynx^{TM} software. Chromatographic analysis lasted 9 min. The LC analysis conditions were: T_{column}=60 ^{o}C, flow=0.3 mL/min, run time=9 min, partial loop with needle overfill. The weak wash was H_2O + 0.1% formic acid whereas the strong wash was the eluent B. The column used for LC analysis was the BEH Shield RP18. The LC analysis was performed using 0.1 mM ammonium acetate in water + 0.01% formic acid as eluent A and 0.1 mM ammonium acetate in MeOH + 0.01% formic acid as eluent B. The elution gradient was: 0 min 5% B, 1.5 min 5% B, 2 min 30% B, 3 min 50% B, 5 min 70%, 6 min 90% B, 7 min 90% B, 7.1 min 5% B, 9 min 5% B.

A triple quadrupole mass spectrometer (TQD) was used for the detection of target analytes. In order to achieve sufficient sensitivity target for the quantitative analysis, data acquisition was performed with ESI in multiple reaction monitoring mode (MRM), recording the transitions between the precursor ion and the most abundant fragment ions. The electrospray ionization was operated in positive mode and the protonated molecular ion

$[M+H]^+$ was selected as the precursor ion for both analytes. Precursor and product ions with their associated collision energies and retention times are summarized in Table 3.1, together with the operating MS/MS parameters. The two most abundant fragment ions were chosen according to the intensity and the signal to noise ratio. The first transition was used for quantification of the target compound, while the second transition, together with the ratio of two transitions, was used for confirmation purposes (qualifier).



Picture 3.2 Ultraperformance liquid chromatography/tandem mass spectrometry system

Table 3.1 Retention time and MS/MS parameters for the target analytes

Analyte	Retention time (min)	Precursor ion (m/z)	Cone Voltage (V)	Capillary Voltage (kV)	Product ions (m/z)	CE (V)
(OFL)	3.14	362.1	28	3	261.2 58.15	27 35

MS/MS Parameters	
Resolution=0.75 Da	Ion energy 1=0.5
Extractor=3 V	Ion energy 2=1
RF Lens=0.1 V	Low mass resolution 1=7.3
High mass resolution 1=14.5	Low mass resolution 2=10.5
High mass resolution 2=14.7	Source temperature=150 °C
Desolvation temperature=500 °C	Infusion flow rate=20 μL/min
Desolvation flow=1000 L/h	Cone gas=10 L/h
Collision gas (Ar)=0.15 mL/min	

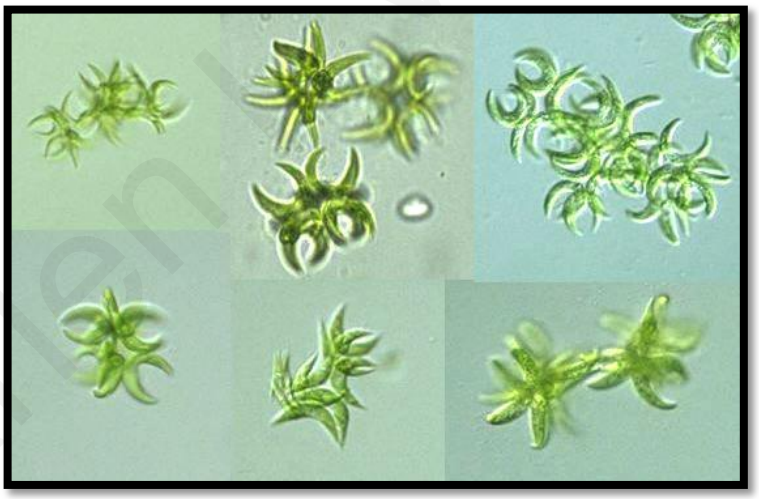
A quality control scheme was applied in parallel with the analysis of the samples from photo(cata)lytic treatment. Ofloxacin samples with concentration of 1-20 mg/L were used for

the quality control. The calibration curve used for calculations had a R^2=0.972. Samples were diluted 1:5 with water prior analysis. The Limit of Detection (LOD) of the method was calculated according to the ISO 11843-2 [279], with a confidence interval of 99% using the standard deviation of a linear regression curve for a concentration range of 0.01-100 µg/L. The LOD and Limit of Quantification (LOQ) for ofloxacin were 4.5 and 9.1 ng/L, respectively.

3.4 Assessment of effects using traditional and advanced bioassays

3.4.1 *Pseudokirchneriella subcapitata*

Algae as primary producers are key organisms in the food chain of aquatic ecosystems, being the primary food source for many planktonic organisms and fish. Algae are autotrophic; hence they can synthesize the required organic compounds for their nutrition using solar energy and simple inorganic molecules, such as CO_2 and H_2O.



Picture 3.3 ***Pseudokirchneriella subcapitata*** [280]

P. subcapitata (Picture 3.3) taxonomy is shown in Table 3.2. It belongs to the freshwater green algae. The test used in this thesis is described by OECD 201 [281] and its objective was to determine the effects of selected matrices and/or APIs on its growth. An exponentially growing culture was exposed to a sample following a batch mode over a period of 72 h. In

spite of the relatively brief test duration, effects over several generations were assessed. Hence this bioassay is considered as a chronic one.

The response was evaluated as a function of the exposure in comparison with the average growth of replicate, unexposed control cultures. Growth and growth inhibition can be quantified by measurements of the algal biomass as a function of time. Algal biomass is defined as the dry weight per volume, e.g., mg algae/L test solution. However, dry weight is difficult to measure and therefore surrogate parameters are usually used. In the present thesis, the optical density (OD) at 670 nm was converted to biomass by an equation provided by the kit manufacturer.

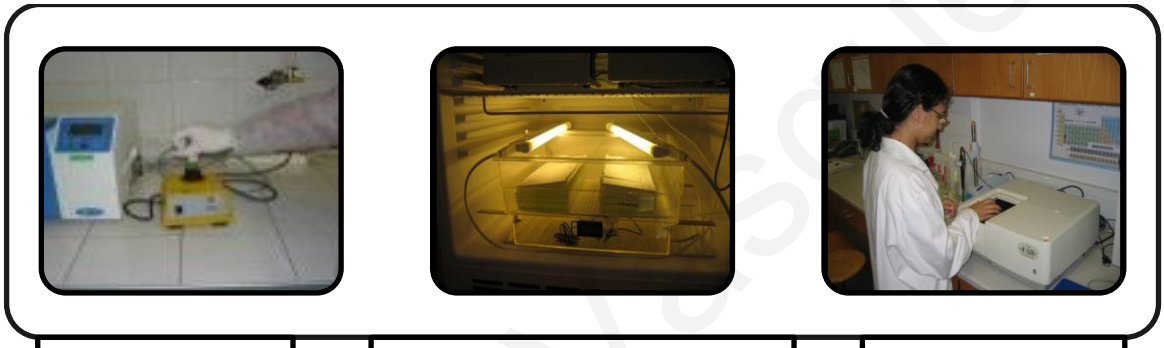
Table 3.2 Taxonomy of *Pseudokirchneriella subcapitata*

Kingdom	*Plantae*
Subkingdom	*Viridaeplantae*
Phylum	*Chlorophyta*
Class	*Chlorophyceae*
Genus	*Pseudokirchneriella*
Species	*Pseudokirchneriella subcapitata*

The tests were performed using the Algaltoxkit F^{TM} with *P. subcapitata* microalgae, as presented in Schematic 3.1. Algal cultural medium was prepared using different nutrients and its pH was adjusted to 8±0.2 °C. The algae beads were then de-immobilized using a matrix dissolving medium and vigorous shaking with a Vortex mixer (Heidolph, UK) and a centrifuge (Sigma, Germany) at 3000 rpm. The algal cultural medium was added to the de-immobilized algae beads in order to create the algal inoculum. The algal inoculum was transferred to the Algal Stock Cell and filled up with algal cultural medium in order to achieve an initial density of 1×10^6 cells/L. 100 mL of five different concentrations causing 5-100% inhibition were prepared in algal cultural medium and 1 mL of the algal inoculum was added to achieve an initial concentration of 1×10^4 cells/L algae.

Each test concentration was tested in triplicate. A preliminary range-finding test was performed for each API. At least three independent experiments were performed for the APIs. All cells were put in a holding tray randomly and a plastic strip was slided in order to leave

some opening near the middle of the long cells for gas exchange. The holding tray was transferred in an incubator with constant uniform sideway illumination of 10000 lux supplied by a cool white fluorescent lamp. The temperature was kept at 25 °C. Positive tests were performed using different concentrations of potassium dichromate ($K_2Cr_2O_7$). Inhibition of the algal growth relative to the control was determined by daily measurement of the OD at 670 nm of the algal suspensions in the long cells during the three days of the test, 24, 48 and 72 h exposure time to the testing sample. The number of cells was calculated using a reference chart provided by the kit manufacturer.



Day 0: Preparation
- Preparation of algal cultural medium and pH adjustment
- Preparation of test solutions
- Pretreatment of samples (filtration) (if applicable)

Day 1: Exposure
- De-immobilization of algae beads
- Preparation of algal inoculum at initial density of 10^6 cells/L
- Preparation of 5 different concentrations for each test solution
- Transfer of 1 mL of algal culture for 100 mL test solution
- Division of test solutions in three long cells
- Calculation of OD at 670 nm
- Incubation at 25 °C under continuous illumination (10000 lux)

Day 2-4: Exposure time 24-72 h
- Calculation of OD at 670 nm
- Calculation of growth rate and relevant parameters

Schematic 3.1 Presentation of the test procedure for *Pseudokirchneriella subcapitata*

The response variable used was defined as the biomass at the end of the exposure period minus the biomass at the start of the exposure period. The dose-response relationship was calculated by a regression analysis and as a result thereof, EC_{50} value on growth was determined. The validity criteria used for the tests, according to OECD [281] were the following:

- The biomass in the control cultures increased exponentially by a factor of at least 16 within the 72 h test.
- The mean coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2 and 2-3, for 72 h tests) in the control cultures did not exceed 35%.
- The coefficient of variation of average specific growth rates during the whole test period in replicate control cultures did not exceed 7%.
- The EC_{50} value ranged between 0.40-0.49 mg/L in the quality control test.

3.4.2 *Lepidium sativum*

Plantae are autotrophic organisms that serve as bioindicators for terrestrial ecosystems. In the case of semi-arid countries, such as Cyprus, which rely on the reuse of treated wastewater for irrigation purposes, the use of such bioindicator to monitor treated wastewater quality should be considered indispensable.

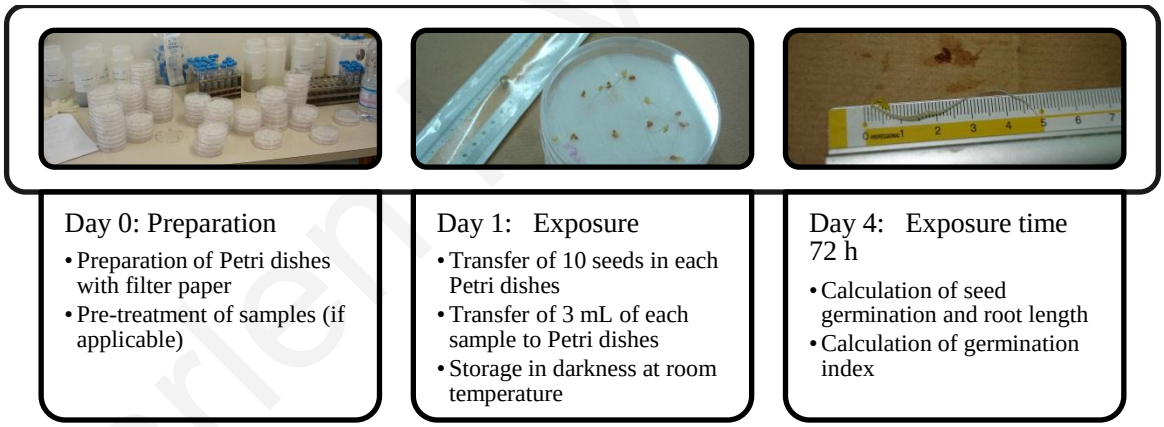
Picture 3.4 *Lepidium sativum* [282]

The taxonomy of *L. sativum* (Picture 3.4) is provided in Table 3.3.

Table 3.3 Taxonomy of *Lepidium sativum*

Kingdom	*Plantae*
Division	*Magnoliophyta*
Class	*Magnoliopsida (Dicotyledonae)*
Order	*Capparales*
Family	*Brassicaceae*
Genus	*Lepidium*
Species	*Lepidium sativum*

Phytotoxicity tests were performed using *L. sativum* (garden cress) seeds. A variation of the Guideline 208 was used [283] in which potential effects of the tested samples on seeding emergence and growth were assessed. A description of the method is presented in Schematic 3.2. Ten seeds were placed in a Petri dish with filter paper that acted as an artificial substrate and exposed to 3 mL of samples at different concentrations for 72 h at 25 °C under dark conditions. Each concentration was assessed in triplicate.



Schematic 3.2 Presentation of the test procedure for *Lepidium sativum*

After this exposure period, the germinated seeds were evaluated for seed germination, root elongation and germination index (GI factor of relative seed germination and relative root elongation) as determined according to the following Equations:

$$Relative\ seed\ germination\ (\%) = \frac{Number\ of\ seeds\ germinated\ in\ sample}{Number\ of\ seeds\ germinated\ in\ control} \times 100$$

Equation 3.4 Calculation of seed germination

$$Relative\ root\ elongation\ (\%) = \frac{Mean\ root\ elongation\ in\ sample}{Mean\ root\ elongation\ in\ control} \times 100$$

Equation 3.5 Calculation of root elongation

$$Germination\ Index\ (\%) = \frac{Relative\ seed\ germination \times Relative\ root\ elongation}{100}$$

Equation 3.6 Calculation of germination index

A dose-response curve was created in order to calculate the EC_{50} value for the samples using appropriate statistical analysis. The validity criteria set is in accordance with the OECD guidelines (OECD, 2006) and for the control tests are as follows:

- The relative seed germination was at least 70%
- No visible phytotoxic effects were noticed (e.g., necrosis, chlorosis)
- The mean survival of emerged seeds was at least 90%

3.4.3 *Daphnia magna*

Daphnids are freshwater organisms that have been used intensively throughout the last three decades for assessing the effects of chemicals in regulatory testing or for measuring the toxicity of water samples. These planktonic crustaceans (Picture 3.5) are widely distributed in ponds and lakes and are representative of many ecologically filter feeding zooplankton [284].

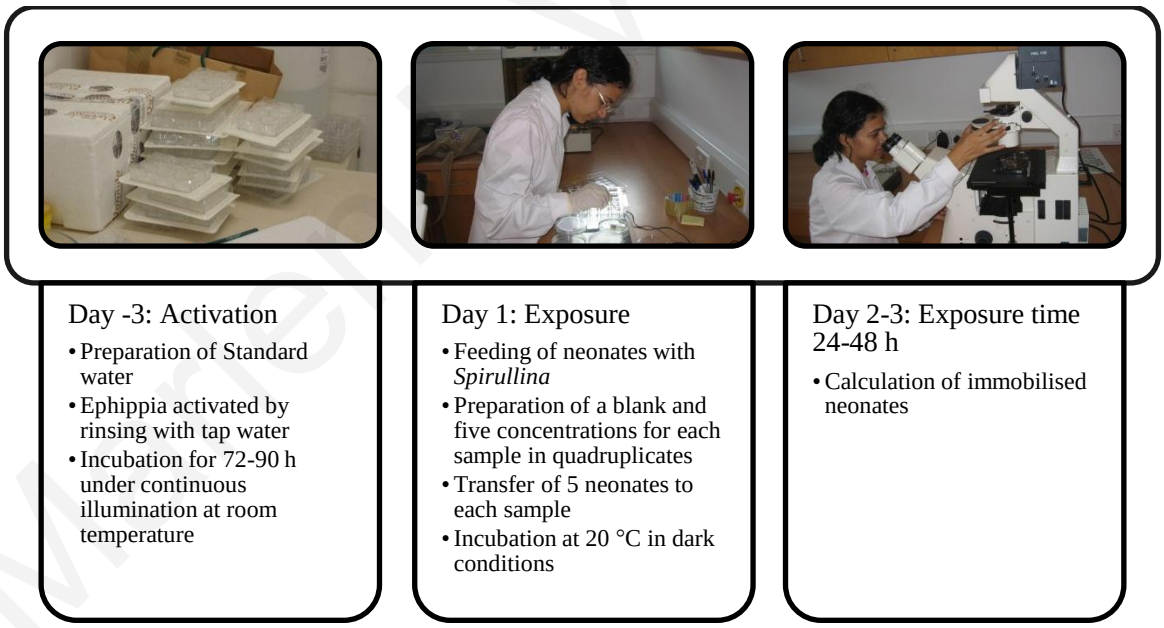
Picture 3.5 *Daphnia magna* nauplius [285]

Its taxonomy is shown in Table 3.4.

Table 3.4 Taxonomy of *Daphnia magna*

Kingdom	*Animalia*
Phylum	*Arthropoda*
Subphylum	*Crustacea*
Class	*Branchiopoda*
Order	*Cladocera*
Family	*Daphniidae*
Genus	*Daphnia*
Species	*Daphnia magna*

The tests were performed using the *Daphtoxkit FTH magna* according to the OECD protocol [286]. The procedure followed is shown in Schematic 3.3 and described in detail below.



Schematic 3.3 Presentation of the test procedure for *Daphnia magna*

Ephippia were activated by rinsing with tap water and hatched for 72-90 h before testing in Standard Freshwater suitable for *Daphnia* at 20-22 ºC under continuous illumination of 6000 lux. The Standard Freshwater was prepared using $NaHCO_3$, $CaCl_2$, $MgSO_4$, and KCl. Two

hours before testing, the neonates were fed using a dilution of *Spirulina* microalgae in order to reduce mortality caused by starvation, which can bias the test results. During the subsequent 48 h of test exposure the organisms were not fed. One hundred and twenty neonates were used to perform the tests. Five daphnids were tested in quadruplicate for each dilution in specific test plates. Five different concentrations of testing substances/matrices were prepared adding Standard Freshwater and a control using only Standard Freshwater was also tested. The multi-well plates were covered and incubated at 20 ºC, in darkness. After 24 and 48 h of incubation, the number of dead and/or immobilized neonates was calculated. Positive tests were performed using different concentrations of potassium dichromate ($K_2Cr_2O_7$).

The dose-response relationship was calculated by a regression analysis and as a result thereof, EC_{50} value referring to immobilization was determined. The validity criteria applied were the following [286]:

- The percentage of mobility in control vessels was less than 10% at the end of the test. Signs of stress or disease, such as discolouration and unusual behaviour, were also considered as immobilization.
- The DO concentration at the end of the test was ≥3 mg/L in the control and test vessels.
- The EC_{50} value for the positive control ranged between 0.6-2.1 mg/L.

3.4.4 *Artemia salina*

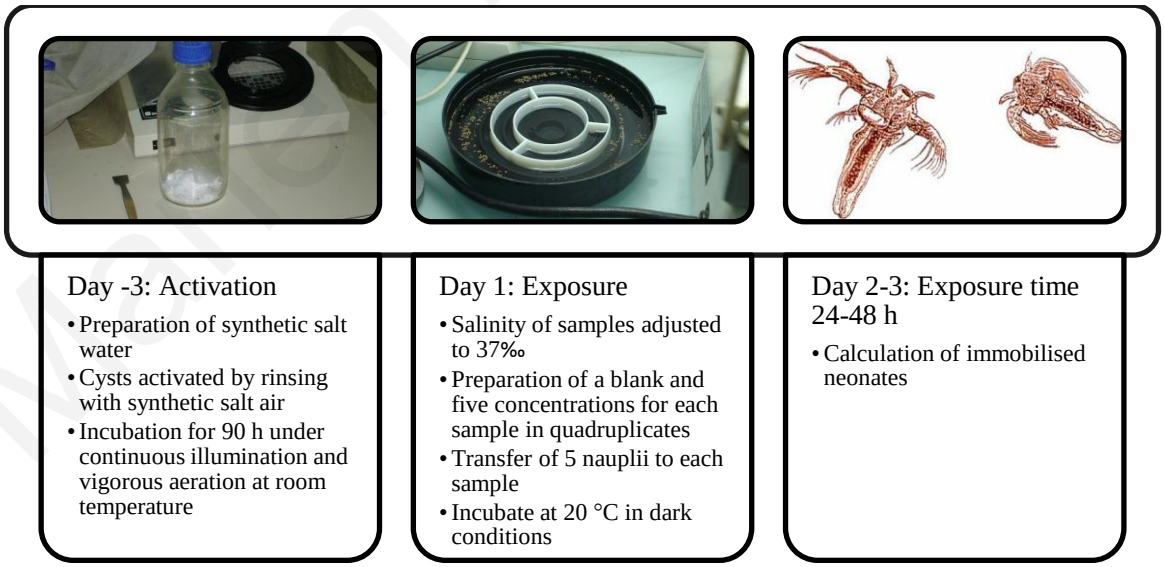
A. salina is a well-known crustacean that is a key organism in extreme environments of high salinity, such as inland saltwater lakes. It has high nutritional properties in lipids and unsaturated fatty acids [287]. It is the main food source of *Phoenicopterus spp*.

In Cyprus, *A. salina* has been reported only in the Larnaka Salt Lake [289]. Larnaka Salt Lake is a Ramsar site due to its ecological importance, making *A. salina* an extremely significant organism in the specific ecosystem, as it is the main nutritional source of the birds present. Its taxonomy is the provided in Table 3.5.

Picture 3.6 *Artemia salina* nauplius [288]

Table 3.5 Taxonomy of *Artemia salina*

Kingdom	*Animalia*
Phylum	*Arthropoda*
Subphylum	*Crustacea*
Class	*Branchiopoda*
Order	*Anostraca*
Family	*Artemiidae*
Genus	*Artemia*
Species	*Artemia salina*



Schematic 3.4 Presentation of the test procedure for *Artemia salina*

In the present thesis *A. salina* cysts were collected from the Larnaka Salt Lake to perform acute toxicity assays (Schematic 3.4). The cysts were collected using sandboxes of different sizes. The cysts were then cleaned and washed using salted water (37‰) and left to dry in the sun. Before testing, cysts were hatched in synthetic salted water (37‰) and nauplii less than 24 h were used to perform the tests.

The procedure followed [124] is similar to the procedure for testing *Daphnia* nauplii with the following differences: all dilutions were prepared using synthetic salted water, salinity of samples was adjusted to 37‰ and the *Artemia* nauplii were not fed before testing. Immobilization of *Artemia* nauplii was evaluated at 24 and 48 h. Both negative control with synthetic salted water and positive control with $K_2Cr_2O_7$ tests were performed in parallel.

The dose-response relationship was calculated by a regression analysis after log-probit transformation of the value pairs and as a result thereof, EC_{50} value referring to immobilization was determined. The validity criteria applied were the same as those applied to *D. magna* (Section 3.4.3). The EC_{50} value for the positive control ranged 1-1.8 mg/L.

3.4.5 *Vibrio fischeri*

V. fischeri (former *Photobacterium phosphoreum*) is a Gram-negative rod-shaped bacterium found globally in marine environments. It is heterotrophic and moves by means of flagella. Free living *V. fischeri* survives on decaying organic matter.

Table 3.6 Taxonomy of *Vibrio fischeri*

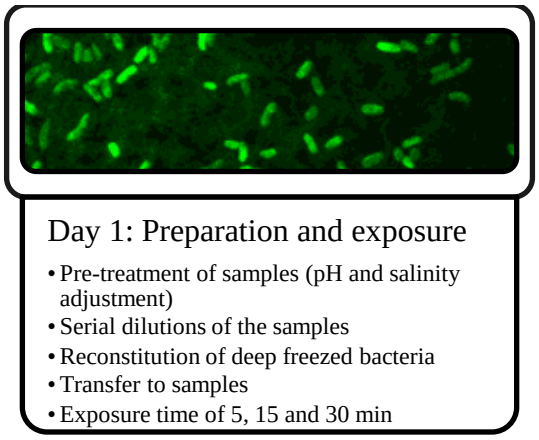
Kingdom	*Bacteria*
Phylum	*Proteobacteria*
Class	*Gamma Proteobacteria*
Order	*Vibrionales*
Family	*Vibrionaceae*
Genus	*Vibrio*
Species	*Vibrio fischeri*

V. fischeri is best known as the specific symbiont in the light-emitting organs of certain squids and fishes, such as *Euprymna scolopes* (bobtail squid), where it produces bioluminescence by expressing the *lux operon*, a small cluster of genes found in several of the *Vibrionaceae* [290]. The taxonomy of *V. fischeri* is shown in Table 3.6.

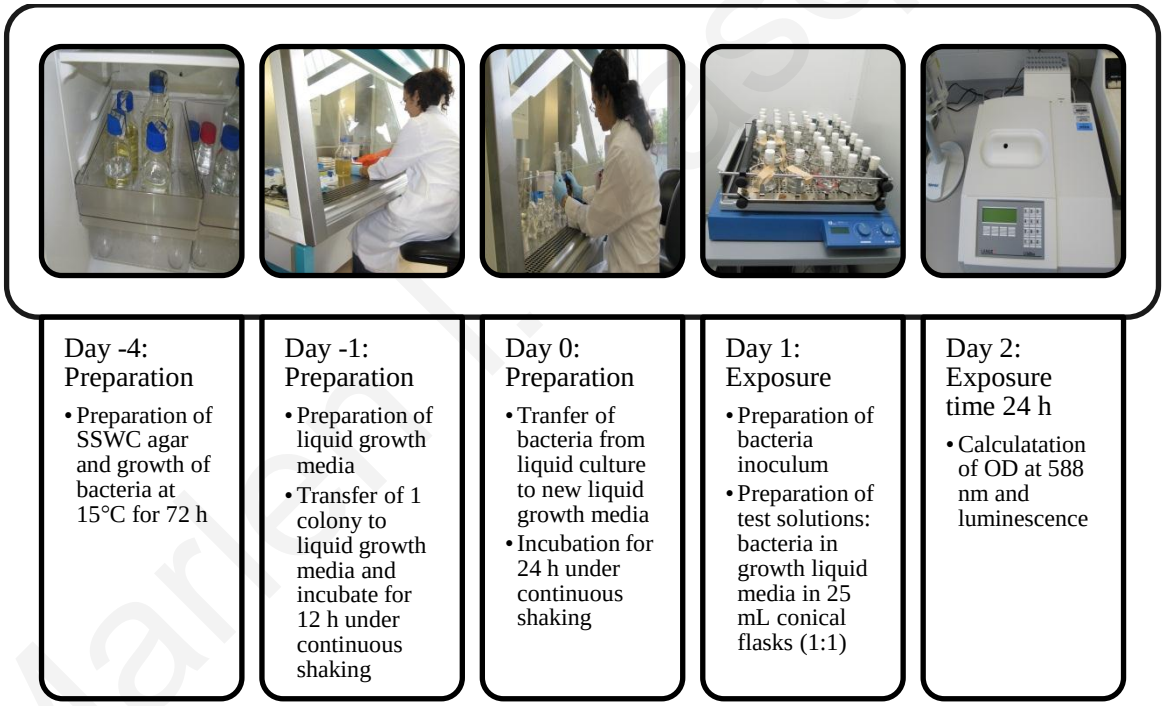
For nearly 25 years now, the bioluminescent bacteria test with the marine bacterium *V. fischeri* has become a basic test for ecotoxicological testing of chemicals, wastewater and eluates from soil and sediment. It can be carried out by using freshly prepared bacteria, as well as liquid-dried or freeze-dried bacterial preparations. The test system gives a rapid method for determining the toxicity of an aqueous solution by determining the inhibition of the luminescence emitted by *V. fischeri* after a given exposure time, usually 15, 30 min or 24 h.

V. fischeri bacteria (NRRL B-11177) were tested to obtain percentile bioluminescence inhibition during 5 and 15 min exposure times. The Microtox® assay was performed in accordance with the operational procedures from Azur Environmental Ltd. Lyophilized bacteria (approx. one million in one preparation), using the Microtox® kit, were reconstituted by adding a reconstitution solution, and then the suspensions were sequentially diluted and tested at 15 °C in parallel to a negative control test (Schematic 3.5). The light transmissions in both negative control and samples were recorded by a luminometer (Microtox® Model 500 Analyzer, UK). All samples were adjusted at pH 8 and salinity 2%. A basic test was conducted with the reference standard phenol for each fresh vial of bacteria to ensure the validity of the test method.

A chronic toxicity assay with *V. fischeri* was applied (Schematic 3.6). The bacteria were grown in petri dishes with Seawater Complete (SWC) agar (15 g NaCl, 3.05 g NaH_2PO_4, 1.049 g K_2HPO_4, 0.102 g $MgSO_4.7H_2O$, 0.25 g $(NH_4)_2HPO_4$, 2.5 g peptone, 0.25 g yeast extract, 1.5 mL glycerol 87%, 7.5 g agar in 500 mL Milli-Q water) for 72 h at 15±0.2 °C. A single culture was selected and grown in suspension (20 mL of SWC medium without adding agar) for 12 h in a dark climatic chamber at 15±1 °C under continuous shaking (180 rpm, orbital diameter 30 mm, KS 501 digital, IKA Werke GmbH & Co. KG, Staufen, Germany). A second cultivation of the desired bacteria density was then prepared in 120 mL of medium and incubated at the same conditions for 24 h.



Schematic 3.5 Presentation of the test procedure for *Vibrio fischeri* acute assay



Schematic 3.6 Presentation of the test procedure for *Vibrio fischeri* chronic assay

The ratio of growth medium:test sample in each experiment was 1:1. Tests were performed in a dark climatic chamber at 15±1 °C under continuous shaking. The OD at 588 nm and bioluminescence were the endpoints of bacterial growth recorded. The OD was measured using a UV spectrophotometer (Ultrospec III, Pharmacia LKB Biosystems GmbH,

Freiburg) and bioluminescence using a luminometer (LumiStox, Hach Lange GmbH, Düsseldorf, Germany).

Each independent experiment was performed in triplicate and included a control (2% NaCl), a positive control (18% NaCl), test samples with adjusted salinity (2%) and a negative control series in which only the growth medium was added.

The inhibition expressed as a percentage was calculated using Equation 3.7.

$$I = 100 - [(I_c - I_s)/I_c \times 100]$$

I: % Inhibition, I_c: bioluminescence of control, I_s: bioluminescence of sample

Equation 3.7 Calculation of inhibition for *Vibrio fischeri*

The dose-response relationship was calculated by a regression analysis and as a result thereof, EC_{50} value on growth was determined. The results of the experiments were considered as valid if the below criteria were met:

- The mean correction factor in the control was in the range of 0.6–1.8.
- The parallel determinations did not deviate more than 3%.

3.4.6 *Pseudomonas putida*

P. putida is a widely distributed Gram-negative rod-shaped saprotrophic soil bacterium, which is very important for the rhizosphere and the plant health in general. It has multiple polar flagella for motility.

Its taxonomy is shown in Table 3.7. It is able to tolerate environmental stresses due to its diverse control of proteins. *P. putida* has a very diverse aerobic metabolism that is able to degrade organic solvents, such as toluene, and also to convert styrene oil to biodegradable plastic Polyhydroxyalkanoates. It can degrade the hydrocarbons of these organic solvents through oxidative reactions and therefore it is considered as one of the most important microbes in bioremediation [292].

Picture 3.7 *Pseudomonas putida* [291]

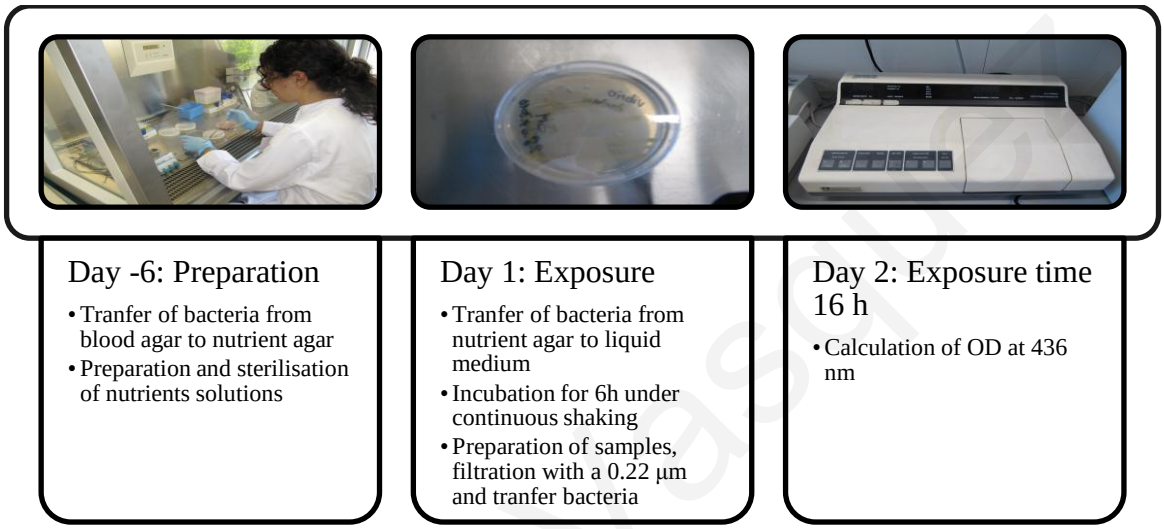
The test method ISO 10712 [293] was used for determining the inhibition of selected samples on the growth of *P. putida*. The bacteria *P. putida* were provided by the Hospital Hygiene Section, Department of Environmental Health Science, Freiburg. The procedure followed is briefly described herein and presented in Schematic 3.7. After cultivation in blood agar, the bacteria were transferred to nutrient agar plates and from there a liquid cultivation of the bacteria was used to perform the bioassay. After an exposure time of 16 h, bacterial growth was determined by measuring the OD at 436 nm using a UV spectrophotometer (Ultrospec III, Pharmacia LKB Biosystems GmbH, Freiburg).

Table 3.7 Taxonomy of *Pseudomonas putida*

Kingdom	*Bacteria*
Phylum	*Proteobacteria*
Class	*Gamma Proteobacteria*
Order	*Pseudomonadales*
Family	*Pseudomonadaceae*
Genus	*Pseudomonas*
Species	*Pseudomonas putida*

During the exposure time, the bacteria were kept in a dark climatic chamber at 20±1 °C with continuous orbital shaking (120 rpm, orbital diameter 30 mm, KS 501 digital, IKA Werke GmbH & Co. KG, Staufen, Germany). The inhibition of bacterial growth was calculated by comparing the OD of the samples with the OD of the control experiments. The pH was measured at the beginning and at the end of each test and ranged between 6.8±0.2.

Before testing, all the samples were filtered with 0.22 μm Milli-Pore filters in order to avoid serious contamination from other microorganisms. Each test was performed in triplicate and consisted of a control experiment in which only growth medium was added, a positive control experiment in which a toxic reference compound (3,5 dichlorophenol) and growth medium were added and the rest in which the test samples and growth medium were added.



Schematic 3.7 Presentation of the test procedure for *Pseudomonas putida*

In order to standardize the method and eliminate device-specific divergence of the measurement, the spectrophotometer used was calibrated (FNU). A well-defined dilution series between 50 and 200 FNUs was prepared and measured in the spectrophotometer used for the test at 436 nm. The FNU and OD were correlated and only extinctions between 0.1 and 0.4 were allowed. A defined bacterial starting concentration of 50 FNU was inoculated for the tests performed. The OD measured at the end of the test was converted into FNU. The inhibition of growth was calculated using the Equation 3.8.

$$I = [(B_c - B_n)/(B_c - B_0)] \times 100$$

I: Inhibition percentage, B_c: Biomass of control experiment after time n, B_n: Biomass at time n (n = exposure time), B_0: Biomass at the beginning of the test.

Equation 3.8 Calculation of inhibition for *Pseudomonas putida*

The dose-response relationship was calculated by regression analysis and as a result thereof, EC_{50} value referring to the growth was determined. The validity criteria used for this test are as follows:

- Growth rate of at least 60 fold had to be achieved at the end of the test.
- The coefficient variation should not exceed 25%.

3.4.7 *Saccharomyces cerevisiae*

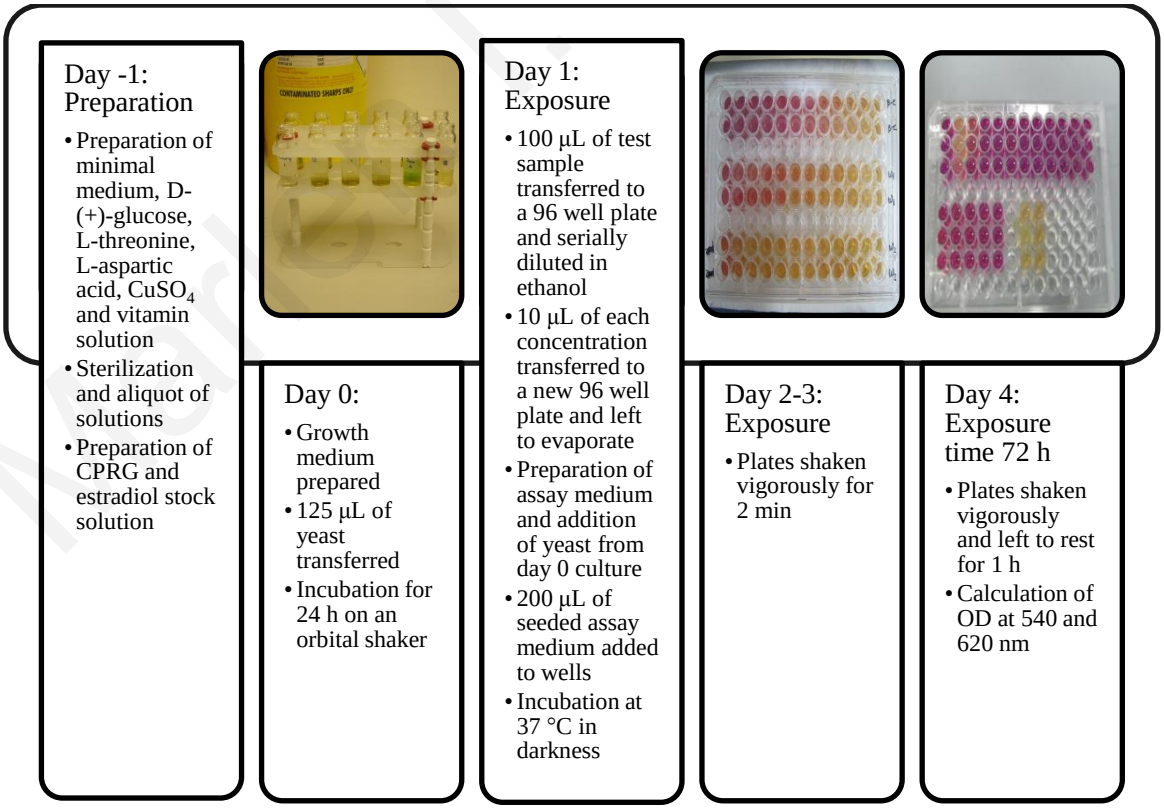
S. cerevisiae (baker's yeast or brewer's yeast) are single cell fungi that can use glucose in aerobic or anaerobic conditions. The natural habitat of the yeast is on the surface of fruits and it has been extensively exploited in bakery and brewery. Apart from this, it is a model organism for the study of eukaryotes.

A recombinant strain of *S. cerevisiae* was used in this thesis to determine the total estrogenic activity in extracts, as shown in Schematic 3.8. The yeast estrogen screen is an *in vitro* transactivation bioassay based on estrogen-dependent synthesis of β-galactosidase. When applied to chemically complex samples, results can be converted to equivalent concentrations of 17β-estradiol (E2) via reference to a suitable E2 standard response curve. Briefly described, the human estrogen receptor (hERα) was integrated into the main chromosome and an expression plasmid carrying estrogen receptor elements (EREs) and the reporter gene *lac-Z*. When an estrogen-like compound enters the cell, the hERα receptor interacts with the EREs and provoke the expression of *lac-Z*, which in turn synthesized β-galactosidase and secreted into the medium. The excreted β-galactosidase metabolized the chlorophenol red-β-D-galactopyranoside (CPRG) producing a colourimetric response from yellow to red measured at 540 nm. This response was used to indicate the presence of estrogenic activity in the samples [294,295]. The protocol used for the implementation of this test was kindly provided by Dr. Edwin Routledge, University of Brunnel.

For the preconcentration of samples, solid phase extraction (SPE) was implemented. SPE cartridges (C-18) were conditioned by passing 2×5 mL of ddH_2O, 2×5 mL of methanol and 2×5 mL ddH_2O at a flow of 1 mL/min using a slight vacuum (J.P. Selecta, Spain). Depending on the sample type, 100 mL of untreated or 1 L treated wastewater were used. The cartridge

was then rinsed with 5 mL ddH_2O and dried to remove excess of water. Elution was performed with 2×5 mL of methanol at 1 mL/min. The extract was evaporated under a gentle nitrogen stream and reconstituted in ethanol.

A minimal medium was prepared by adding KH_2PO_4, $(NH_4)_2SO_4$, KOH, $MgSO_4$, $Fe_2(S0_4)_3$, L-leucine, L-histidine, adenine, L-arginine-HCl, L-methionine, L-tyrosine, L-isoleucine, L-lysine-HCl, L-phenylalanine, L-glutamic acid, L-valine, and L-serine to ddH_2O. D-(+)-glucose, L-threonine, L-aspartic acid, $CuSO_4$ solutions were prepared. A vitamin solution consisting of thiamine, pyridoxine, pantothenic acid, inositol and biotin solution in ddH_2O was prepared. All solutions were divided in aliquots and sterilized accordingly by heating at 121 °C for 10 min or by filtering with a 0.2 µm pore size filter. Finally, a solution with the chromogenic substrate chlorophenol red-ß-D-galactopyranoside (CPRG) was prepared. A 17ß-estradiol stock solution of 0.2 µM (54.48 µg/L) in absolute ethanol was prepared for the standard curve. All glassware used was carefully cleaned since contamination may give rise to false positives. It was rinsed twice with absolute ethanol. Concentrated (10×) yeast stocks were prepared and stored at -20 °C in cryogenic vials.



Schematic 3.8 Presentation of the test procedure for the yeast estrogen screen

For the implementation of the test on day 0 the growth medium was prepared by adding 5 mL glucose solution, 1.25 mL L-aspartic acid solution, 0.5 mL vitamin solution, 0.4 mL L-threonine solution, and 125 µL $CuSO_4$ solution to 45 mL minimal medium. The growth medium was transferred to a sterile conical flask and 125 µL of 10× concentrated yeast stock. Incubation at 28°C followed for approximately 24 h on an orbital shaker, or until turbid. On day 1, 100 µL of each sample were transferred to a 96-well microtitre plate and serially diluted in absolute ethanol. 10 µL aliquots of each concentration were transferred to another 96-well optically flat bottom microtitre plate. 10 µL absolute ethanol (or appropriate solvent) was added to negative control wells. Samples were left in the assay plate to evaporate to dryness. The assay medium was prepared by adding 0.5 mL CPRG to 50 mL fresh growth medium. This medium was seeded with 4×10^7 yeast cells from the 24 h culture prepared on Day 0. 200 µL of this seeded assay medium were added to wells using a multichannel pipette. Each plate contained at least one row of negative controls and each assay a 17ß-estradiol standard curve. The plates were sealed with autoclave tape and shaken vigorously for 2 min on a titre plate shaker. The plates were incubated at 32 °C in a naturally ventilated heating cabinet. On day 2 the plates were shaken vigorously for 2 min, to mix and disperse the growing cells and returned to 32 °C incubator. On day 4 the plates were again shaken and left for approximately 1 h to allow the yeast to settle. The plates were read at an absorbance of 540 nm and 620 nm using a plate reader. To correct for turbidity Equation 3.9 was applied to the data:

$$Corrected\ value\ =\ OD_{sample\ (540\ nm)} - [OD_{sample\ (620\ nm)} - OD_{blank\ (620\ nm)}]$$

Equation 3.9 Calculation for yeast estrogen screen

3.4.8 Biodegradability test

Biodegradation occurs with enzymatic action and involves living organisms. Molecular degradation is promoted by enzymes and can occur under aerobic and anaerobic conditions, leading to complete or partial removal of organic compounds from the environment. The biodegradability of chemicals is one of the most important transformations that occur in the environment and can provide very important information regarding their fate in the environment. The investigation of the biodegradation potential of some chemicals is even mandatory, such as for detergents, and it is a crucial step in risk assessment.

Table 3.8 Set-up for the implementation of the Closed Bottle test

	Quality control	Negative control$_1$	Negative control$_2$	Test suspension	Toxicity control
Mineral medium	x	x	x	x	x
Inoculum	x	x	x	x	x
Test substrates	-	-	-	x	x
$TiO_2/UV_{(64min)}$	-	-	x	-	-
Sodium acetate	x	-	-	-	x
No of test vessels	2	2	2	18	18

The method of Closed Bottle test (OECD 301 D) was selected to determine whether the samples under examination were readily biodegradable [296]. A relatively small amount of microorganisms from a mixed population ($10^2 – 10^5$ colony forming units (CFU)/L) was kept in completely full, closed bottles for 28 d and the dissolved oxygen concentration was recorded daily. In order to have at the beginning of the test a Theoretical Oxygen Demand (ThOD) of 5 mg/L the chemical structure of the test substances was taken into account. A mineral medium, strongly aerated was used for the preparation of all samples. One mL of filtered inoculum derived from secondary treated wastewater effluent (Kenzingen, Germany; 13000 population equivalents) was used for each litre of medium. Each test consisted of four different series, as shown in Table 3.8.

The series consisted of: (a) a negative control$_1$ series, with only inoculum and no chemical substance added in, (b) a negative control$_2$ series, with inoculum and TiO_2 at 1 g/L, which was irradiated for 64 min (the maximum period of the photocatalytic treatment) and filtered, (c) a quality control series, in which the inoculum and a reference compound readily biodegradable was added (sodium acetate corresponding to a ThOD of 5 mg/L), (d) a series with inoculum and the test substrates, and (e) a toxicity control series in which inoculum, our test substrates and sodium acetate were added. The toxicity control series allowed demonstrating whether the bacteria are killed or suppressed due to the exposure to the test substrates. All series contained the same mineral salt solution. Samples were transferred into glass bottles with oxygen-sensor spots (Planar Oxygen-Sensitive foils, SP – PSt3, PreSens GmbH, Regensburg, Germany). A schematic presentation of the setup is described in Schematic 3.9.

pH measurements were obtained at the beginning and at the end of the test. The temperature was measured daily and was kept at 20±1 °C using a dark climatic chamber. The oxygen concentration was measured with a Fiber Optic oxygen meter (Fibox 3, PreSens

GmbH, Regensburg, Germany). Two parallel tests were run for each series. Data were recorded and analysed using the software FibSoft (PreSens, GmbH, Germany).

The uptake of oxygen by microorganisms for the biodegradation, corrected by the uptake in the negative control$_1$ (for photolytically treated samples) and negative control$_2$ (for photocatalytically treated samples) series run in parallel was expressed as a percentage of ThOD.

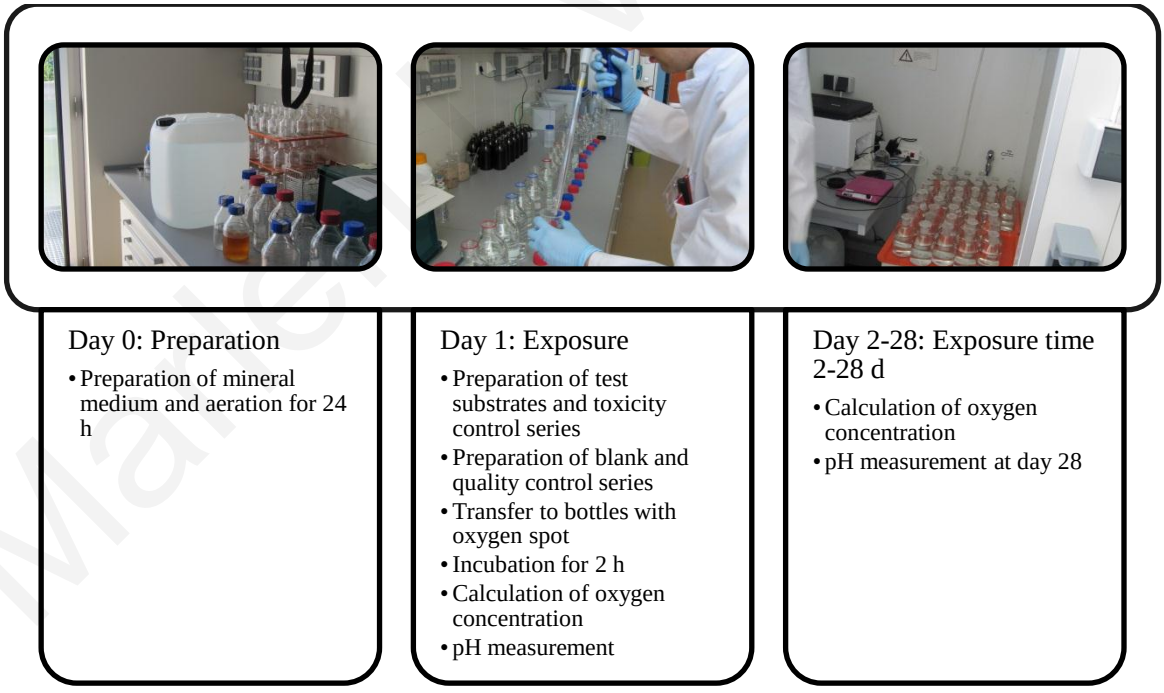
Equations 3.10-11 were used to calculate the percentage of biodegradation:

$$BOD = (O_2 \text{ uptake by test substance} - O_2 \text{ uptake by control}) / \text{test substance}$$

Equation 3.10 Biological Oxygen Demand calculation in the Closed Bottle test

$$\% \text{ Biodegradation} = (BOD/ThOD) \times 100$$

Equation 3.11 Calculation of biodegradation percentage



Schematic 3.9 Presentation of the test procedure for the Closed Bottle

This test is considered as quite advantageous because of its simple application, the possibility to study poorly soluble and volatile compounds and the low testing concentrations

required. This allows getting below possible effect threshold levels that are toxic to bacteria. Disadvantageous is on the one hand, the low density of inoculation of a few mL of cleaned wastewater per litre, and on the other hand, the oxygen reduction that is caused by its consumption due to nitrification. For this reason, the test application is limited to the study of slowly degradable and/or nitrogen-rich substances.

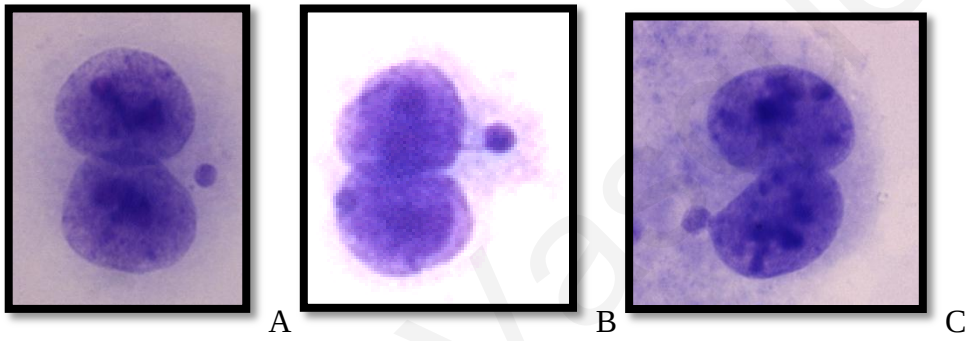
The validity criteria used for this test were the following [296]:

- The difference of extremes replicate values of the removal of the test chemical at the plateau and at the end of the test had to be less than 20%.
- The percentage degradation of the reference compound by day 14 had to be greater than 60%.
- Degradation greater than 25% in the toxicity test needed to occur within 14 d.
- Oxygen depletion in the inoculum negative control needed to be greater than 1.5 mg dissolved oxygen/L after 28 d.
- The residual concentration of oxygen in the test bottles had to be greater than 0.5 mg/L at any time.

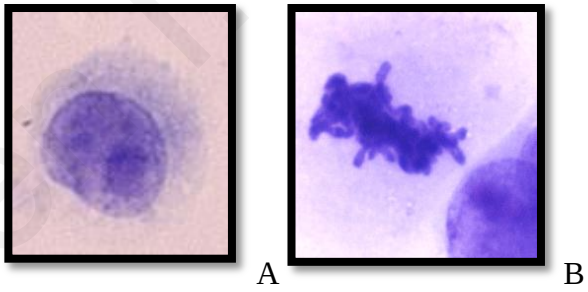
3.4.9 Cytokinesis-block micronucleus cytome assay

The assay can be used for measuring genotoxicity, cytostasis and cytotoxicity. DNA damage can be scored in the cytoplasm of interphase cells calculating three different biomarkers; the number of micronuclei (MNi), nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs). The number of MNi is considered as a reliable index of both chromosome breakage and chromosome loss. MNi are expressed in dividing cells that either contain chromosome breaks lacking centromeres and/or whole chromosomes that are unable to travel to the spindle poles during mitosis. The centromeres are the DNA region where both chromatids are held together and on which kinetochores, the region on which spindle fibres are associated during cells division, are attached side-by-side. At telophase, a nuclear envelope forms around the lagging chromosomes and fragments, which gradually assume the morphology of an interphase nucleus with the exception that they are smaller than the main nucleus. The number of NPBs is used as a biomarker of dicentric chromosomes resulting from telomere end-fusions or DNA misrepair. NBPs are usually formed when centromeres of

dicentric chromosomes are drawn to different poles of the cell at anaphase. Dicentric chromosomes are formed when two chromosome segments (either from different chromosomes or different chromatids of the same chromosome) are bonded or when DNA is not repaired properly and dicentric ring chromosomes are formed. The number of NBUDs can be used as a biomarker of gene amplification. Nuclear budding is a recently discovered mechanism of MNi formation by which amplified sequences of DNA are eliminated during S phase of mitosis. NBUDs have the same morphology as MN with the difference that they are linked to the nucleus by a stalk of nucleoplasmic material (Picture 3.8) [297,298].



Picture 3.8 Micronucleus (A), nucleoplasmic bridges (B) and nuclear buds (C)

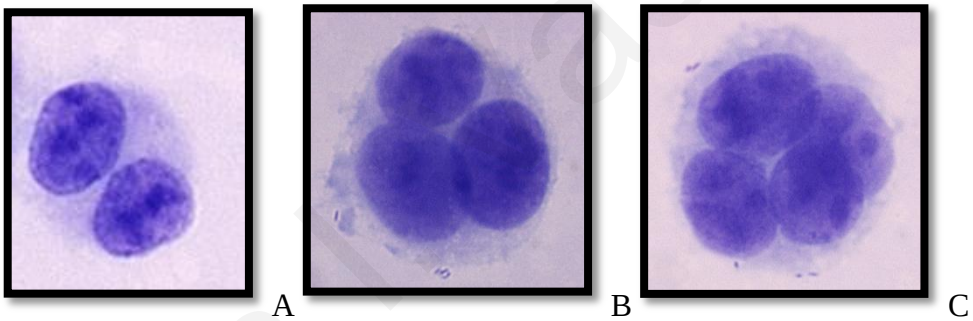


Picture 3.9 Necrotic (A) and apoptotic (B) cell

Furthermore, the assay can be used for measuring necrosis, apoptosis and cytostasis (Picture 3.9). It is considered as a cytome assay as it addresses complex and dynamic cellular processes (structure and function) underlying physiological processes such as viability, mitosis status and chromosomal damage. It detects the activity of clastogenic chemicals causing structural chromosomal aberrations and aneugenic chemicals interacting in the mitotic cell division cycle and allows genotoxic, cytotoxic and cytostatic events to be captured in one assay. It has been proven to be an effective tool for the study of cellular and nuclear

dysfunction by *in vitro* or *in vivo* aging, micronutrient deficiency or excess, genotoxin exposure and genetic defects in genome maintenance [297].

The MNi can only be assessed in dividing eukaryotic cells and specifically in cell populations in which the kinetics of cell division is well understood or controlled. It is important to identify cells that have completed one nuclear division only, as there is uncertainty regarding the fate of MNi following more than one division. For this purpose the method selected was the cytokinesis-block micronucleus cytome assay (CBMN). In the CBMN assay, cells that have completed one nuclear division are blocked from performing cytokinesis (the process following mitosis) using cytochalasin B (cytoB). CytoB inhibits actin assembly, and thus prevents separation of daughter cells after mitosis leading to the formation of binucleated cells (Picture 3.10).



Picture 3.10 Binuclear (A), trinuclear (B) and tetranuclear (C) cells

The protocol used was developed in cooperation with Prof. Klaus Kümmerer and his research group, Universtitätklinikum Freiburg. The protocols of Fenech and OECD [297,298] were taken into account. The cell line used was the HepG2 from human hepatocellular carcinoma obtained from ATCC (HB 8065) (Maryland, USA). The hepatic cells were chosen as they were found to be more representative of xenobiotic metabolism. HepG2 cells were grown as a monolayer in DMEM, supplemented with 15% fetal calf serum and antibiotics [penicillin (100 U/L) and streptomycin (0.1 mg/L)].

The cultures were incubated at 37 °C in an atmosphere containing 5% CO_2 and 95% humidity and regularly checked for possibly mycoplasma contamination. Before seeding cells were slowly passed through a needle's injection to minimize agglomerations of cells. The cell density was evaluated by dying cells with erythrocinB (1:1) and using a Neubauer chamber.

An initial concentration of 0.4×10^6 cells/L was used for each trial. The cells were incubated for 24 h.

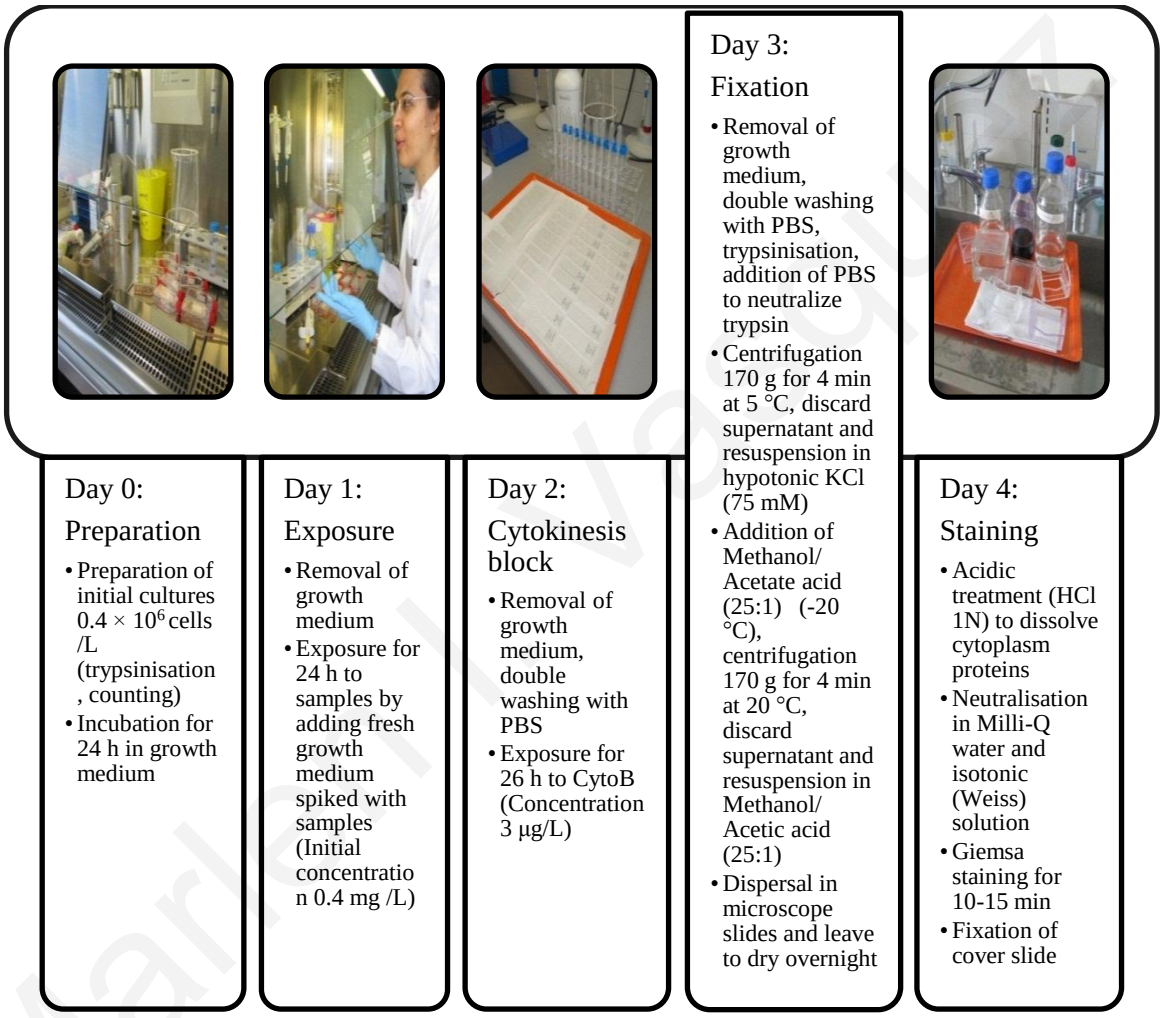
On day 1, the growth medium was replaced with new growth medium spiked with the test samples and left to incubate for 24 h. On day 2, the growth medium was removed and the cells were washed twice with PBS. New growth medium with spiked CytoB (3 μg/L) was added to the cells and incubated for 26 h. The 26 h was the optimised exposure time that produced the highest amount of binucleated cells, a proportion greater than 80 %.

On day 3, the growth medium was removed and cells were washed twice using PBS. Cells were trypsinised and neutralized with PBS. The cells were centrifuged at 170 g for 4 min at 20 °C. Supernatant was removed and the cell pellet was resuspended in PBS with the use of a needle's injection. The cells were again centrifuged using the parameters mentioned above and the supernatant discarded. The pellet was resuspended for 6 min using 700 μL KCl causing a hypotonic treatment. 4 mL of methanol: acetic acid (25:1) at -20 °C were added for 5 min to cause cell fixation and the cells were centrifuged for 4 min at 70 g at 20 °C. The supernatant was discarded and cells were resuspended in 1 mL of methanol. Approximately 500 μL were transferred to slides and left to dry.

Prior staining slides were transferred to a slide carrier. The slides were transferred for max 1 min in HCl (1N) for protein dissolution and then neutralized in distilled H_2O for 1 min and Weiss buffer for 2 min. The slides were transferred to freshly made Geimsa stain for approximately 15 min. Excess stain was removed by washing in distilled H_2O and Weiss buffer. The slides were left to dry overnight and slide covers were fixed on each slide. The procedure followed is described diagrammatically in Schematic 3.10. Control and positive control experiments, with the use of Milli-Q and 400 μM EMS respectively, were run in parallel. Each experiment was assessed in triplicate.

Following preparation of the slides with the exposed cells, the assessment of the MNi took place. The slides were independently coded and then evaluated using a light microscope with a 400× magnification. At least 1000 binuclear cells were assessed from each slide for the presence of MNi. Mononucleated, trinucleated and tetranucleated cells were also counted in a total of 500 cells in order to calculate the Nuclear Division Index (NDI) and the cytostatic

percentage. The NDI provides a measure of proliferative status of the viable cell fraction and information on the cytostatic and cytotoxic effects of the test substance. In the framework of this thesis genotoxic (MNi formation) and cytostatic (ratios of mono-, bi- and multinucleated cells) biomarkers were investigated. Cytotoxic effects were derived by comparing values in treated and control tests. Apoptotic (programmed death) and necrotic cells were not included in the MNi assessment.



Schematic 3.10 Presentation of the test procedure for the cytokinesis-block micronucleus cytome assay

Concentrations of test substances that could produce cytotoxicity, precipitation in the culture medium and/or changes in the pH and osmolality were avoided. At least three concentrations were evaluated in triplicates. The highest concentration tested did not produce more than 50±5% cytotoxicity. The highest concentration did not exceed 0.01 M and 5 mg/L.

The formulae used are shown in Equations 3.12-14.

$$\text{Cell density} = \text{Total counted number}/\text{number of squares} \times \text{dilution} \times 10^4$$

Equation 3.12 Cell density calculation

$$\text{NDI} = (M_1 + 2M_2 + 3M_3 + 4M_4)/N$$

$M_{1\text{-}4}$: Number of mono-, bi-, tri-, tetra-nucleated cells respectively, N: Number of cells scored

Equation 3.13 Nuclear Division Index calculation

$$\%\ \text{Cytostasis} = 100 - [100 \times (\text{NDI} - 1)/(\text{NDI}_c - 1)]$$

NDI_c: NDI of control experiment

Equation 3.14 Cytostasis percentage calculation

The scoring criteria applied are as follows [297]:

- Necrotic (pale cytoplasm, presence of numerous vacuoles, damaged cytoplasmic membrane, lower staining intensity of the nucleus) and apoptotic cells (presence of chromatin condensation within the nucleus or nuclear fragmentation, greater staining intensity of the nucleus) were not scored.
- Binucleated cells with undamaged nuclear membranes and a common discrete cytoplasmic boundary were scored. Their nuclei were of the similar size, did not overlap and their boundaries were distinguishable and had the same staining pattern and intensity.
- The MNi were morphologically the same with the nuclei, but smaller in size (1/16 to 1/3 of the nuclei diameter). They were non-refractile, unconnected to the nuclei and with a separate boundary that may touch but not overlap the nuclei.
- The same scorer was maintained throughout the study in order to reduce interscorer variability.

The quality criteria applied are as follows [298]

- Cells being scored had to complete one nuclear division.
- Regular shaped cells, with intact cytoplasmic membrane and similar nuclei size were scored.
- A control experiment reproducibly with low MNi frequencies that did not exceed the 25 MNi/1000 cells had to be met.
- Coefficient of variation had to be less than 20%.

CHAPTER 4. ASSESSMENT OF EFFECTS OF WASTEWATER USING A BATTERY ASSAY

4.1 Background information

The present study aimed at evaluating an application of the WET approach using multi-bioassays for the assessment of the effluents of three STPs in Cyprus. The treated wastewater from these STPs is used mostly for irrigation and for groundwater recharge or disposed into the sea during the rainy seasons. A set of six bioassays was used including acute testing with *D. magna* and *V. fischeri* and chronic testing with unicellular freshwater green micro-algae *P. subcapitata*, as suggested by the WET approach [124]. Furthermore, as alternative tests, an acute test with *A. salina*, an acute test with the plant seeds of *L. sativum* and an estrogenicity test using a recombinant yeast assay were performed.

The monitoring of the quality of the treated wastewater in Cyprus is an obligation resulting from the Cyprus law regarding the STPs serving an equivalent population up to 2000 inhabitants [147]. In the present study the monitoring was performed for STPs serving a population equivalent in the range of 15000-150000, for which the quality limits are being set yearly by the Department of Environment. The limits during the period 2007-2010 are presented in Table 4.1.

Table 4.1 Quality limits for treated wastewater according to the Cyprus (Law 106 (I)/2002) [147]

Parameters	STP I-III Values	Frequency of sampling (/year)
BOD_5 (mg/L)	10	4
COD (mg/L)	70	4
SS (mg/L)	10	4
Conductivity (μS/cm)	2200[1]	4
TN (mg/L)	15	4
TP (mg/L)	10	4
Cl^- (mg/L)	300	4
Oils (mg/L)	5	4
Zn (mg/L)	1[2]	2
Cu (mg/L)	0.1	2

[1] For STP III 1700 μS/cm.
[2] For STP I 0.1 mg/L. For STP II for release into sea 0.1 mg/L.

Parameters	STP I-III Values	Frequency of sampling (/year)
Pb (mg/L)	0.15	2
Cd (mg/L)	0.01	2
Hg (mg/L)	0.05	2
Cr (mg/L)	0.1	2
Ni (mg/L)	0.2	2
B (mg/L)	1[3]	2
Faecal coliforms /100 mL	50[4]	4
Faecal parasites eggs	0	4
Residual Cl (mg/L)	1[5]	4
pH	6.5 – 8.5	4
Toxicity	75% of samples must comply with the following:[6] Bioluminescence inhibition TU_{50}<1 and/or TU_{20}<1.5 Immobilization for *Daphnia* TU_{50}<1 Growth inhibition for algae TU_{50}<1	1

[3] For STP II 0.75 mg/L.
[4] For STP I, 5 *E. coli* /100 mL and for STP II for release to dam located nearby 5 *E. coli* /100 mL.
[5] For STP II for release into the sea 0.5 mg/L.
[6] Applied only for STP III and STP II in case of release to a dam.

The general characteristics of the STPs during the study period are summarized in Table 4.2. The treatment consists of pre-treatment, preliminary treatment, primary sedimentation, secondary (biological) treatment, sand filtration and chlorination. Pre-treated wastewater includes a physical process of settling in a primary clarifier. Secondary treatment occurs in pre-denitrification (anaerobic) and nitrification (aerobic) tanks and (i) oxidation ditches at STP I, (ii) conventional activated sludge including aeration tanks and secondary settling tanks at STP II and (iii) two secondary clarifiers at STP III.

Table 4.2 General characteristics of the three sewage treatment plants studied in the present study

	STP I	STP II	STP III
Average daily flow (m^3/d)	15000	25000	16000
Population equivalent	130100	223600	74900
Hydraulic retention time (h)	24	14	20-25
Process	Activated sludge	Activated sludge	Nitrification-denitrification
Wastewater source	Domestic	Domestic, industrial	Domestic

Table 4.3 provides some extra information regarding the physical characteristics of STP I and III. The corresponding characteristics of STP II were considered confidential by the company running the plant and therefore are not available.

Table 4.3 The general operating characteristics of the sewage treatment plants

Characteristics		STP I		STP III	
		summer	autumn	summer	autumn
Flow rate	FR	16493	16540	10118	8114
Treatment units		CAS	CAS	ND	ND
Design	HRT	10.3	10.3	20	25
parameters	F/M	0.07	0.07	0.09	0.09
Operational	Qc:	15	15	21	19
parameters	SVI	950	950	126	143
	SRR	100	100	100	100
	Sludge return	9624	14919		
	SW	1251	799	253	202

FR: flow rate (m^3/d), HRT: hydraulic retention time (h), F/M: food to microorganisms ratio, Qc: sludge age (d), SVI: sludge volume index (mg/L), SRR: Sludge return ratio (%), SW: sludge waste (m^3)

The primary treatment of STP I consists of a screen, bar racks, an aerated grit removal chamber and a primary clarifier. The secondary (biological) treatment has oxidation ditches, secondary settlement banks and an effluent storage reservoir. The tertiary treatment stage of the process consists of a sand filtration and a chlorination unit.

Screenings, grid chamber, skimmer tanks and large solid removal, and a primary sedimentation tank are the main steps of the primary treatment of STP II. A conventional secondary activated sludge is applied, consisting of an aeration tank and secondary settlement tanks. A combination of primary and secondary (activated) sludge is processed by dewatering and anaerobic digestion. Finally, the biologically treated wastewater undergoes sand filtration and chlorination.

The primary treatment of STP III includes bar racks and a grid removal chamber, followed by two primary sedimentation tanks. During the secondary treatment, phosphorus biological removal, nitrification and denitrification take place in four tanks. The biological unit consists of two denitrifying tanks, two nitrifying tanks and secondary clarifiers with the recycling sludge going to the inlet of the first denitrification tank. The tertiary treatment is performed by sand filtration and chlorination.

It should be mentioned that during the last visit to the STPs in autumn 2011, extension works were taking place and the STPs nowadays are able to serve a larger population equivalent. No advanced treatment process though is applied.

4.2 Results

It is important to note that during the presentation of the results, abbreviations are used for simplicity as follows: **STP I-IN** denotes composite sample taken from the inlet at STP I, **STP I-STE** refers to composite sample taken after the secondary treatment at STP I, **STP I-OUT** is used for composite sample taken from the outlet at STP I. The same notation is used for STP II and STP III samples.

4.2.1 Physicochemical investigation of wastewater

Prior to the assessment of effects with the use of bioassays, a statistical physicochemical investigation for the wastewater was performed for STP I and III using the daily and weekly data sets provided by the administrators of the STPs. STP II was not evaluated because the data sets were considered confidential by the operators and as mentioned before, were not made available to the author.

The methodology applied is presented in the Schematic 4.1. All data sets' analysis was performed using the SPSS software (IBM SPSS v.19).

Schematic 4.1 Methodology for statistical analysis of physicochemical parameters

A descriptive statistics analysis was used to organize the data. The parameters used for the statistical analysis of the STP I and III-INs were the temperature (°C), pH, TP (mg/L), TN

(mg/L), BOD_5 (mg/L), COD (mg/L), TSS (mg/L) and conductivity (mS/cm). The Kolmogorov test was used to identify deviation from normal distribution. The original data demonstrated values of skewness from -0.1400 to 2.424 and kurtosis from -1.2 to 6.956 indicating that some parameters were not close to the normal distribution. The parameters were log-transformed reducing the skewness to the range of -1.585 to 1.818 and the kurtosis to the range of -1.484 to 4.004 indicating that the data were in normal or close to normal distribution Table 4.4. Ammonia concentration (mg/L) was excluded from the analysis as it is included in the total nitrogen concentration and the kurtosis was maintained high after the log-transformation.

Table 4.4 Descriptive statistics for the log-transformed parameters for the inlet of the sewage treatment plants I and II (STP I and III-INs)

	N	Minimum	Maximum	Mean	Std. Deviation	Skewness		Kurtosis	
	Statistic	Statistic	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Std. Error
log[Temperature]	381	1.15	1.48	1.3374	0.07518	0.116	0.125	-1.048	0.249
log[pH]	378	0.85	0.89	0.8716	0.00679	-0.480	0.125	-0.293	0.250
log[TP]	134	0.95	1.41	1.2564	0.07785	-1.263	0.209	2.743	0.416
log[TN]	136	1.72	2.00	1.8799	0.04382	-0.681	0.208	3.027	0.413
log[BOD_5]	69	2.53	2.91	2.6497	0.06784	1.818	0.289	4.004	0.570
log[COD]	346	2.47	2.97	2.8629	0.09006	-1.585	0.131	2.455	0.261
log[TSS]	361	1.88	2.71	2.2814	0.11824	0.922	0.128	2.500	0.256
log[Conductivity]	380	0.16	0.73	0.4294	0.16386	-0.253	0.125	-1.484	0.250
Valid N (listwise)	20								

The visualization of the data through boxplots indicates that the quality of STPs I and III-INs were different (data not shown). In order to examine this hypothesis a K-means cluster analysis was applied due to the large number of cases. The number of clusters was set to 2-4. The maximum iterations were set to 20 and the missing values were excluded pairwise. An ANOVA table was developed which is presented in Table 4.5.

The data set was best split into two different clusters. When a higher number of clusters were investigated, the STP III cases were divided among the clusters; whereas the cases from STP I remained in one cluster. No specific trend e.g. seasonal variation was observed, when the STP III cases were divided into more than one cluster. From the F-values it can be concluded that the parameters which contribute the most to the formation of the clusters are the conductivity, the COD and the pH of the INs (inlet samples).

Table 4.5 ANOVA analysis for the Z-scores of the log-transformed values for the inlet of the sewage treatment plants I and III (STP I and III-INs)

	Cluster		Error			
	Mean Square	df	Mean Square	df	F	Sig.
Zscore: log[Temperature]	64.129	1	0.833	379	76.945	0.000
Zscore: log[pH]	120.154	1	0.683	376	175.895	0.000
Zscore: log[TP]	22.550	1	0.837	132	26.949	0.000
Zscore: log[TN]	10.138	1	0.932	134	10.880	0.001
Zscore: log[BOD]	31.096	1	0.551	67	56.455	0.000
Zscore: log[COD]	183.019	1	0.471	344	388.676	0.000
Zscore: log[TSS]	39.955	1	0.891	359	44.819	0.000
Zscore: log[Conductivity]	327.159	1	0.137	378	2385.505	0.000

The F tests should be used only for descriptive purposes because the clusters have been chosen to maximize the differences among cases in different clusters. The observed significance levels are not corrected for this, and thus, cannot be interpreted as tests of the hypothesis that the cluster means are equal.

The final cluster centers are presented in Table 4.6. 142 cases were grouped in cluster 1 and 243 in cluster 2. 100% (142) of the cases of STP III were grouped in cluster 1 and 97.93% (241) of the cases of STP I were grouped in cluster 2.

Table 4.6 Final cluster centers of the Z-scores of the log-transformed values for the inlet of the sewage treatment plants I and III (STP I and III-INs)

	Cluster	
	1	2
Zscore: log[Temperature]	-0.54133	0.31093
Zscore: log[pH]	-0.73929	0.42996
Zscore: log[TP]	-0.59677	0.28199
Zscore: log[TN]	-0.41560	0.17936
Zscore: log[BOD]	1.05078	-0.42889
Zscore: log[COD]	-1.01096	0.52322
Zscore: log[TSS]	0.43301	-0.25561
Zscore: log[Conductivity]	-1.22873	0.70068

The descriptive statistics of the STP I and III-STEs revealed a normal distribution after the application of the Komgorov test. The skewness ranged from -0.881 to 1.349 and the kurtosis from -1.121 to 0.803. The temperature, pH, TP, COD, TSS and nitrate concentrations were evaluated since these were monitored at both plants. The data was transformed to the z-scores prior the cluster analysis.

The data sets were split into two clusters; 98.33% (235) cases of STP I were grouped in cluster 1 and 100% (98) of STP III were grouped in cluster 2, indicating that the samples from the two STEs cannot be considered of the same quality (Table 4.7). All values of STP III were lower than the ones of STP I, for all the parameters evaluated.

Table 4.7 Final cluster centers of the Z-scores for the secondary treated effluent of the sewage treatment plants I and III (STP I and III-STEs)

	Cluster	
	1	2
Zscore[Temperature]	0.07902	-0.18206
Zscore[pH]	0.60289	-1.42725
Zscore[TP]	0.60823	-0.33558
Zscore[COD]	0.44747	-1.14571
Zscore[TSS]	0.50529	-1.19673
Zscore[NO_3]	1.18703	-0.65353

The ANOVA, shown in Table 4.8 helped to identify that the pH was the most critical parameter in splitting the clusters, followed by the TSS, the nitrate and the COD concentrations.

Table 4.8 ANOVA analysis for the Z-scores for the secondary treated effluent of sewage treatment plants I and III (STP I and III-STEs)

	Cluster		Error			
	Mean Square	df	Mean Square	df	F	Sig.
Zscore[Temperature]	4.849	1	0.989	335	4.905	0.027
Zscore[pH]	283.957	1	0.137	328	2067.743	0.000
Zscore[TP]	27.555	1	0.800	133	34.429	0.000
Zscore[COD]	166.104	1	0.487	322	340.900	0.000
Zscore[TSS]	193.502	1	0.395	318	490.315	0.000
Zscore[NO_3]	107.055	1	0.220	136	486.207	0.000

The F tests should be used only for descriptive purposes because the clusters have been chosen to maximize the differences among cases in different clusters. The observed significance levels are not corrected for this, and thus, cannot be interpreted as tests of the hypothesis that the cluster means are equal.

For the analysis of the STP I and III-OUTs the same procedure was followed and during the descriptive statistics analysis the data sets indicated that are normally or very close to

normal distribution since the skewness ranged between -0.520 to 1.137 and the kurtosis between -1.648 to 0.911.

Table 4.9 ANOVA analysis for the Z-scores for the outlet of sewage treatment plants I and III (STP I and III-OUTs)

	Cluster		Error			
	Mean Square	df	Mean Square	df	F	Sig.
Zscore[Temperature]	104.896	1	0.729	384	143.804	0.000
Zscore[pH]	23.187	1	0.941	374	24.649	0.000
Zscore[TP]	88.510	1	0.608	223	145.678	0.000
Zscore[TN]	83.449	1	0.630	223	132.400	0.000
Zscore[NO_3]	327.334	1	0.078	354	4188.475	0.000
Zscore[BOD_5]	4.678	1	0.947	69	4.941	0.030
Zscore[COD]	194.216	1	0.471	365	412.661	0.000
Zscore[TSS]	164.043	1	0.546	359	300.532	0.000
Zscore[Conductivity]	357.287	1	0.062	380	5725.514	0.000

The F tests should be used only for descriptive purposes because the clusters have been chosen to maximize the differences among cases in different clusters. The observed significance levels are not corrected for this, and thus, cannot be interpreted as tests of the hypothesis that the cluster means are equal.

The cluster analysis split the data sets into two clusters in which cluster 1 had all the cases of STP III-OUT (146) and cluster 2 had all the cases of STP I-OUT (240). The most influential parameters are the conductivity and the nitrate concentration, shown in Table 4.9. The COD and TSS influenced though to a lesser extent, the quality of the OUTs, as well.

The final cluster centers are shown in Table 4.10. It can be seen that all the parameters of the STP III-OUTs are lower than the parameters of the STP I-OUTs. Regarding STP III, the conductivity and the nitrate concentrations are less than 1 standard deviation from the distribution mean, followed by the COD and the TSS that are close to 1 standard deviation from the distribution mean. Regarding STP I, the conductivity, the nitrate and the total nitrogen, and total phosphorus concentrations deviated 0.5-1 standard deviation from the mean.

Table 4.10 Final cluster centers of the Z-scores for the outlet of the sewage treatment plants I and III (STP I and III-OUTs)

	Cluster	
	1	2
Zscore[Temperature]	-0.66837	0.40659
Zscore[pH]	-0.31169	0.19785
Zscore[TP]	-0.54106	0.72705
Zscore[TN]	-0.53015	0.69958
Zscore[NO_3]	-1.24165	0.74053
Zscore[BOD_5]	-0.39607	0.16635
Zscore[COD]	-0.98810	0.53557
Zscore[TSS]	-0.86706	0.52409
Zscore[Conductivity]	-1.25730	0.74390

To summarize, it may be suggested that the cluster analysis revealed a significant differentiation of the STP I and III-INs, -STEs and -OUTs. Regarding the STP I and III-STEs and -OUTs, STP III was found to deviate with negative standard deviations and STP I with positive standard deviations for all parameters evaluated. This trend was not so obvious for the STP I and III-INs, in which the BOD_5 and the TSS were higher in STP III, than in STP I. The clustering in different groups indicated that different quality of wastewater enter and leave each STP, which was not the original hypothesis. Hence, the assessment of the effects of the wastewater from each of the STPs examined is presented separately, since no homogeneity among the samples was found to exist.

4.2.2 Assessment of effects using a multi-species approach

A set of five species from different taxonomy (bacteria, unicellular micro algae, crustacean, plant seeds), representing both the aquatic and terrestrial environment, were exposed to the samples collected from the STPs at different dilutions in order to determine a set of acute and chronic toxicity end-points (*i.e.*, bioluminescence inhibition, cell growth inhibition, immobilization, seed germination index inhibition) of the effluents as described in detail in Chapter 3. Negative control tests were performed by adding 0.5 μL micronutrients to Milli-Q water. Positive control tests were performed with $K_2Cr_2O_7$ solutions for *D. magna*, *P. subcapitata* and *A. salina*, and with phenol solutions for *V. fischeri*.

4.2.2.1 *Pseudokirchneriella subcapitata*

The results of the toxicity tests performed with *P. subcapitata* are shown in Figure 4.1 (vivarium-grown organisms) and Figure 4.2 (kits). Each sample was tested to different concentrations of wastewater (7.5-100%) as already described in Chapter 3.

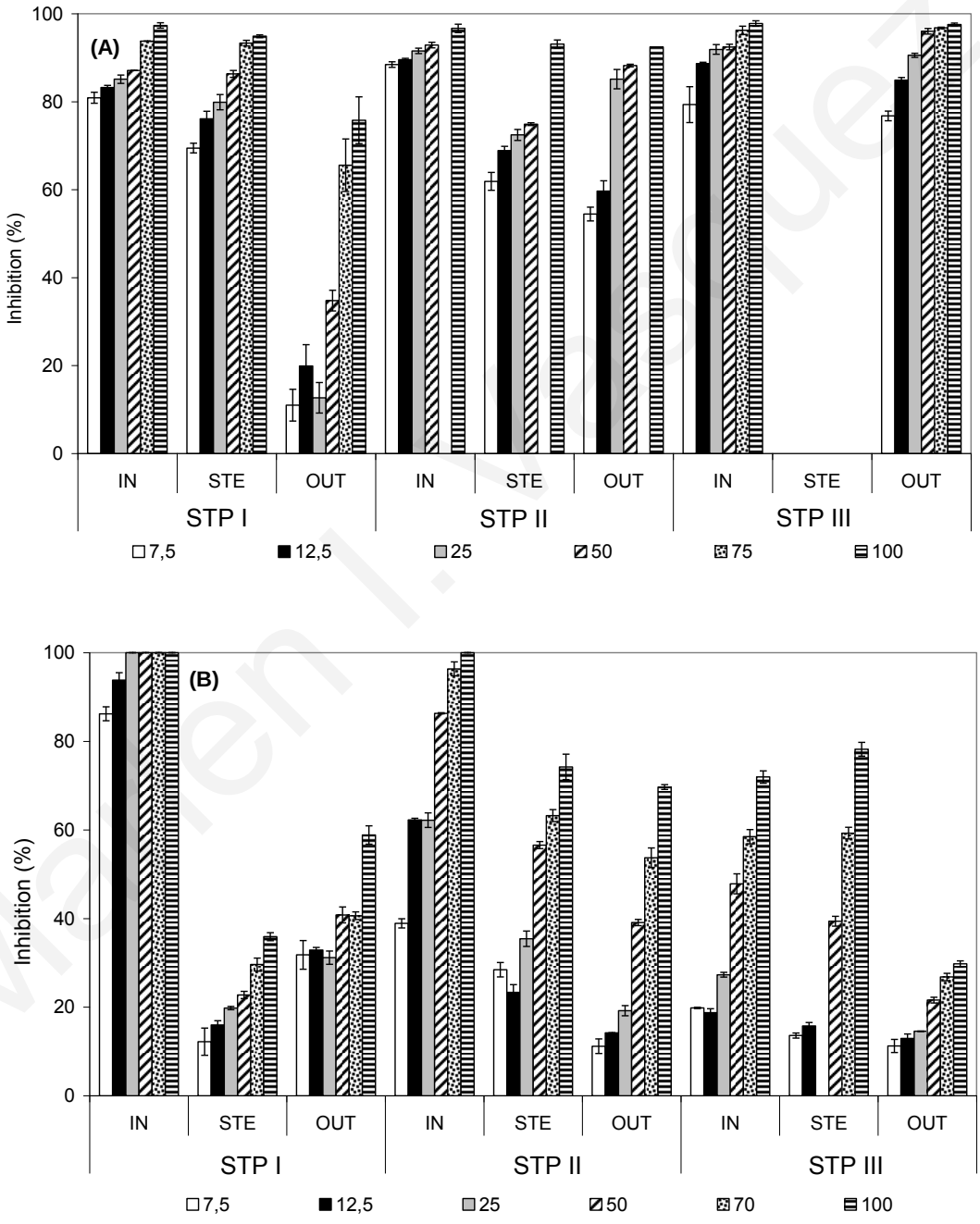


Figure 4.1 Cell growth inhibition percentage for *Pseudokirchneriella subcapitata* exposed to inlet (INs), secondary treated (STEs) and outlet (OUT) samples collected in summer (A) and winter (B) for 96 h

Regarding the summer sampling (Figure 4.1 (A)), the INs from all STPs were found to be severely toxic. The toxicity during primary and secondary treatment was not significantly removed and the STP I-II STEs continued to be toxic. It should be noted the STP III-STE was not evaluated for vivarium grown organisms due to the conditions that prevailed in the clarifier tank during the summer sampling day. Tertiary treatment caused a reduction of toxicity of the STP I-OUTs; whereas the toxicity of STP II and III-OUTs remained at the same levels as the toxicity of the INs. The results obtained with the Algaltox kits were in accordance with the results obtained using the vivarium-grown organisms (Figure 4.2).

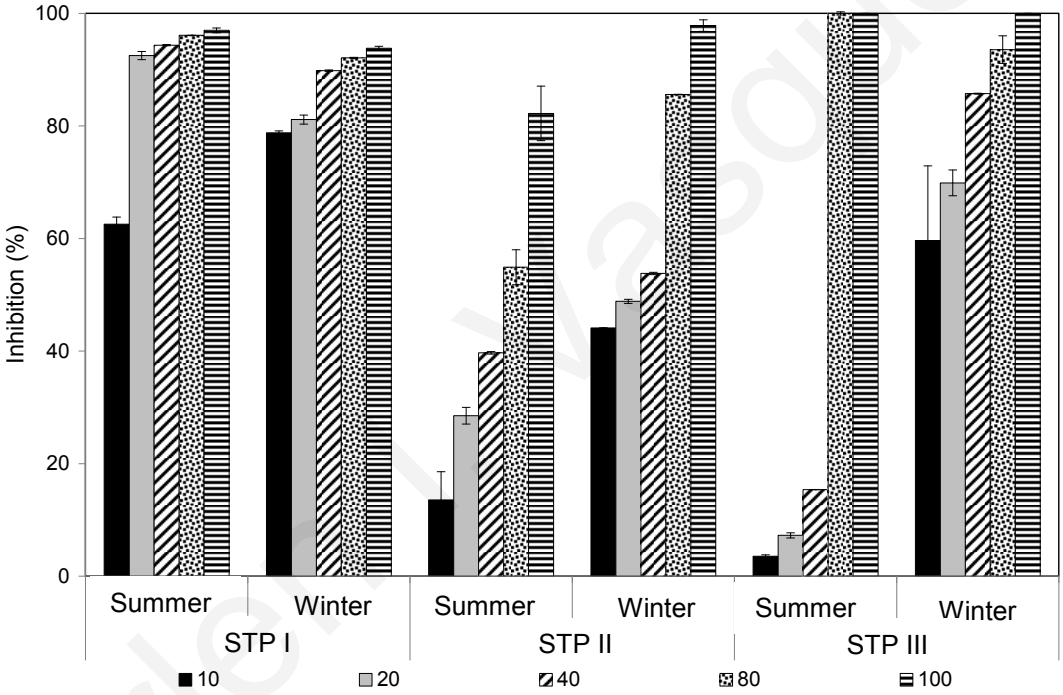


Figure 4.2 Cell growth inhibition percentage for *Pseudokirchneriella subcapitata* (AlgalTox kit) exposed to secondary treated (STEs) samples collected in summer and winter sampling for 72 h

Regarding the winter sampling (Figure 4.1 (B)), the INs were also found to be toxic. The toxicity of the INs decreased as follows: STP I-IN>STP II-IN>STP III-IN. The secondary treatment reduced toxicity primarily during STP I treatment and to a lesser extent during STP II and III treatment processes. The OUTs toxicity did not follow the same trend as the STEs and was higher in STP I; being at the same range in STP II and lower in STP III. Chlorination by-products may be a reason for the increase of the toxicity of the OUTs. The results obtained by the Algaltoxkit demonstrated the same range of response for STE II and III; whereas the toxicity of STE I was higher (Figure 4.2).

4.2.2.2 *Lepidium sativum*

Figure 4.3 (A) shows the germination index profiles of the *L. sativum* seeds exposed to the OUTs tested at two dilutions (50 and 100%) for 72 h (summer sampling). The germination index was inhibited in the order of STP I=STP II>STP III.

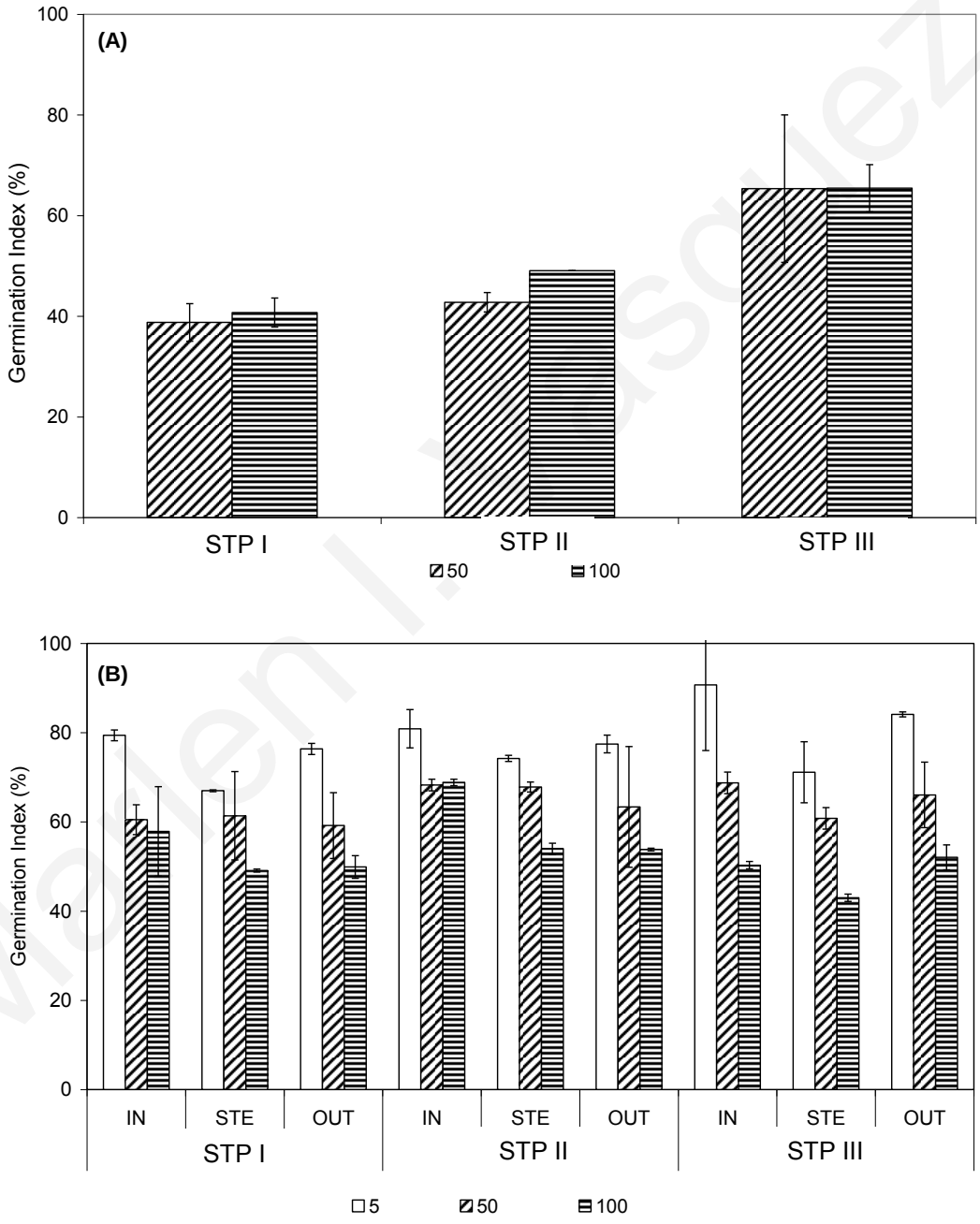


Figure 4.3 Germination Index (%) of *Lepidium sativum* exposed to outlet (OUTs) samples collected in summer (A) and to inlet (INs), secondary treated (STEs) and outlet (OUTs) samples in winter (B) for 72 h

As illustrated in Figure 4.3 (B), during the winter sampling, slight differences in the toxicity were observed, among the samples collected from the same STP and also among the samples from different STPs, and comparing to the other species tested, indicates species robustness. A slight decreasing toxicity trend was observed as follows: INs>STEs>OUTs samples.

4.2.2.3 *Daphnia magna*

The INs collected from the 3 STPs in summer exhibited a severe toxic effect to *D. magna* grown in vivarium (100% immobilization) even at the highest dilution ratio (7.5% of sample volume). After filtration through 0.45 μm Millipore filter paper to remove suspended solids, the samples still exhibited 100% immobilization. A severe toxicity effect of the INs collected in winter was also observed by all samples (data not shown).

The toxicity results of the OUTs tested to *D. magna* grown in vivarium are shown in Figure 4.4. The sample from STP I gave slight toxicity (20 and 35% immobilization at 75 and 100% sample ratios respectively) after 48 h of exposure time while the sample from STP II resulted in severe toxicity (100% immobilization) at the same test ratios as those for STP I during the summer samplings. The STP III-OUTs were found to be the most toxic resulting in 100% immobilization even when it was tested at 7.5% dilution during the summer sampling (Figure 4.4 (A)).

During the winter samplings, toxicity to *D. magna* was observed in STP II and STP I-OUTs, as presented in Figure 4.4 (B). The toxicity was higher than the one observed at 24 h in all samples.

The STEs taken in summer were found to be toxic for all three STPs, as shown in Figure 4.5 (A). The highest concentrations (80% and 10%) were severely toxic. Greater dilution of samples of STP I and II-STEs reduced the toxicity significantly; whereas the STP III-STEs were found to be severely toxic even when substantially diluted (10%).

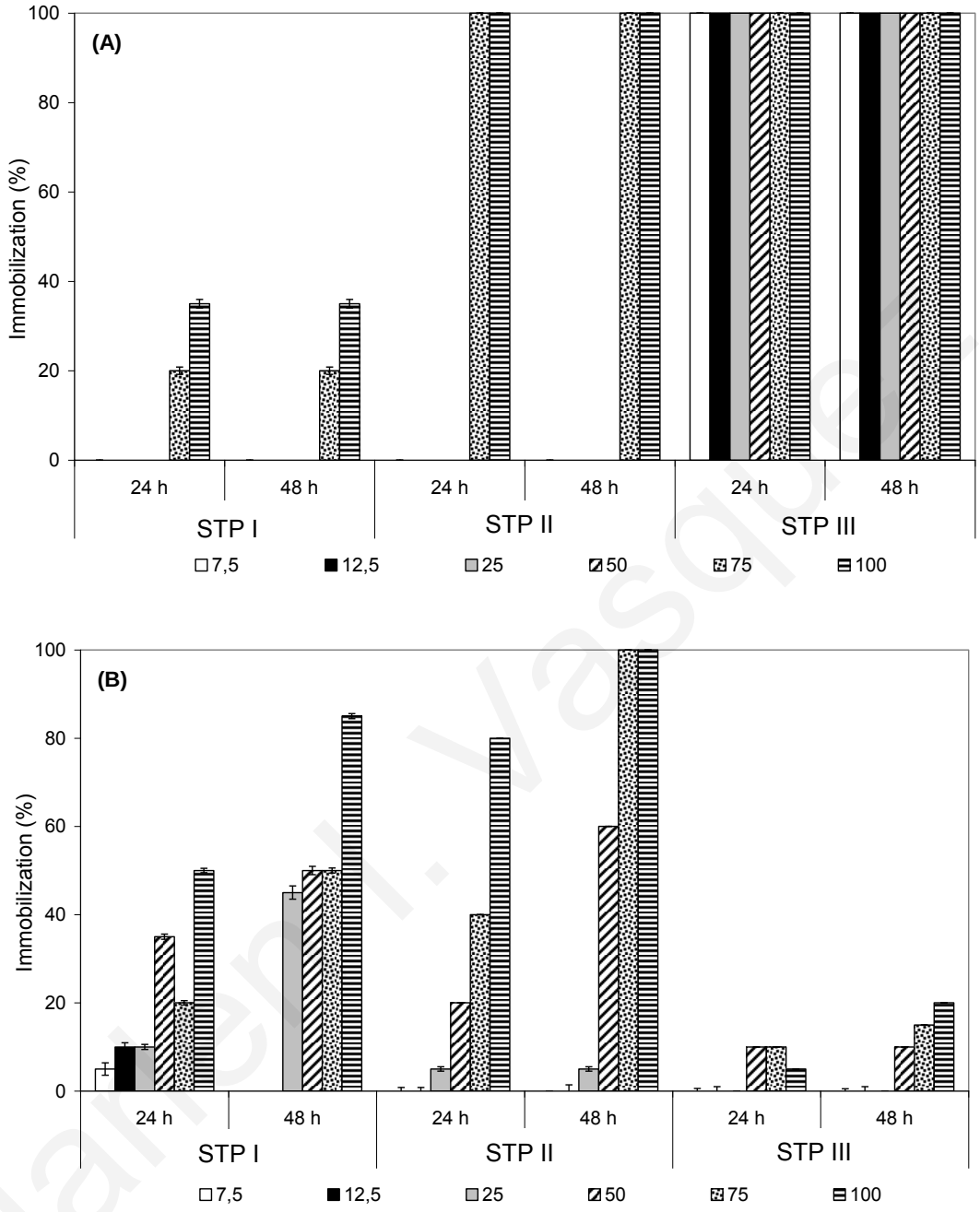


Figure 4.4 Immobilization percentage for *Daphnia magna* exposed to outlet (OUTs) samples collected in summer (A) and winter (B)

During winter, the toxicity of STP II-STEs was high, as presented in Figure 4.5 (B); whereas the toxicities of STP I and STP III were moderate. The effect was reduced significantly when the samples were slightly diluted. The order of toxicity decreased as follows STP II>STP III>STP I.

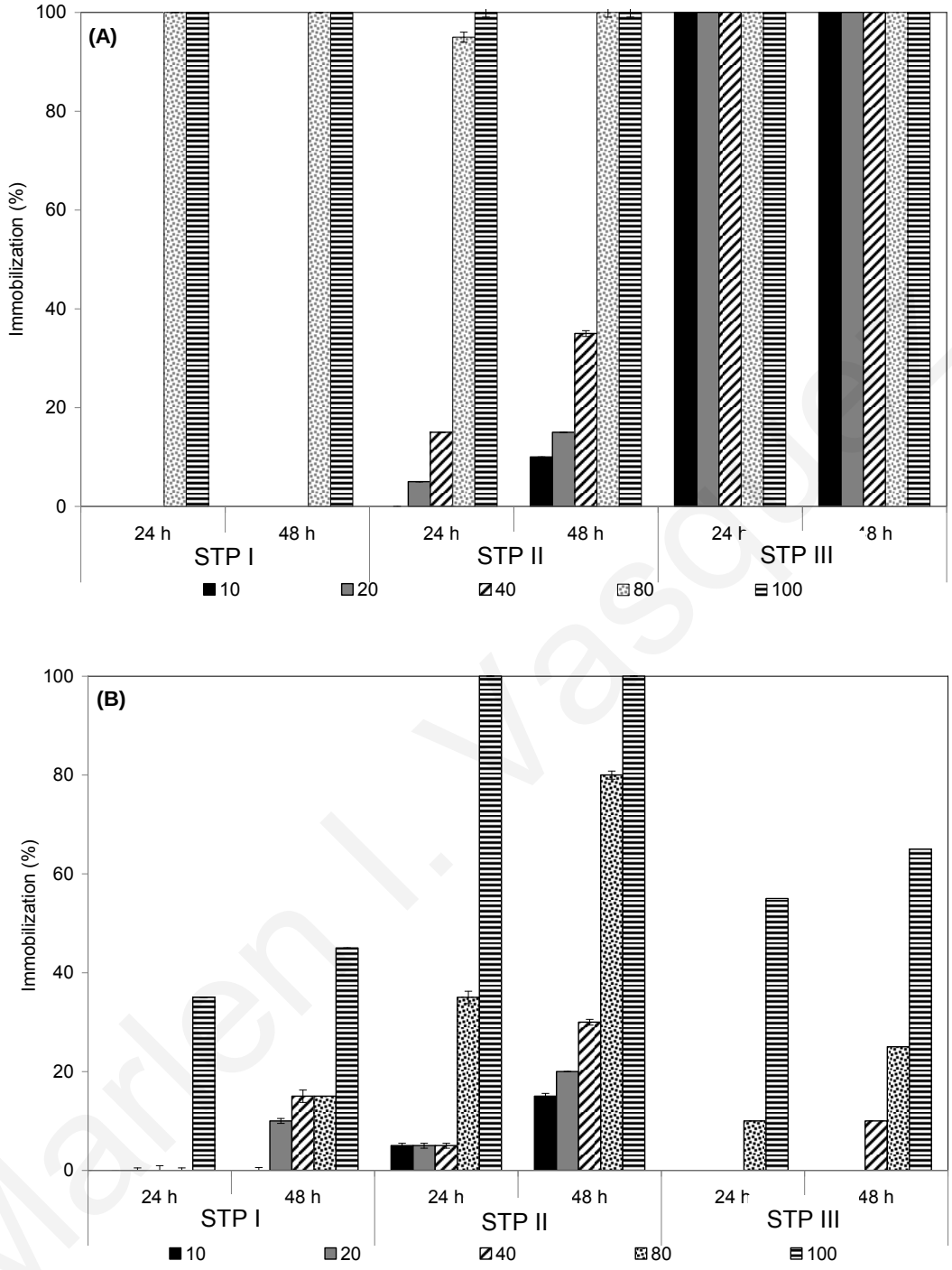


Figure 4.5 Immobilization percentage for *Daphnia magna* (Daphtox kit) exposed to secondary treated (STEs) samples collected in summer (A) and winter (B)

4.2.2.4 *Artemia salina*

As shown in Figure 4.6 (A), none of the STP I-INs and STP I-OUTs for the summer sampling resulted in toxicity to *A. salina* kits. Both STP II-INs (70% immobilization when the sample was not diluted) and STP III-INs (>75% immobilization starting from 50% sample volumes tested) displayed toxic effects after 48 h of exposure time; whereas no toxicity was observed in STP II and STP III-OUTs.

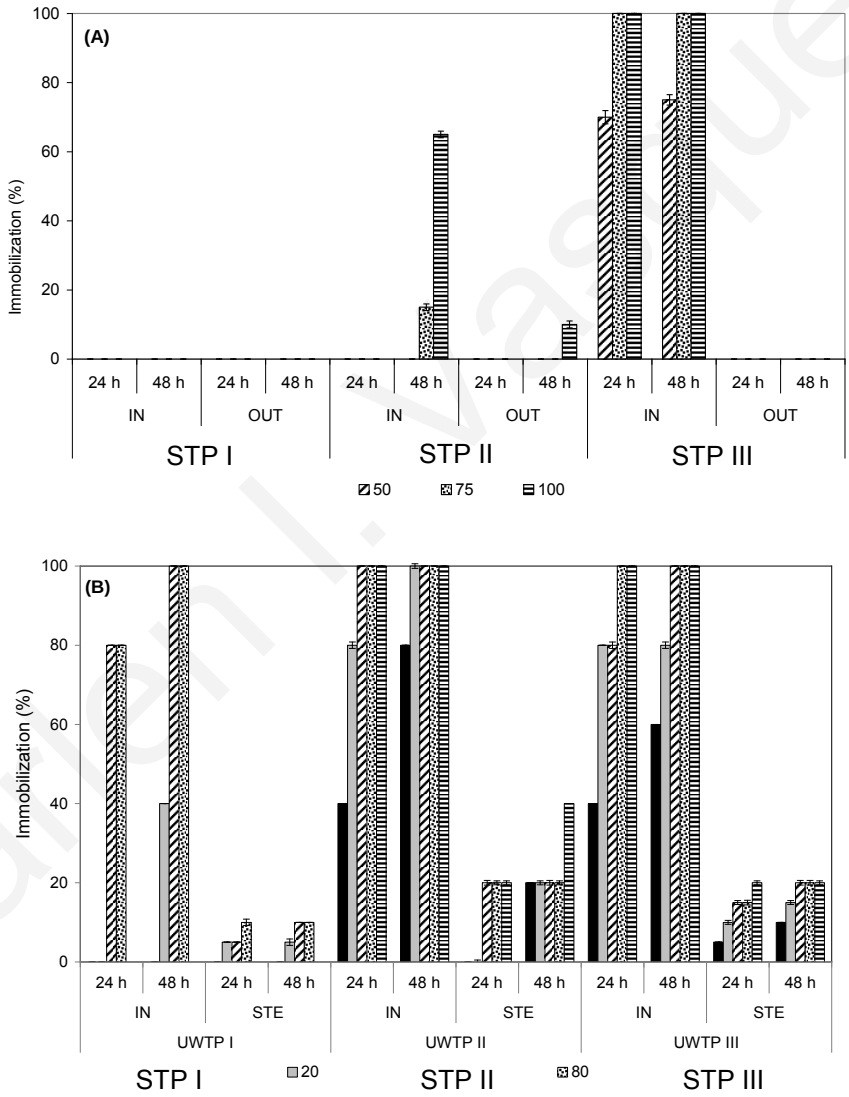
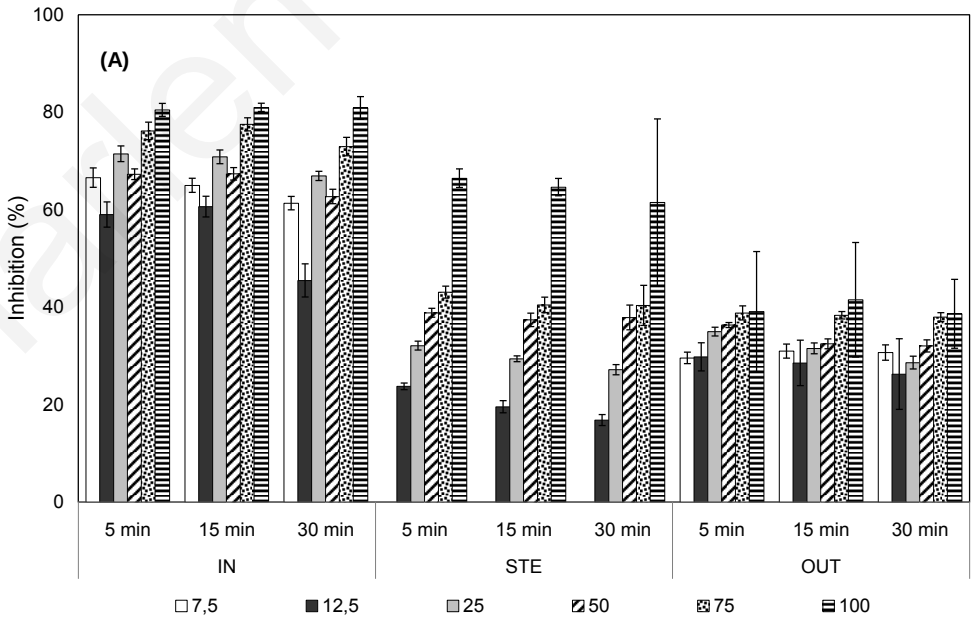


Figure 4.6 Immobilization percentage of *Artemia salina* (kits) exposed to inlet (INs) and outlet (OUTs) samples collected in summer (A), and *Artemia salina* (field collected) exposed to inlet (INs) and secondary treated (STEs) samples collected in winter (B)

The INs collected during the winter sampling from the STPs exhibited severe toxicity (100% immobilization) at the highest dilution (7.5%); whereas OUTs from the STPs did not show any significant toxicity to *A. salina* (data not shown). The use of the *A. salina* collected from Larnaka salt lake demonstrated the same toxicity pattern regarding the INs, which were severely toxic (Figure 4.6 (B)). The STEs from the three STPs were non-toxic causing a 10-20% immobilization percentage.

4.2.2.5 *Vibrio fischeri*

The results of the toxicity tests performed with *V. fischeri* with the samples collected during summer are illustrated in Figure 4.7 A, B and C. In all STPs the toxicity decreased in the order of INs>STEs>OUTs. Among STPs, the toxicity of the INs, STEs and OUTs decreased in the order STP I>STP II>STP III. Toxicity to *V. fischeri* did not vary significantly with exposure time (5-30 min). It is worth noting that hormetic effects, as bioluminescence stimulation, were observed in STP II-STEs, when exposed to the concentration of 7.5% (data not shown), indicating the occurrence of growth stimulating compounds in the STEs like nutrients.



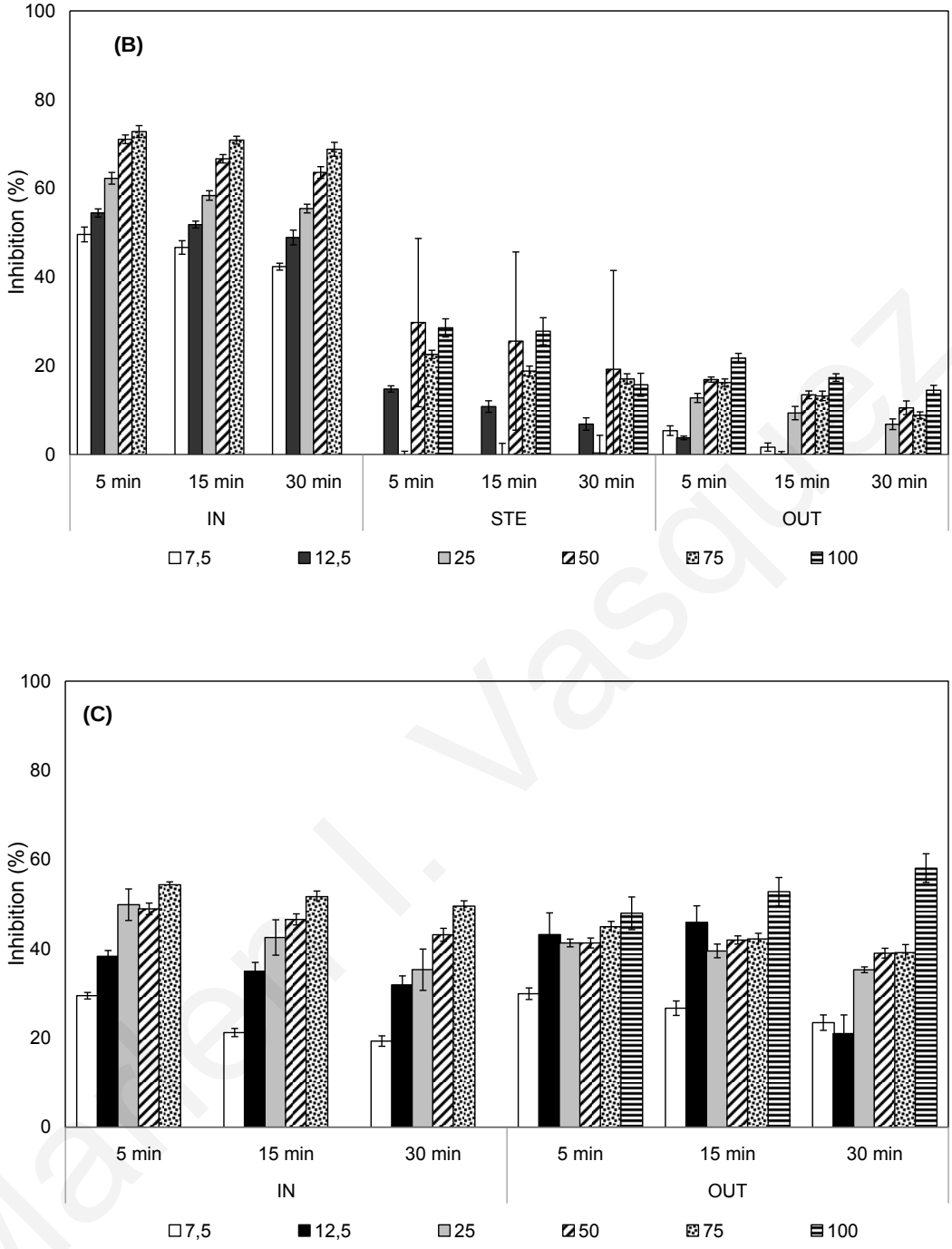


Figure 4.7 Inhibition percentage for *Vibrio fischeri* luminescence exposed to samples collected in summer samples from the inlet (IN) and the outlet (OUT) of sewage treatment plant I (A), II (B) and III (C)

The toxicity of the STP I and II-STEs remained more or less at the same levels after chlorination (OUTs) (Figure 4.7 A, B). Almost no difference was observed in the toxicity level between STP III-INs and -OUTs (Figure 4.7 C). It should be noted that the toxicity of INs and OUTs samples from all the STPs did not change significantly (ca. 20-30 %) between

different dilutions; whereas the toxicity of the STP I and II-STEs had a wider range of variation when diluted (20-70% inhibition). The robustness of this species to the wastewater composition may be attributed to the short exposure time, which might be insufficient to lead to toxicity effect observations.

The wastewater quality characteristics, provided in Table 4.11-Table 4.12, indicate that the inlet wastewater pollutants' load is high and at the same time variable enough to raise concerns related to the capability of the conventional activated sludge system to have a stable operation to comply with the discharge requirements.

The values of COD and BOD_5 parameters decrease gradually during the treatment process and the final effluents' characteristics generally comply with the quality limits provided in Table 4.1. However, the quality limit of the final effluent is not met at STP I-OUT, regarding the conductivity parameter. Furthermore, COD, total phosphorous and total nitrogen parameters slightly exceeded the limits in STP I-OUT and the BOD_5 level was above the limit set at STP II-OUT for the autumn sampling. This can be attributed to operational problems such as high sludge return concentration as expressed by the high SVI ratio, which can result in low viability of the microorganisms ratio [299].

It is also worth noting that the ammonia concentration which is related to the toxicity [124,299] was found to be high in the STP I, III-OUTs; whereas the total nitrogen was found to be in compliance with the limits set for STP III. The metal concentrations for STEs were in the range of 0.065-0.03 for Cd, 0.035-0.229 for Ni, 0.789-1.569 for Zn and 0.06-0.166 for Pb. Ammonia concentration was generally higher in STP I than STP III, as expected as nitrification/denitrification was not applied at STP I. In addition, it should also be mentioned that the STPs examined do not have balance tanks in order to regulate the amount and the loads of wastewater entering to the STPs.

Table 4.11 Characteristics of the sewage treatment plant samples during summer and winter samplings

Parameters	STP I						STP II						STP III					
	summer			Winter			summer			winter			summer			winter		
	IN	STE	OUT	IN	STE	OUT	IN	STE	OUT	IN	STE	OUT	IN	STE	OUT	IN	STE	OUT
pH	7.4	7.4	7.5	7.6	7.5	7.7	7.7		8.2	8.4		8.5	7.3		7.7	7.4	7.0	7.6
Temperature (ºC)	28.3	28.4	27.7	22.6	22.8	22.4	20.2		19.3	18.2		18.3	22.4		14.5	21.2	24.2	13.8
Conductivity (µS/cm)	2970	3630	4500	3380	3260	3400	1788		1702	1685		1521	1603		1319	1661		1370
BOD_5 (mg/L)	420	21	6	440	36	4	249		3	370		14	159		3	170		2
COD (mg/L)	833	99	69	852	100	74	483		49	477		46	566		30	607	42	40
SS (mg/L)	184	39	5	178	40	6	135		1	150		1	128		1	132	9	3
TDS (mg/L)													1000		300	1030		300
TP (mg/L)	18.2	8.6	7.9	16.2	11.1	10.2							18.9		3.8	25.0		2.6
PO_4-P (mg/L)													16.3		3.7	25.0		2.3
TN (mg/L)	81.25	47.5	13.8	75.0	47.5	18.7							55.0		9.6	97.0		12.0
NH_4-N (mg/L)	66.0	40.2	11.8	60.0	38.4	14.0				32.0		2.0	53.7		6.6	79.1		7.5
HS^- (mg/L)													2.4			0.20		
Total Hardness as mg/L $CaCO_3$															238			292

Table 4.12 Characteristics of the samples collected from sewage treatment plant I and III

Parameters	Frequency	STP I						STP III					
		Range			Mean value (SD)			Range			Mean value (SD)		
		IN	STE	OUT	IN	STE	OUT	IN	STE	OUT	IN	STE	OUT
pH	d	7.2-7.6	7.2-7.6	7.2-7.8	7.5 (0.1)	7.5 (0.1)	7.5 (0.1)	6.8-8.0	6.8-7.4	6.9-8.0	7.4 (0.2)	7.0 (0.1)	7.4 (0.3)
Temperature (ºC)	d	16.4-29.9	13.9-29.5	12.1-29.5	23.3 (3.9)	22.3 (4.8)	22.0 (5.2)	14.0-27.5	13.7-27.6	2.8-25.4	20.2 (2.8)	21.1 (3.0)	15.4 (5.2)
Conductivity (µS/cm)	d	2800-5670	3150-4650	3230-4520	3580 (600)	3800 (400)	3800 (400)	685-2240	-	1037-1558	1685 (163)	-	1358 (66)
BOD_5 (mg/L)	1 /w	86-460	13-42	3-27	411 (53)	29 (8)	12 (7)	390-1120	-	5-17	564 (167)	-	9 (3)
COD (mg/L)	3 /w	140-912	11-146	33-118	798 (100)	94 (24)	70 (20)	151-3198	20-273	6-65	660 (299)	49 (28)	32 (11)
TSS (mg/L)	3 /w	68-199	11-69	3-69	175 (20)	41 (10)	19 (13)	76-3240	4-175	1-13	272 (282)	23 (25)	3 (2)
TDS (mg/L)	3 /w	-	-	-	-	-	-	800-3500	-	100-1900	1381 (473)	-	518 (262)
TP (mg/L)	2 /w	8.3-26.0	7.1-11.1	5.0-11.0	18.9 (2.5)	8.7 (0.8)	8.4 (1.2)	8.9-26.0	-	0.8-19.6	16.9 (4.4)	-	5.2 (3.4)
PO_4-P (mg/L)	2 /w	-	-	-	-	-	-	0.3-25.0	0.5-20.9	0.2-16.7	12.0 (4.6)	5.8 (4.2)	4.7 (3.3)
TN (mg/L)	2 /w	22-84	22-60	3-25	76 (8)	44 (9)	14 (6)	8-125	-	3-23	74 (18)	-	7 (5)
NH_4-N (mg/L)	d	16-69	14-65	3-19	63 (7)	35 (8)	11 (4)	15-101	0-18	0-16	54 (14)	3 (4)	3 (4)
HS^- (mg/L)	1 /m	-	-	-	-	-	-	0.10-15.00	-	-	1.90 (1.96)	-	-
Total Hardness as mg/L $CaCO_3$	d	-	-	-	-	-	-	-	-	218-393	-	-	295 (39)

The overall results concerning the toxicity to the producers, consumers and decomposers se can be summarized in Table 4.13.

Table 4.13 The toxicity classification of the WET (100% tested) approach results on the tested samples versus bioassays

Bioassays	Sampling points	STP I	STP II	STP III
P. subcapitata	IN	**T**	**T**	**T**
	STE	**T**/ST	**T**/M	M
	OUT	M	**T**/M	**T**/ST
P. subcapitata (kit)	IN	**T**	**T**	**T**
	STE	**T**	M	M
L. sativum	IN	ST	ST	ST
	OUT	ST	ST	ST
D. magna	IN	**T**	**T**	**T**
	OUT	ST/M	**T**/M	**T**/ST
D. magna (kit)	IN	**T**	**T**	**T**
	STE	**T**/ST	M	**T**/M
A. salina	IN	ST	ST	**T**
	OUT	ST	ST	ST
A. salina (field collection)	IN	**T**	**T**	**T**
	STE	ST	ST	ST
V. fischeri	IN	M	M	M
	STE	M	ST	-
	OUT	ST	ST	M

Toxic (T): 75-100% toxicity; Moderate (M): 50-75% toxicity; slightly toxic (ST): <50% toxicity

A number of observations can be made regarding inter- and among species sensitivities. The vivarium grown organisms and the corresponding kit demonstrated similar toxicity, demonstrating their interchangeability. *A. salina* though, was an exception. The population collected from Larnaka saltmarsh was more sensitive than the organisms of the kit used.

The species can be ranked according to increasing sensitivity as follows: *L. sativum*>*V. fischeri*>*A. salina* (kit)>*A. salina* (field collected)>*D. magna=P. subcapitata.* The species sensitivity indicated that the terrestrial species were less sensitive, followed by the marine species. The freshwater species were more sensitive than the marine species.

Regarding the toxicity evaluation, the STPs -INs were undoubtedly found to cause the greatest toxicity. The STEs and OUTs toxicity were in the same range, decreasing the possibility of chlorination by-products being the most significant causes of the toxicity observed. Furthermore, the STEs and OUTs samples taken in winter, compared with those taken in summer, presented a lower toxic effect, indicating variability during the year. Among the STPs the toxicity increases as follows STP I>STP II>STP III. The nitrification/ denitrification procedure applied in STP III seem to improve wastewater quality and decrease its toxicity.

4.2.3 Hazard classification of wastewaters using a battery assay

After the assessment of effects using a multi-species approach described in the previous section, a toxicity investigation was performed for the STP I, II and III. Samples were taken seasonally. The species used were *P. subcapitata*, *D. magna*, *A. salina* and *V. fischeri.*

The effective concentration responsible for the inhibition in 50% of the population tested (EC_{50}) was calculated for the tests performed using regression analysis. The EC_{50} value for *P. subcapitata* was calculated by fitting the logistic, the S or the growth equation to the measured data (IBM SPSS, v. 19). The EC_{50} values for *D. magna* and *A. salina* were calculated using the Probit analysis (IBM SPSS, v. 19). The EC_{50} value for *V. fischeri* was calculated using the MicrotoxOmni software (SDI Europe, Hampshire, UK). A detailed description of the statistical analysis is provided in Chapter 5.

The EC_{50} values calculated for each species were transformed to Toxic Units (TU) permitting a comparison among species (Equation 4.1).

$$TU = \frac{100}{EC_{50}}$$

Equation 4.1 Toxic Unit conversion

In order to understand the meaning of the resulting TUs, a hazard classification was implemented. In the context of this study a hazard is defined as a source of potential danger to

the environment and hazard assessment as the evaluation of inherent properties of a sample to cause harm [300]. This should be differentiated from risk assessment which encloses the probability that a hazard will occur. The WET tests serve mainly to identify hazard, being a first stage in ecological risk assessment. The toxicity was ranked as suggested by Persoone et al. [301], in which samples are categorized in five classes according to the highest toxic response shown by at least one of the tests applied (Table 4.14).

Table 4.14 Hazard classification system for wastewaters discharged into the aquatic environment

TU	Toxicity	Class	Score
<0.4	No acute toxicity	Class I	0
0.4<TU<1	Slight acute toxicity	Class II	1
1<TU<10	Acute toxicity	Class III	2
10<TU<100	High acute toxicity	Class IV	3
TU>100	Very high acute toxicity	Class V	4

A weight score was calculated for each hazard class to indicate the quantitative importance of the toxicity in the class using Equation 4.2-Equation 4.3.

$$Class\ weight\ score = \frac{(\Sigma\ all\ test\ scores)}{n}$$

$$n = number\ of\ tests\ performed$$

Equation 4.2 Class weight score calculation

$$Class\ weight\ score\ in\ \% = \frac{Class\ weight\ score}{maximum\ class\ weight\ score} \times 100$$

Equation 4.3 Class weight score in percentage calculation

All INs samples were found to be severely toxic even when only a 5% concentration of sample was evaluated, consequently were ranked as Class V. The calculation of an EC_{50} value was possible only for *A. salina* through sample dilution. The INs had TUs for the 24 h exposure time ranging from 2.6-4.2, 4.03-5.52 and 3.7-4.2 in STP I, II and III, respectively. The TUs for the 48 h exposure time were ranging between 3.3-5.8, 7.1-10 and 4.5-5.0 in STP I, II and III, respectively. The toxicity of the INs increased in the order STP I>STP III>STP II.

The TUs of the STE are presented in Figure 4.8-Figure 4.10. The STEs of STP I (Figure 4.8) had a seasonal behaviour with the toxicity found during autumn and winter being lower and the toxicity during spring and summer being severely high.

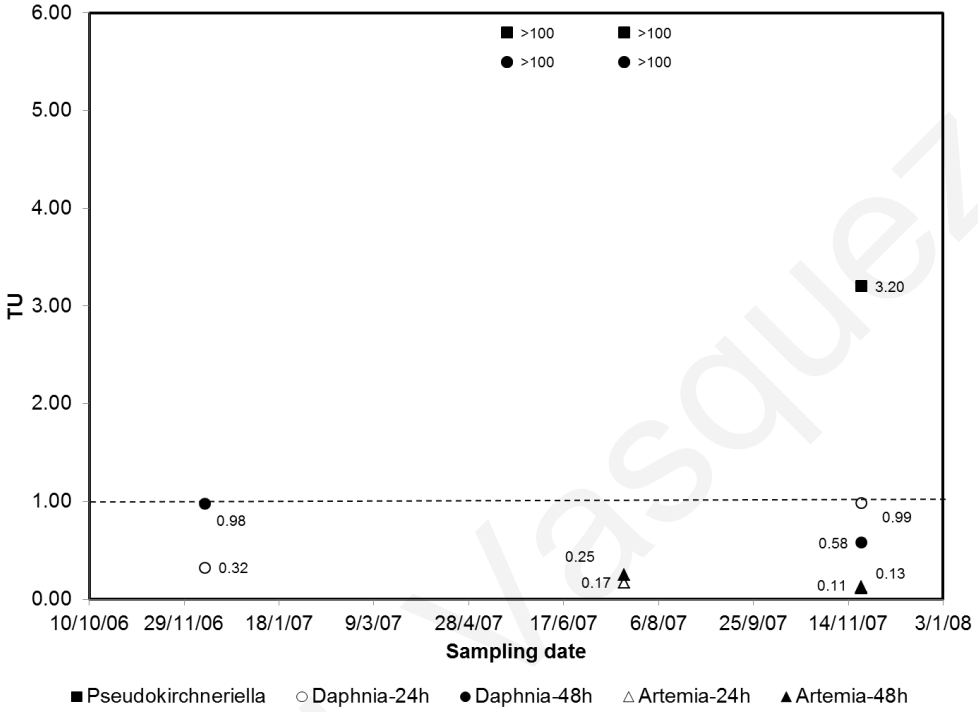


Figure 4.8 Toxicity evaluation of the secondary treated effluent (STEs) of sewage treatment plant I

The toxicity pattern was similar for the freshwater species; whereas *A. salina* was not affected by the STP I-STEs at any time when exposed both at 24 and 48 h. The STP I-STE was considered toxic for *P. subcapitata* in all tests performed but the toxicity intensity followed the seasonal behaviour observed.

The toxicity of the STP II-STEs is presented in Figure 4.9. It can be easily seen that all samples were found to be toxic to the species used with the exception of *A. salina*. The winter sampling showed lowest toxicity. The autumn sampling demonstrated the greatest range of toxicity (0.01 to >100 TU), indicating a species-specific type of toxicity. The chronic test with *P. subcapitata* was the most sensitive, followed by *D. magna* (48 h), *D. magna* (24 h), *A. salina* (48 h) and *A. salina* (24 h).

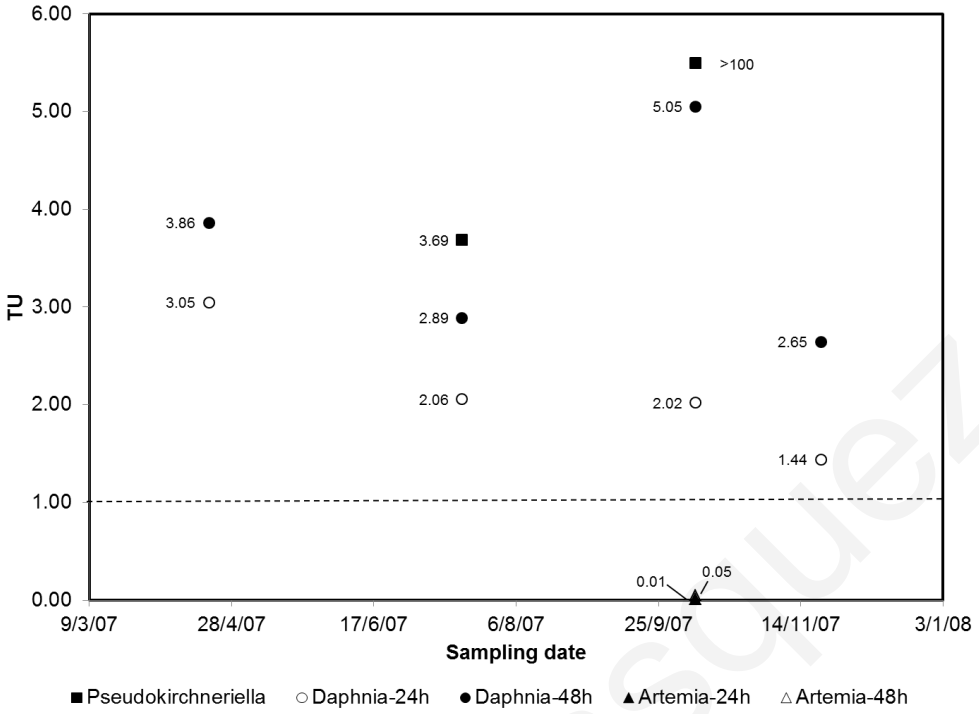


Figure 4.9 Toxicity evaluation of secondary treated effluent (STEs) of sewage treatment plant II

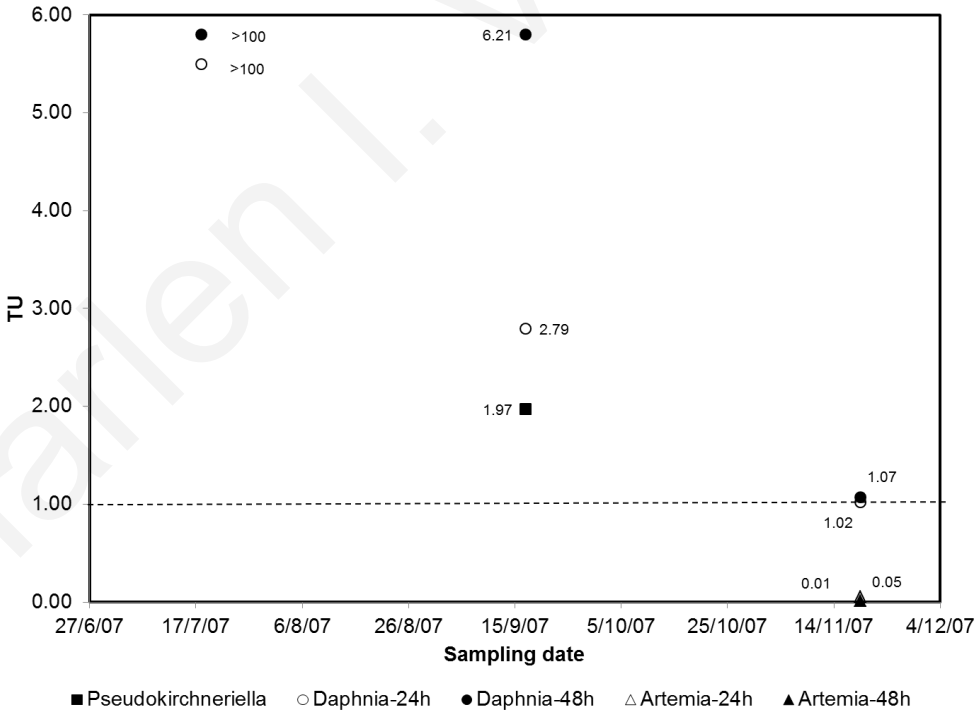


Figure 4.10 Toxicity evaluation of secondary treated effluent (STEs) of sewage treatment plant III

The toxicity of the STP III-STEs presented in Figure 4.10 shows a clear trend in which toxicity decreases from summer with TUs >100, autumn 1.97-6.21, to winter 0.01-1.07. *D.*

magna was found to be the most sensitive, followed by *P. subcapitata*. *A. salina* was not affected by the STEs samples.

In summation, the STEs were considered toxic to *P. subcapitata* and *D. magna*; whereas *A. salina* was not affected. The toxicity intensity had a variation throughout the year and among the STPs. Samples taken during summer were severely toxic in the three STEs. The initial assumption that the STEs would not be toxic due to the significant reduction of the biological load and the absence of disinfection products formed in the OUTs was rejected. For this reason the monitoring during the following year was performed in OUTs.

The toxicity of the OUTs was monitored during 2008 and the toxicity evaluation is presented in Figure 4.11-Figure 4.13. The toxicity of the STP I-OUTs showed low acute toxicity to *D. magna* (0.01-0.96 TUs), *A. salina* (0.01-0.16 TUs) and *V. fischeri* (0.1-1.52). However, chronic toxicity for *P. subcapitata* was observed during spring sampling (Figure 4.11).

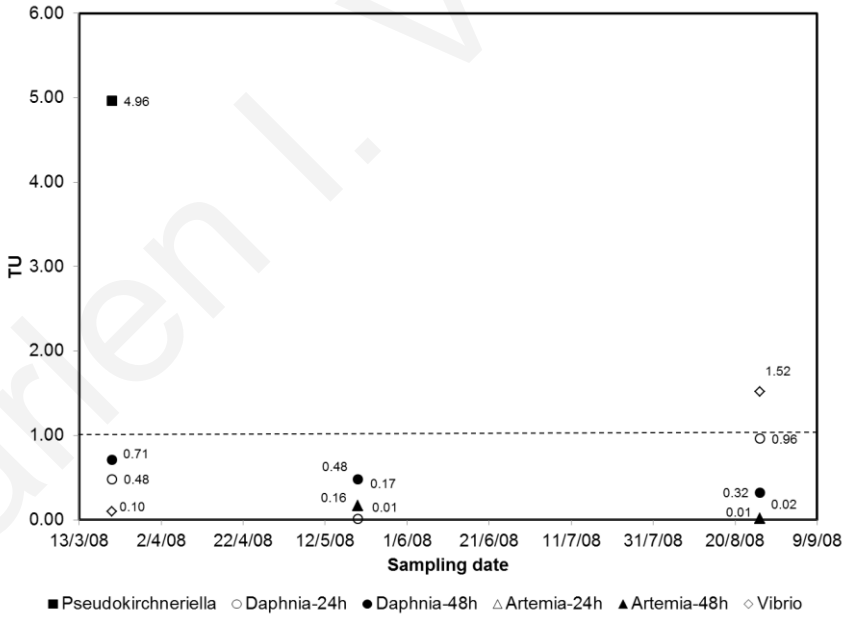


Figure 4.11 Toxicity evaluation of outlet (OUTs) samples of sewage treatment plant I

The toxicity of the STP II-OUTs is presented in Figure 4.12. The toxicity in acute and chronic bioassays was moderate and constant throughout the year with the exception of the winter sample that exhibited no toxicity to the species evaluated. The chronic test with *P. subcapitata* demonstrated the highest toxicity (2.99 TUs) which was in the same range of the

toxicity for *D. magna* after a 48 h exposure time. The range of toxicity observed was from 0.01 to 2.99.

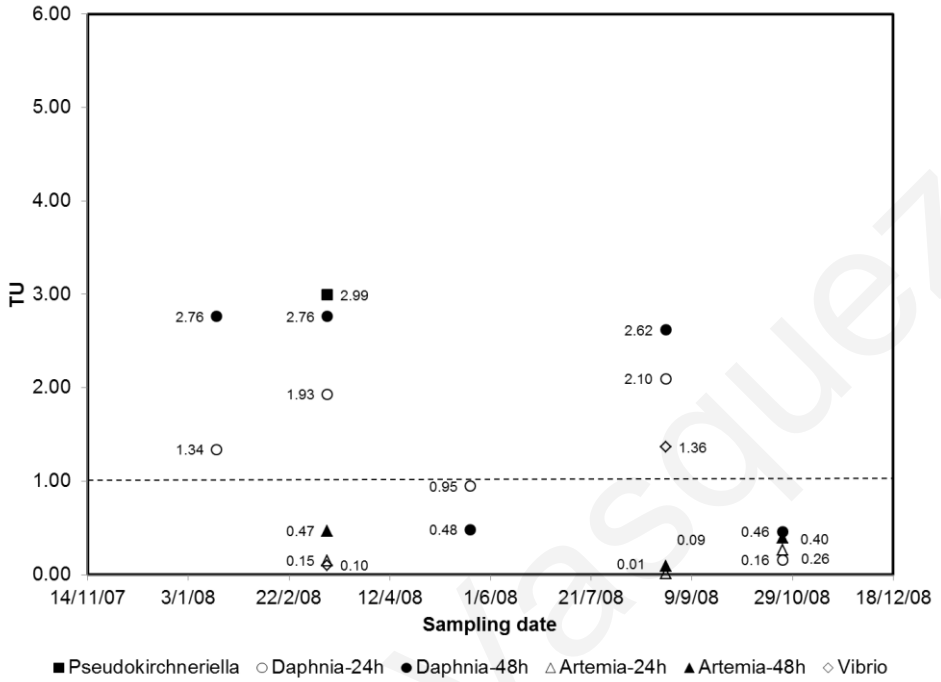


Figure 4.12 Toxicity evaluation of outlet (OUTs) samples of sewage treatment plant II

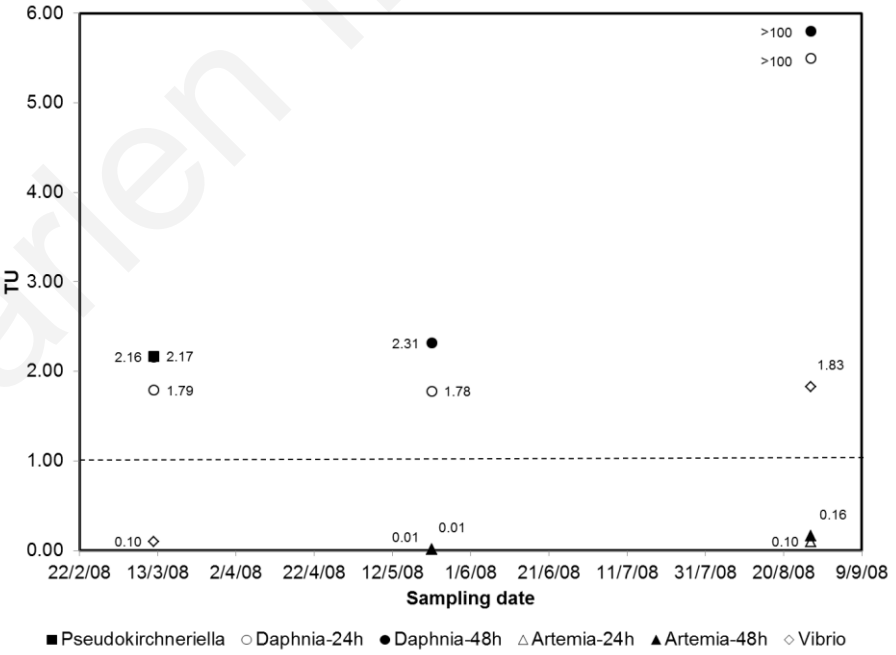


Figure 4.13 Toxicity evaluation of outlet (OUTs) samples of sewage treatment plant III

The STP III-OUT was evaluated and its results are shown in Figure 4.13. The toxicity of spring and summer samplings were in the same range; whereas the early autumn samples were more toxic. The freshwater microorganisms were negatively affected by all samples tested. The toxicity to *P. subcapitata* was the same as the toxicity to *D. magna* (48 h). *A. salina*, on the contrary, was not inhibited by the STP III-OUTs.

In order to compare the toxicity assessed among the INs, STEs and OUTs the hazard classification scheme previously described was applied. Table 4.1 summarizes the results for the assessment of effects for the species evaluated. As the sensitivity to freshwater and marine microorganisms was quite different, a separate hazard classification for the freshwater (F) and the marine (M) microorganisms was developed.

Table 4.15 Hazard scoring of sewage treatment plants wastewater

	Inlet (IN) (%)			**Secondary treated effluent (STE) (%)**			**Outlet (OUT) (%)**		
	T	**F**	**M**	**T**	**F**	**M**	**T**	**F**	**M**
STP I	100	100	100	50	71	0	30	71	16
STP II	100	100	100	46	55	0	55	77	25
STP III	100	100	100	55	68	0	38	57	16

T: total, F: freshwater organisms, M: marine organisms

The STPs receive severe toxic INs to both freshwater and marine microorganisms. During secondary and tertiary treatment this toxicity is reduced. Based on the methodology applied though, the reduction is not substantial as a hazard percentage of 46-55% for the STEs and 30-55% for the OUTs were calculated when all bioassays were taken into account simultaneously. The separate hazard scoring revealed a higher hazard for freshwater species ranging between 55-71% in STEs and 57-77% in OUTs. On the other hand, the hazard for the marine species was very low 0% for the STEs and 16-25% for the OUTs.

The methodology applied allows for a comparison among the STPs. During secondary treatment the toxicity is removed more efficiently in the order of STP III>STP I>STP II; whereas during tertiary treatment in the order of STP II>STP III>STP I. The hazards to

freshwater organisms of the STEs increase in the order of STP II>STP III>STP I and of the OUTs STP III>STP I>STP II.

Along with the toxicity evaluation, the physicochemical parameters of the INs, STEs and OUTs were monitored and compared to the mean values of the year, to investigate whether during the sampling dates extreme conditions were present. Regarding the INs all parameters were in the range of mean value ± one standard deviation for the three STPs investigated. Regarding the STEs, the parameters were in the range of the mean value ± one standard deviation for STP II and STP III. The STP I STEs however, deviated during the spring and summer samplings. During the spring sampling, conductivity (4.24 mS/cm) was in the range of the mean (3.76) ± two standard deviations (0.43); whereas during the summer sampling phosphorus concentration (11.1 mg/L) was in the range of the mean (8.72) ± three standard deviations (0.74), indicating higher deviation from mean values. Regarding the OUTs, the parameters were in the range of the mean value ± one standard deviation for the three STPs investigated.

To conclude this Section, bivariate comparisons were performed for all the parameters normally distributed. The Kolmogorov-Smirnov test (IBM SPSS, v. 19) was applied to recognize deviation from normal distribution. The Pearson's correlation coefficient was calculated to investigate correlations between the variables. A perfect negative correlation describes the relationship in which one variable increases as the other decreases. A perfect positive correlation describes the relationship in which both variables move in tandem. Perfect negative correlation denoted as -1, no correlation or non-linear as 0 and perfect positive correlation as 1 is presented in Table 4.16. No statistically significant correlation between toxicity and characterization parameters was observed using the Pearson's correlations coefficients.

The Pearson's correlations coefficients are presented in Table 4.16. The COD was correlated with the majority of parameters (temperature, conductivity, BOD_5, TSS, nitrates, total nitrogen and phosphorus) positively, besides pH, for which a negative correlation was calculated. The TSS could be correlated positively with conductivity, BOD_5, COD and phosphorus and negatively with the pH. The greatest positive correlation was found between the COD and the TSS (0.910) and between the BOD_5 and the total nitrogen (0.905); whereas the greatest negative correlation was found among the pH and the conductivity (-0.878). The

COD and the phosphorus concentrations (0.741) and the nitrate with the phosphorus concentration (0.73) were correlated positively with a $p<0.01$. The temperature was correlated positively with the COD concentration, demonstrating a seasonal behaviour of the three STPs.

Pearson's correlation coefficients were calculated to low toxicity samples, in which no statistically different distribution from the normal distribution was observed ($p<0.05$). The toxicity to *D. magna* at 24 h could be correlated negatively to the conductivity (-0.752, $p<0.05$), to the nitrate (-0.652, $p<0.05$) and to phosphorus (-0.863, $p<0.01$) concentrations. The same trend was observed when the toxicity to *D. magna* at 48 h was examined. Furthermore a negative correlation to the COD was calculated (-0.536, $p<0.05$) (data not shown).

The Spearman rank-order correlation, a more conservative approach, was applied to the data sets (Table 4.17), in order to overcome non normality of some parameters (*D. magna*, ammonia). The toxicity to *D. magna* at 24 h was negatively correlated with the nitrate (-0.583), nitrite (-0.86) and phosphorus (-0.633) concentrations ($p<0.05$). When the toxicity to *D. magna* at 48 h was examined, only the negative correlation to phosphorus (-0.686) was present ($p<0.01$). The toxicity to *A. salina* at 24 h was positively correlated to the conductivity (0.9, $p<0.05$). The toxicity to *A. salina* at 48 h was positively correlated to the conductivity (1, $p<0.01$) and to the ammonia concentration (0.812, $p<0.05$). The toxicity to *P. subcapitata* was positively correlated to the nitrate concentration (0.824, $p<0.05$).

Table 4.16 Pearson's correlation coefficient for the chemical parameters

		Temperature	pH	Conductivity	BOD_5	COD	TSS	Nitrates	Nitrogen Total	Phosphorus
Temperature	Pearson Correlation	1	-0.476	0.490	0.089	0.676*	0.552	-0.252	-0.093	0.422
	Sig. (2-tailed)		0.118	0.106	0.783	.016	0.063	0.586	0.843	0.346
	N	12	12	12	12	12	12	7	7	7
pH	Pearson Correlation	-0.476	1	-0.878**	-0.443	-0.657*	-0.688*	0.536	-0.657	0.706
	Sig. (2-tailed)	0.118		0.000	0.150	0.020	0.013	0.215	0.109	0.076
	N	12	12	12	12	12	12	7	7	7
Conductivity	Pearson Correlation	0.490	-0.878**	1	0.471	0.584*	0.648*	-0.131	0.181	-0.722
	Sig. (2-tailed)	0.106	0.000		0.123	0.046	0.023	0.780	0.698	0.067
	N	12	12	12	12	12	12	7	7	7
BOD_5	Pearson Correlation	0.089	-0.443	0.471	1	0.559*	0.632*	-0.171	0.905**	0.082
	Sig. (2-tailed)	0.783	0.150	0.123		0.030	0.012	0.637	0.000	0.823
	N	12	12	12	15	15	15	10	10	10
COD	Pearson Correlation	0.676*	-0.657*	0.584*	0.559*	1	0.910**	0.527	0.613	0.741**
	Sig. (2-tailed)	0.016	0.020	0.046	0.030		0.000	0.064	0.059	0.004
	N	12	12	12	15	18	18	13	10	13
TSS	Pearson Correlation	0.552	-0.688*	0.648*	0.632*	0.910**	1	0.402	0.621	0.595*
	Sig. (2-tailed)	0.063	0.013	0.023	0.012	0.000		0.173	0.055	0.032
	N	12	12	12	15	18	18	13	10	13
Nitrates	Pearson Correlation	-0.252	0.536	-0.131	-0.171	0.527	0.402	1	-0.071	0.730**
	Sig. (2-tailed)	0.586	0.215	0.780	0.637	0.064	0.173		0.846	0.005
	N	7	7	7	10	13	13	13	10	13
Nitrogen Total	Pearson Correlation	-0.093	-0.657	0.181	0.905**	0.613	0.621	-0.071	1	0.234
	Sig. (2-tailed)	0.843	0.109	0.698	0.000	0.059	0.055	0.846		0.516
	N	7	7	7	10	10	10	10	10	10
Phosphorus	Pearson Correlation	0.422	0.706	-0.722	0.082	0.741**	0.595*	0.730**	0.234	1
	Sig. (2-tailed)	0.346	0.076	0.067	0.823	0.004	0.032	0.005	0.516	
	N	7	7	7	10	13	13	13	10	13

*. Correlation is significant at the 0.05 probability level (2-tailed), **. Correlation is significant at the 0.01 level (2-tailed).

Table 4.17 Spearman's rho correlation coefficient for the toxicological and chemical parameters

			Daphnia-24 h	Daphnia-48 h	Artemia-24 h	Artemia-48 h	Pseudokirchneriella	Conductivity	Nitrate	Nitrite	NH_4-N	Phosphorus
Spearman's rho	Daphnia-24 h	Correlation Coefficient	1.000	0.948**	-0.025	-0.025	0.296	-0.211	-0583*	-0.860*	-0.242	-0.633*
		Sig. (2-tailed)	.	0.000	0.949	0.949	0.519	0.510	0.037	0.013	0.384	0.020
		N	18	18	9	9	7	12	13	7	15	13
	Daphnia-48 h	Correlation Coefficient	0.948**	1.000	0.076	0.042	0.333	-0.077	-0.530	-0.262	-0.260	-0.686**
		Sig. (2-tailed)	0.000	.	0.847	0.915	0.465	0.811	0.063	0.571	0.349	0.010
		N	18	18	9	9	7	12	13	7	15	13
	Artemia-24 h	Correlation Coefficient	-0.025	0.076	1.000	0.933**	1.000**	0.900*	0.395	-0.211	0.638	0.357
		Sig. (2-tailed)	0.949	0.847	.	0.000	.	0.037	0.333	0.789	0.173	0.385
		N	9	9	9	9	3	5	8	4	6	8
	Artemia-48 h	Correlation Coefficient	-0.025	0.042	0.933**	1.000	1.000**	1.000**	0.419	-0.211	0.812*	0.333
		Sig. (2-tailed)	0.949	0.915	.000	.	.	.	0.301	0.789	0.50	0.420
		N	9	9	9	9	3	5	8	4	6	8
	Pseudokirchneriella	Correlation Coefficient	0.296	0.333	1.000**	1.000**	1.000	0.821	0.824*	-0.272	0.471	0.464
		Sig. (2-tailed)	0.519	0.465	.	.	.	0.089	0.044	0.728	0.346	0.354
		N	7	7	3	3	7	5	6	4	6	6
	Conductivity	Correlation Coefficient	-0.211	-0.077	0.900*	1.000**	0.821	1.000	0.090	0.148	0.651*	-0.714
		Sig. (2-tailed)	0.510	0.811	0.037	.	0.089	.	0.848	0.751	0.022	0.071
		N	12	12	5	5	5	12	7	7	12	7
	Nitrate	Correlation Coefficient	-.0583*	-0.530	0.395	0.419	0.824*	0.090	1.000	0.748	0.024	0.834**
		Sig. (2-tailed)	0.037	0.063	0.333	0.301	0.044	0.848	.	0.053	0.947	0.000
		N	13	13	8	8	6	7	13	7	10	13
	Nitrite	Correlation Coefficient	-0.860*	-0.262	-0.211	-0.211	-0.272	0.148	0.748	1.000	-0.519	0.222
		Sig. (2-tailed)	0.013	0.571	0.789	0.789	0.728	0.751	0.053	.	0.233	0.632
		N	7	7	4	4	4	7	7	7	7	7
	NH_4-N	Correlation Coefficient	-0.242	-0.260	0.638	0.812*	0.471	0.651*	0.024	-0.519	1.000	0.256
		Sig. (2-tailed)	0.384	0.349	0.173	0.050	0.346	0.022	0.947	0.233	.	0.475
		N	15	15	6	6	6	12	10	7	15	10
	Phosphorus	Correlation Coefficient	-0.633*	-0.686**	0.357	0.333	0.464	-0.714	0.834**	0.222	0.256	1.000
		Sig. (2-tailed)	0.020	0.010	0.385	0.420	0.354	0.071	0.000	0.632	0.475	.
		N	13	13	8	8	6	7	13	7	10	13

**. Correlation is significant at the 0.01 level (2-tailed), *. Correlation is significant at the 0.05 probability level (2-tailed).

4.2.4 Estrogenicity screening using a recombinant yeast assay

An estrogenicity screening was performed by collecting 100 mL of STP II-INs and 1000 mL of STP II-OUTs samples (n=4) which were filtered and passed through a solid-phase extraction cartridges, as already described in Chapter 3. A standard curve with 17β-estradiol was prepared (Figure 4.14). The concentrations of 17β-estradiol in the wells ranged from 1.33 to 340 ng/L. The LOD and LOQ were calculated from the standard curve. The LOD was 1.33 ng/L and the LOQ was 2.65 ng/L.

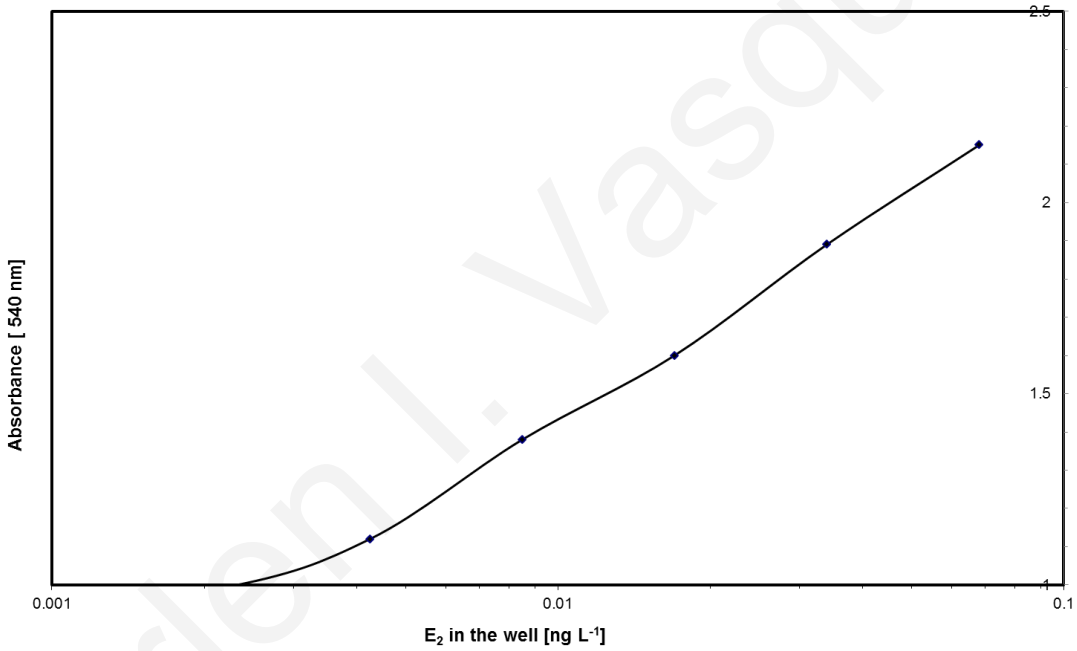
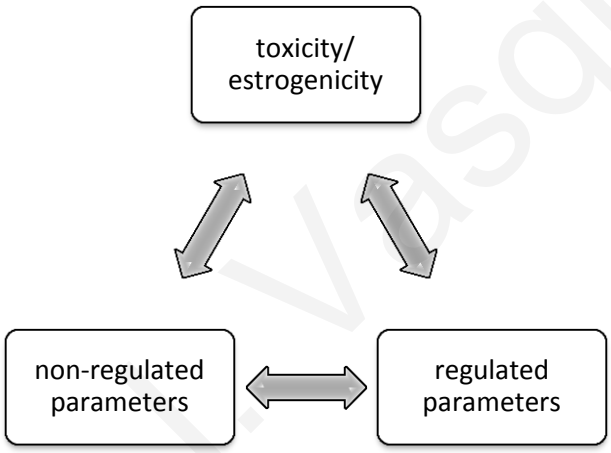


Figure 4.14 Standard curve for β-estradiol

All the STP II-OUTs evaluated had a corrected absorbance lower than 1 corresponding to a 17β-estradiol equivalent concentration lower than 2.65 ng/L. However, a color response was observed in all the tested samples, indicating that estrogenic compounds were present in the range of the LOD-LOQ (1.33-2.56 ng/L). Regarding the STP II-INs, a maximum 17β-estradiol equivalent of 25.6 ng/L was observed, demonstrating that the INs possessed estrogenic equivalent compounds that were removed during treatment.

4.3 Discussion

As presented herein, the main findings of this work include: (i) the concentrations of conventional pollution parameters regulated by the legislation, were not always found to be met in the OUTs (ii) among STP I, II and III, the quality of the INs, STEs and OUTs were not found to be similar (iii) severe toxicity was present in some OUTs. Concerns about the legislation implementation scheme applied in Cyprus are being in a recent journal publication, which was prepared in the framework of this thesis, by Fatta-Kassinos et al. [302] and illustrated in the Schematic 4.2.



Schematic 4.2 Key elements for hazard assessment of wastewater reuse practices

A WET approach was implemented for identifying and delineating treated effluents of concern by hazard ranking. This approach is in accordance with the EU Water Framework Directive which refers to the use of algae, *D. magna* and fish toxicity as food chain indicators for the monitoring of surface water quality. A higher plant was used as an irrigation water quality indicator as suggested by Brain et al. [303] and Pedrero et al. [304] and *A. salina* and *V. fischeri* as indicators of marine environments as suggested by Rizzo et al. [305]. Seasonal and between species variation in toxicity was observed, suggesting that the frequency of the evaluation of the toxicity according to the discharge permitting law is insufficient. Regarding seasonal variation, the findings are in accordance with previous studies, in which higher toxicity was observed during summer season due to less dilution and/or different composition of the wastewater. The variation between species may imply

a composition more toxic to one species than to another. For instance, algae were found to be more sensitive to herbicides and fungicides; whereas daphnids to pesticides [306].

D. magna and *P. subcapitata* were found to be very sensitive and the toxicity seems to be related to the process efficiency [299]. Although it has been clearly proved that *P. subcapitata* is more sensitive than *D. magna* to chemically treated wastewater samples [305,307,308]. The discrepancy observed between the results of *D. magna* and *P. subcapitata* tests exposed to the STP samples can be attributed to the wastewater characteristics and in particular to the presence of nutrients, which could be increased after nitrification, in the effluent [309]. *V. fischeri* and *A. salina* were less sensitive. Their use though should not be excluded since *V. fischeri* bioassay is a rapid method providing information for changes in wastewater quality characteristics quickly [127,139,140] and *A. salina* species could be used for assessing the quality of INs. *L. sativum* was not found to be sensitive for the specific samples and the use of other plants may be needed in future studies.

Physicochemical parameters are still the fundamental criteria to define the use of effluent after treatment. However, a recent study of Mendonça et al. [309] concluded that chemical evaluation of samples did not always correspond to the effects observed towards the tested organisms. The reverse situation was also observed, when samples had effects towards the tested organisms but no indication of potential hazard derived from the chemical evaluation. To some extent these findings were observed in the present study, in which the correlations found between some species and chemical parameters were not able to explain the toxicity present implying a more complex interaction scheme. For example low toxic samples to *D. magna* were negatively correlated with COD, conductivity, nitrate and phosphorus concentrations probably indicating a nutrient deficiency. These are controversial to other studies in which increased hardness, salinity and total dissolved solids have been related to adverse effects on daphnids [141,310]. In another study using industrial wastewater a direct relationship between COD of industrial wastewater and toxicity was shown [311]. A more complex situation seems to prevail that could not be unrevealed by monitoring the “traditional” chemical parameters, in which the combination of regulated parameters and/or unregulated parameters may cause the effects observed.

Much attention has been given to natural and synthetic steroidal hormones, which are shown to induce biological effects on some organisms at the level part per trillion concentration. In the present study low estrogenicicity was detected at STP II-INs and -OUTs. The OUTs concentrations were not quantified due to their low concentrations but the range is similar to the findings in the literature [312]. This is in accordance with an earlier study by Holbrook et al. [295] in which 51-67 % of estrogenic compounds were removed during sewage treatment. Chlorination, as a step for tertiary treatment (OUT) has been found to reduce the amount of estrogenic compounds, however in some cases this was coupled with an increase of the toxicity of effluents [312]. It is not a win-win situation though, since it was recently documented that the decrease of estrogenicity can cause an increase of the antiestrogenicity during chlorination processes [313]. The assay applied cannot identify the specific compounds responsible for the estrogenicity of the samples; however most likely is that the majority of activity is caused by the presence of 17β-estradiol, the synthetic 17α-ethinylestradiol and its metabolites estrone and estriol [314].

For future studies, innovative approaches in which a combination of *in vitro* bioassays for the determination of cytotoxic and genotoxic potential of wastewater could be implemented, in order to determine the presence and potential impacts of pollutants in the wastewater [315]. Methodologies for chronic toxicity, persistence and bioaccumulation need further development [316]. These are mandatory in cases of countries like Cyprus facing water scarcity and demanding alternative and safe water resources.

The application of multivariate techniques, such as the cluster analysis facilitated the interpretation of complicated multi-parametric data with seasonal and spatial variation collected in the present study. The recognition of the most critical parameters affecting the quality were recognised and correlated to other parameters to understand their effects. A substantial variability of the quality of the effluents was observed for the chemical parameters regulated. More importantly though the presence of various micropollutants and other non-regulated parameters may also vary on a daily basis especially during the touristic season, which is quite extended in Cyprus. The performance of the STPs was also found to vary during the sampling periods adding to the necessity of carrying out systematic toxicity assays.

Seasonal or monthly monitoring of chemical and toxicological assessment should be considered for all STPs in which reuse practices are in place. Furthermore, some stricter and more integrated reuse and recharge guidelines should be considered including a greater range of parameters and recharge quantities regarding the water bodies to be used in. Since the chemical parameters data sets demonstrated completely different quality of STPs wastewater, the requirements and limits should be differentiated, as well as the reuse practices implemented. For instance, the volume to be used for recharge purposes may be set according to the condition of the water body affected by this action. An example of such an approach is provided by Asano and Cotruvo presenting the criteria for groundwater recharge in California. Among others, wastewater used for recharge should meet all drinking water maximum concentration levels and less than 50% of the affected water volume should be used for recharge purposes [317]. In fact, the trend to overcome variability and Type II errors (false negative) is to increase the number of testing and reduce the number of dilutions tested to one (the instream waste concentration or the whole effluent sample with no dilution). The initial required monitoring frequency is quarterly for United States and Canada. However most water specialists acknowledge that more frequent monitoring would improve effluent representativeness [300].

In order to fully comprehend the ecological effects of releasing treated wastewater to the environment though, further stages are needed such as an exposure and effect characterization and assessment and risk characterization. Tools such as long-term laboratory or field bioassays and toxicity identification evaluation should be considered for further studies. For instance quantitative and probabilistic studies, in which duration and magnitude of actual exposures of resident communities to effluent could be implemented [300]. Food chain effects may also need to be accounted since they represent a separate exposure route. Irrigation should be included as an additional exposure route for chemicals in terrestrial ecosystems, in order to assess potential risks derived. Special behaviour of wastewater should be studied since many regulators and scientists recognize that concentration-response patterns will not always follow the traditional pattern, especially when complex mixtures are examined. A hormetic curve or inverted U-shaped curves are some examples of these deviations. In some cases the release of treated effluent may not be detrimental if a hormetic curve can best fit the dose-response behaviour of the treated effluents, meaning that low concentrations may be beneficial. An inverted U-shaped, in

which toxicity is observed at both ends of the U, ascribed to endocrine disrupting chemicals, may also be a non-traditional dose-response behaviour [300] that requires more investigation. Chemical analyses, biological assessments of receiving water and toxicity identification evaluations would complement the weight of evidence approach for decision-making.

Fortunately, the technology needed to improve wastewater quality exists and taking into account the socioeconomic conditions the most cost-effective method can be selected. Countries of similar water scarcity problems are already considering in implementing quaternary treatment by the end of the decade in order to improve wastewater quality and ensure its safe reuse [318]. The need to develop advanced treatment processes such as ozonation [276], ultrasonic irradiation [308,319] and photocatalytic oxidation [305,307] just to name a few, in order to hinder the release of micropollutants included in the effluents organic matter is apparent. Their evaluation with toxicity bioassaying is crucial since the transformation products [312] generated during the processes may also exhibit toxicity.

To conclude the 2002 Hyderabad Declaration on Wastewater Use in Agriculture could be restated "without proper management, wastewater use poses serious risks to human health and the environment" [136].

4.4 Conclusions

This work was designed to test a number of bioassays, the objective being the application of the whole effluent toxicity approach to sewage treatment plant effluents reused for irrigation and water bodies' replenishment. Five species from different taxonomies (decomposer, producers and consumers) and environmental habitats (fresh and marine water, terrestrial plant seeds) were exposed to the samples collected from different process steps at various dilutions. Estrogenicity screening was performed. Variation regarding seasons and species was observed. Increased toxicity during summer season was identified; whereas low estrogenicity was detected during rainy season. A toxicity hazard classification procedure identified higher hazard to freshwater species than to marine

species. This is positive, as recharge of water bodies is done mainly during the rainy season, when reclamation for agricultural purposes is low. On the other hand, the use of treated effluents with toxicity may enclose unknown risks that should be investigated.

Variation in chemical parameters data sets and in the sewage treatment plant process efficiency were observed. However the toxicity could not be satisfactory correlated to the traditional parameters evaluated, highlighting the complexity of real matrices. However, even if the toxicity results could not be completely explained, some chemical parameters such as conductivity, ammonia and heavy metal concentrations were found to be of concern and should be evaluated in future studies.

CHAPTER 5. ASSESSMENT OF EFFECTS OF SINGLE ACTIVE PHARMACEUTICAL INGREDIENTS USING A BATTERY ASSAY

5.1 Background information

All types of urban wastewater discharge and reuse practices, including irrigation of landscape and agricultural areas, groundwater replenishment, discharge into inland surface water and sea, cause the release of organic xenobiotic substances into the environment. Fresh surface water bodies are most often used both for receiving treated effluents but also for providing water for the production of drinking water. Therefore, many of these organic compounds including APIs, which constitute one of the most diverse chemical classes of compounds, find their way into the urban water cycle.

In the present work, the eight APIs selected were assessed with acute and chronic ecotoxicity tests using *V. fischeri*, *D. magna* and *P. subcapitata*. These standard tests provide a first step in the assessment of effects of APIs [107,166]. In this thesis the need to assess each API separately was a prerequisite to better understand the effects when all APIs were subsequently exposed simultaneously in complex mixtures, as presented in Chapter 6.

5.2 Results

5.2.1 Acute toxicity assessment using the bacteria *Vibrio fischeri*

At least four independent experiments were performed for each API. Each experiment was performed in triplicate. At least nine concentrations were tested in each experiment, according to the 90% basic test for pure compounds method, provided by the manufacturer.

The response measured was the bioluminescence at 5 and 15 min. The I_0 and I_t, representing the initial bioluminescence and the bioluminescence at time *t* were recorded via a luminometer as described in Section 3. Data was analysed using the MicrotoxOmni software (SDI Europe, Hampshire, UK). Prior analysis, the data were adjusted using a correction factor f_k using the Equation 5.1.

$$f_k = \frac{I_0}{I_t}$$

Equation 5.1 Correction factor calculation

in which f_k: correction factor, I_0 : the initial bioluminescence, I_t: the bioluminescence at t time. A corrected I_{ct} was calculated by multiplying the I_0 with the f_k.

The percentage inhibition of the bioluminescence was calculated using the Equation 5.2.

$$\%\, I = 100 \times \frac{I_{ct} - I_t}{I_{ct}}$$

Equation 5.2 Bioluminescence inhibition percentage calculation

The average inhibition of at least three measurements was used to calculate the Γ value using the following equation.

$$\Gamma = \frac{\bar{I}}{(100 - \bar{I})}$$

Equation 5.3 Inhibition transformation

The $\log \Gamma$ and $\log C$ were used for the calculations of the EC values and are presented in the following Sections.

5.2.1.1 β-blockers

Atenolol, metoprolol and propranolol were examined. The dose-response curves of atenolol are presented in Figure 5.1. The slopes for 5 and 15 min exposure times were 4.73 and 3.48, respectively. For the same exposure times the f_k were 1.153 and 1.104. Furthermore, the estimating equations for the curves were $\log C = 0.2100 \times \log \Gamma + 1.966$ and $\log C = 0.2816 \times \log \Gamma + 1.896$ with coefficients of determination (R^2) of 0.99 and 0.98 for the 5 and 15 min exposure times, respectively.

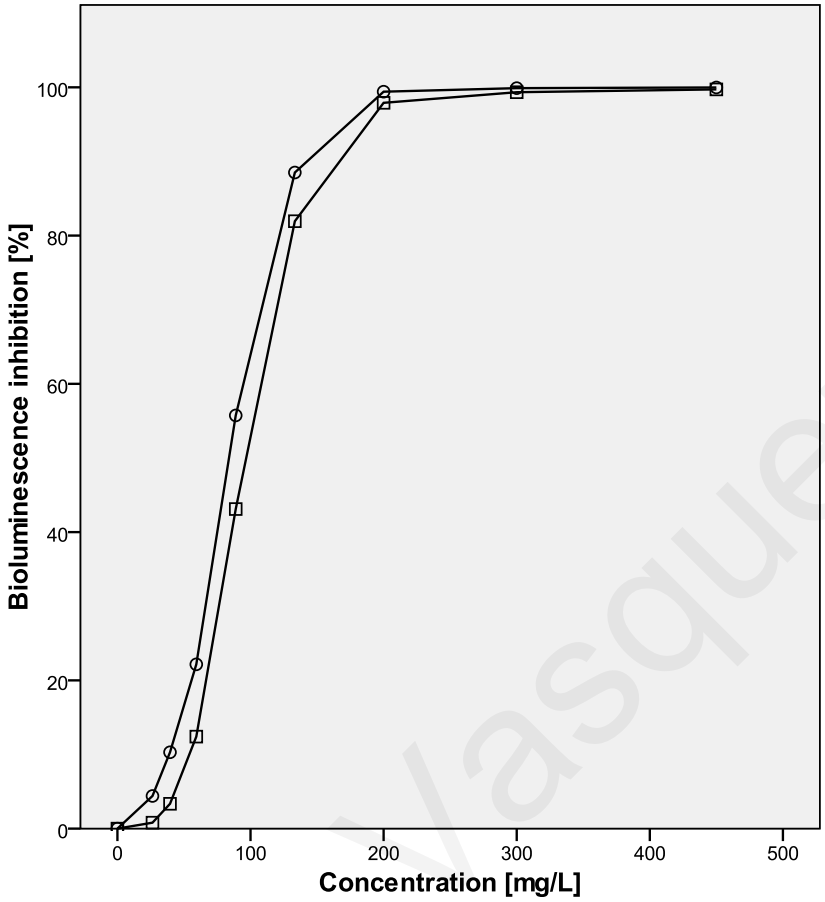


Figure 5.1 Inhibition percentage of the bioluminescence of *Vibrio fischeri* after an exposure time of 5 (circle) and 15 (square) minutes to atenolol

The EC_1 values were 35.23 and 21.58 mg/L and the EC_{50} values were 92.39 mg/L (95% confidence range: 82.20 to 103.8 mg/L) and 78.69 mg/L (95% confidence range: 64.42 to 96.12 mg/L) for 5 and 15 min exposure times, respectively.

The dose-response curves of metoprolol are presented in Figure 5.2. The slopes were 1.03 and 0.97 for the 5 and 15 min exposure times, respectively. The f_k were 0.902 and 0.805 and the estimating equations were $\log C = 0.9654 \times \log \Gamma + 3.204$ and $\log C = 1.005 \times \log \Gamma + 3.263$ with R^2 of 0.99 and 0.98 for the 5 and 15 min exposure times, respectively.

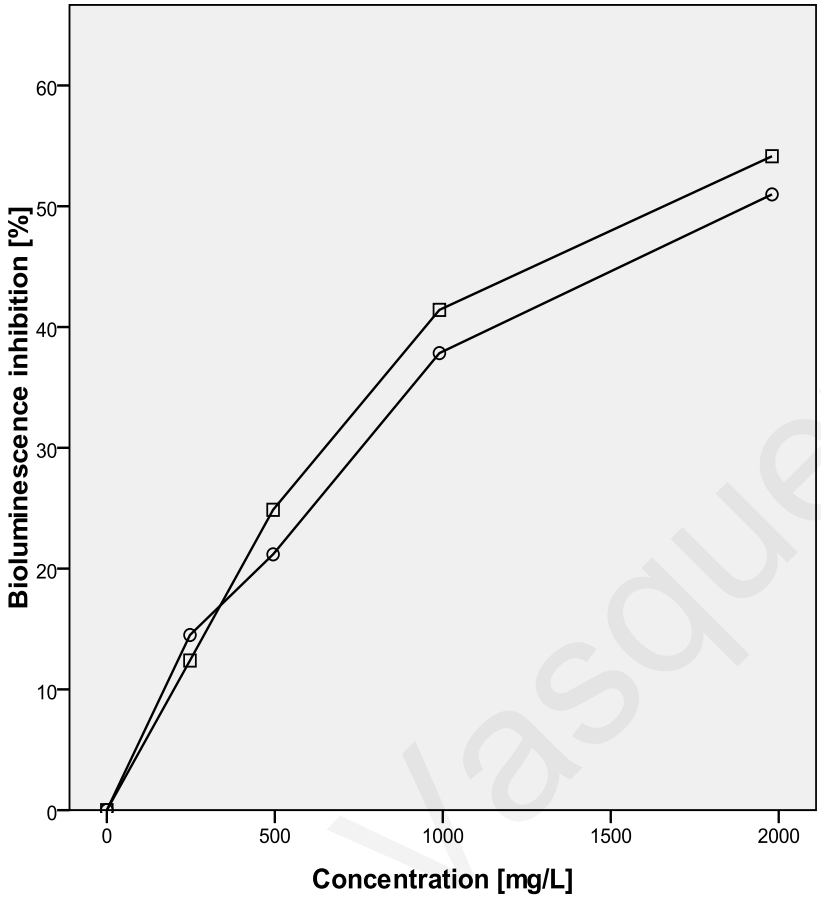


Figure 5.2 Inhibition percentage of the bioluminescence of *Vibrio fischeri* after an exposure time of 5 (circle) and 15 (square) min to metoprolol

The EC_1 value of 5 min exposure time was 18.94 mg/Land 18.09 mg/L for 15 min exposure. The EC_{50} values were 1600 mg/L (95% confidence range 1429 to 1792 mg/L) and 1834 mg/L (95% confidence range 1533 to 2193 mg/L), respectively.

The dose-response curves for propranolol, as shown in Figure 5.3, had slopes of 1.235 and 1.425 for the 5 and 15 min exposure, respectively. The f_k were 1.407 and 1.148 and the estimating equations were $\log C = 0.7748 \times \log \Gamma + 1.878$ and $\log C = 0.6528 \times \log \Gamma + 1.734$ with R^2 of 0.96 and 0.93 for the 5 and 15 min exposure, respectively.

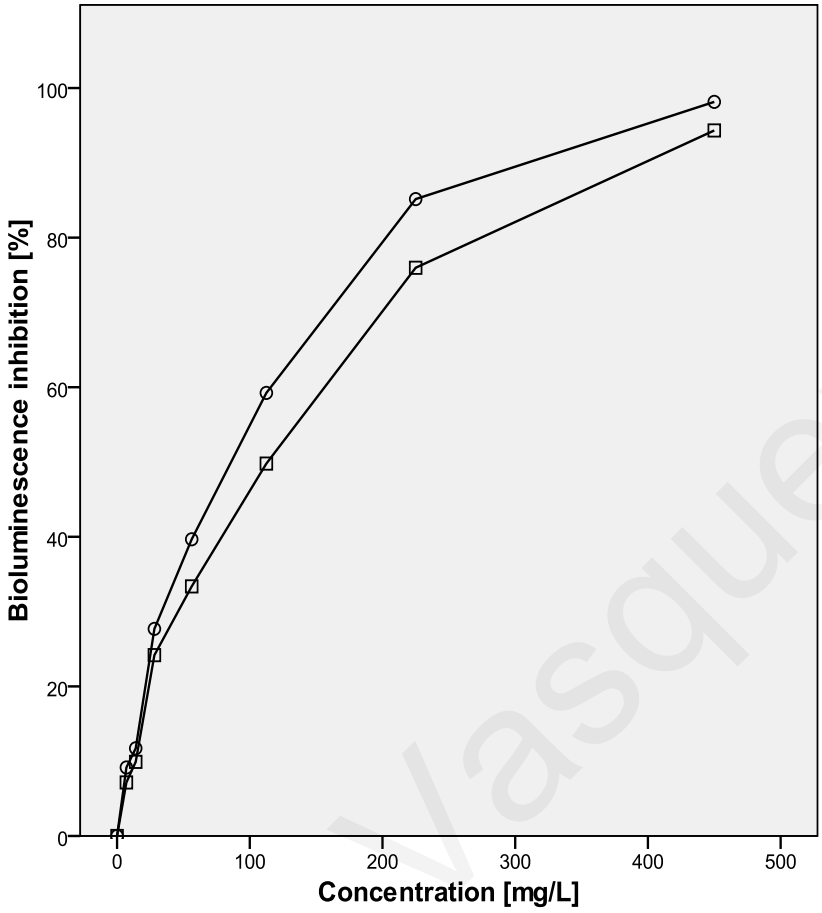


Figure 5.3 Inhibition percentage of the bioluminescence of *Vibrio fischeri* after an exposure time of 5 (circle) and 15 (square) min to propranolol

The EC_1 values were 2.15 and 2.70 mg/L; whereas the EC_{50} values were 75.43 mg/L (95% confidence range: 53.78 to 105.8 mg/L) and 54.20 mg/L (95% confidence range: 35.58 to 82.57 mg/L) for the 5 and 15 min exposure times, respectively.

For the β-blockers examined, a comparison of the dose-response curves for the 5 min exposure time is provided in Figure 5.4. Atenolol and propranolol demonstrated statistically non different EC_{50} values; whereas metoprolol had a much higher value. The same stands true, after the comparison among the EC_{50} values for the 15 min exposure (data not shown).

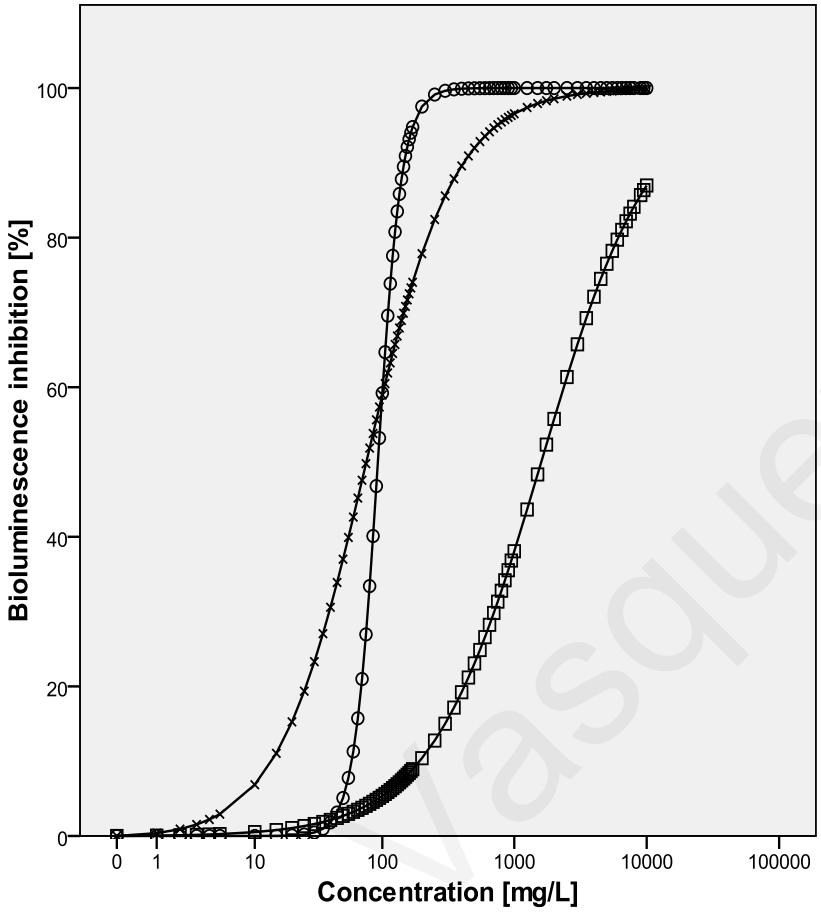


Figure 5.4 Dose-response curves for the percentage bioluminescence inhibition of *Vibrio fischeri* for atenolol (circle), metoprolol (square) and propranolol (cross) at 5 min exposure time

Propranolol was found to cause effects at lower concentrations compared to atenolol and metoprolol. The slopes for metoprolol and propranolol inhibition were similar; whereas the slope for atenolol was higher, demonstrating a steeper curve.

5.2.1.2 Non-steroidal anti-inflammatory APIs

The dose-response curves for diclofenac are presented in Figure 5.5. The curves for diclofenac had slopes of 1.033 and 1.486 and the f_k were 0.9241 and 0.8680 for the 5 and 15 min exposure time, respectively. In the same order, the estimating equations were $\log C = 0.9162 \times \log \Gamma + 1.783$ and $\log C = 0.6494 \times \log \Gamma + 1.607$ with R^2 of 0.95 and 0.96.

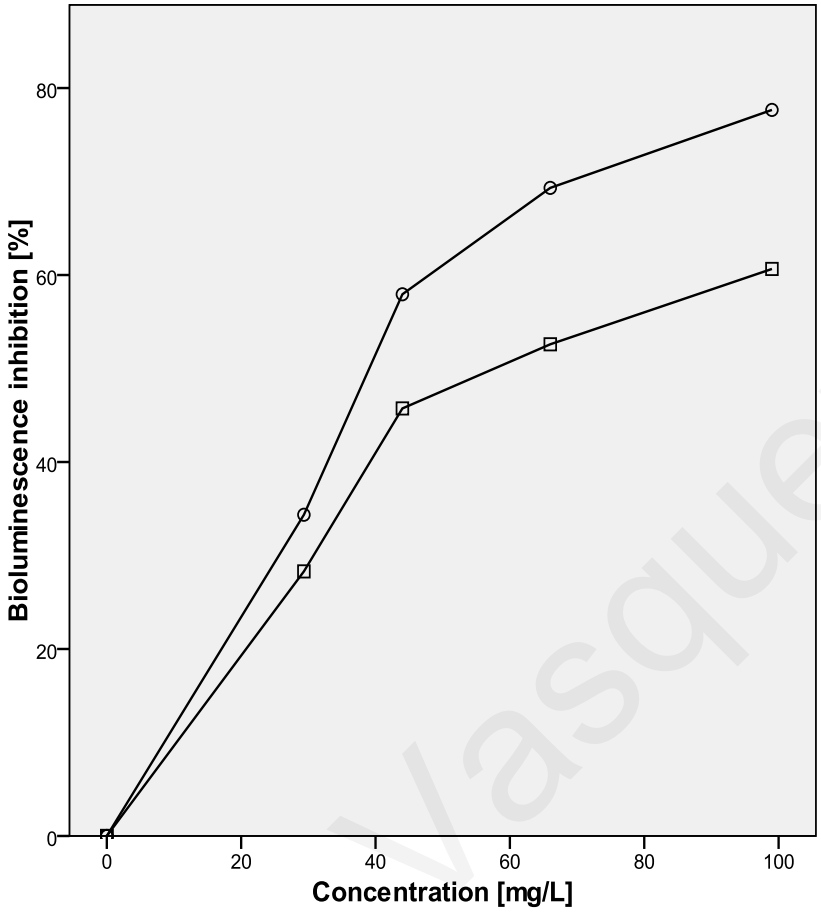


Figure 5.5 Inhibition percentage of the bioluminescence of *Vibrio fischeri* after an exposure time of 5 (circle) and 15 (square) min to diclofenac

The EC_1 values were 0.90 and 2.05 for the 5 and 15 min exposure times, respectively. The EC_{50} values were 60.74 mg/L (95% confidence range: 54.46 to 67.74 mg/L) and 40.47 mg/L (95% confidence range: 36.58 to 44.77 mg/L) for the 5 and 15 min exposure times, respectively.

The dose-response curves for ibuprofen are shown in Figure 5.6. The slopes for 5 and 15 min exposure times were 1.027 and 1.080 and the f_k were 1.089 and 0.8991, respectively. In the same order, the estimating equations were $\log C = 0.9671 \times \log \Gamma + 1.623$ with R^2 of 0.99 and $\log C = 0.9226 \times \log \Gamma + 1.639$ with R^2 of 1.

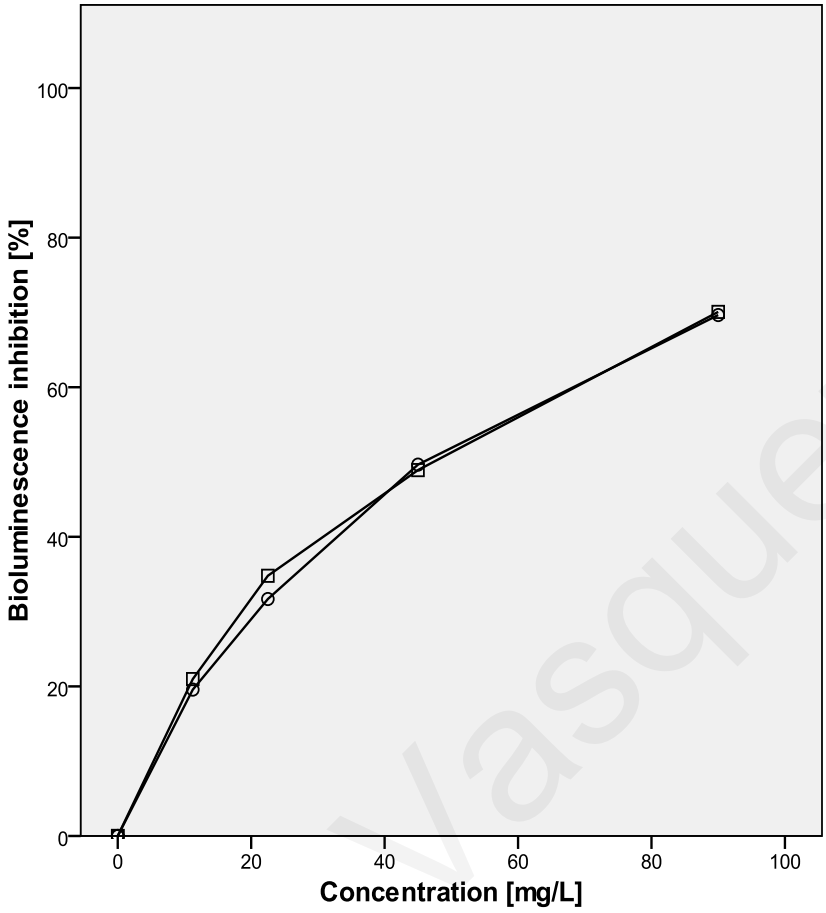


Figure 5.6 Inhibition percentage of the bioluminescence of *Vibrio fischeri* after an exposure time of 5 (circle) and 15 (square) min to ibuprofen

After a 5 min exposure time the EC_1 value was 0.49 mg/L and after a 15 min was 0.63 mg/L. The EC_{50} values were 41.99 mg/L (95% confidence range: 33.93 to 51.97 mg/L) and 43.60 (95% confidence range: 37.44 to 50.76 mg/L) for the 5 min and the 15 min exposure times, respectively.

Regarding non-steroidal anti-inflammatory APIs, ibuprofen was found to be slightly more toxic than diclofenac. The EC_{50} value is statistically different when exposed for 5 min; which was not the case when exposed for 15 min.

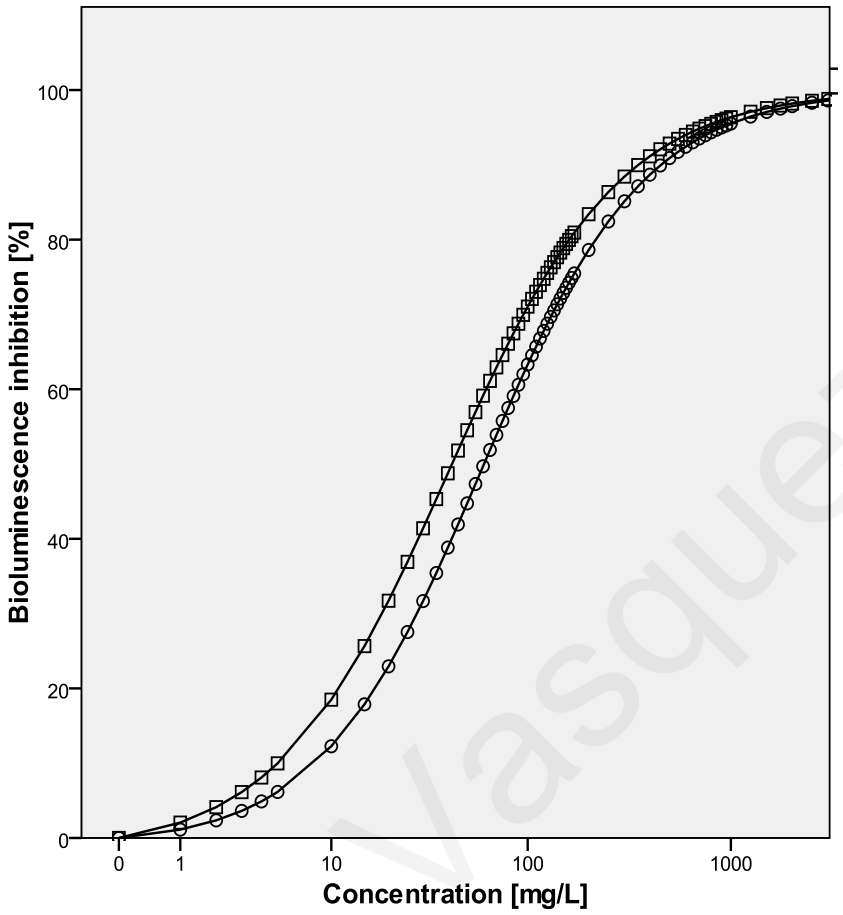


Figure 5.7 Dose-response curves for the percentage of the bioluminescence inhibition of *Vibrio fischeri* for diclofenac (circle) and ibuprofen (square) at 5 min exposure time

As shown in Figure 5.7, the dose-response curves for diclofenac and ibuprofen after an exposure time of 5 min have similar slopes with diclofenac being more to the right, hence demonstrating lower toxicity. The dose-response curves for diclofenac and ibuprofen after an exposure time of 15 min did not differ markedly with the ones after an exposure time of 5 min (data not shown).

5.2.1.3 Antibiotics

The dose-response curves for erythromycin, ofloxacin and sulfamethoxazole are shown in Figures 5.8-5.10. The slopes for erythromycin were 1.980 and 1.941 and the estimating equations were $\log C = 0.5050 \times \log \Gamma + 2.637$ and $\log C = 0.5134 \times \log \Gamma + 2.471$ for the

5 and 15 min exposure time, respectively. For both equations the R^2 was 1 and the f_k were 1.187 and 1.169 for the 5 and 15 min exposure time respectively (Figure 5.8).

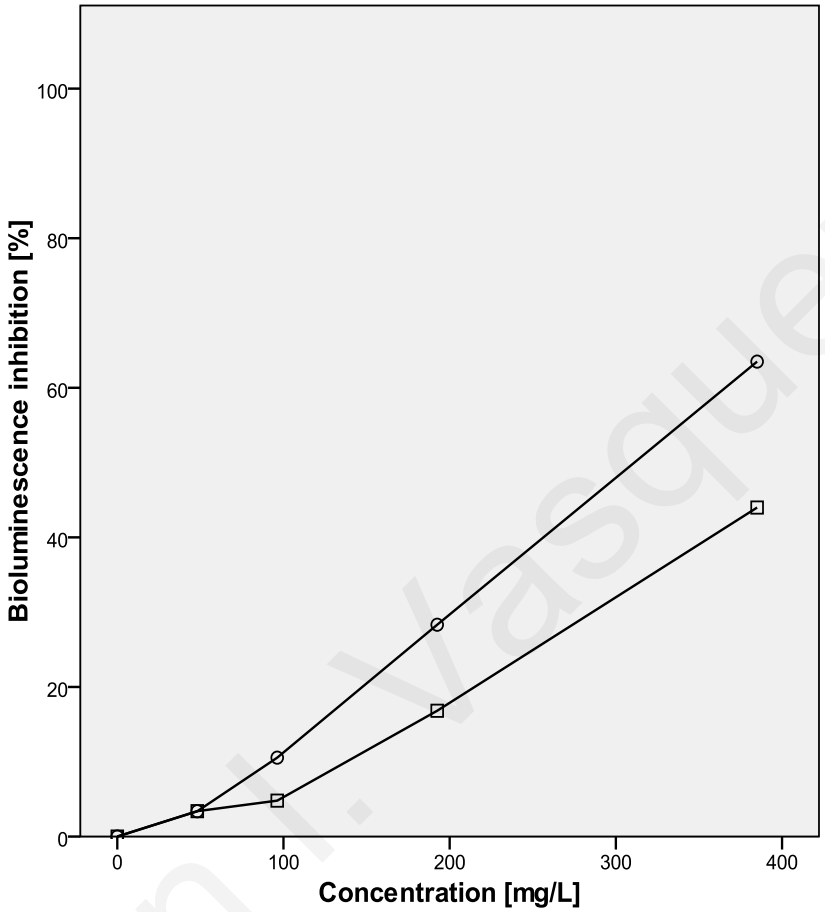


Figure 5.8 Inhibition percentage of the bioluminescence of *Vibrio fischeri* after an exposure time of 5 (circle) and 15 (square) min to erythromycin

The EC_1 values were 42.58 and 27.95 mg/L for the 5 and 15 min exposure time, respectively. The EC_{50} values were 433.8 mg/L (95% confidence range: 397.9 to 472.9 mg/L) and 295.9 mg/L (95% confidence range: 174.3 to 502.5 mg/L) for the 5 and 15 min exposure time, respectively.

The dose-response curves for ofloxacin are shown in Figure 5.9. The slopes were 1.550 and 1.271 for 5 and 15 min exposure time, respectively. The corresponding estimating equations were $\log C = 0.6431 \times \log \Gamma + 2.987$ and $\log C = 0.7074 \times \log \Gamma + 2.899$. The R^2 and f_k were 1 and 1.188 and 1 and 0.8993 for the 5 and 15 min exposure time, respectively.

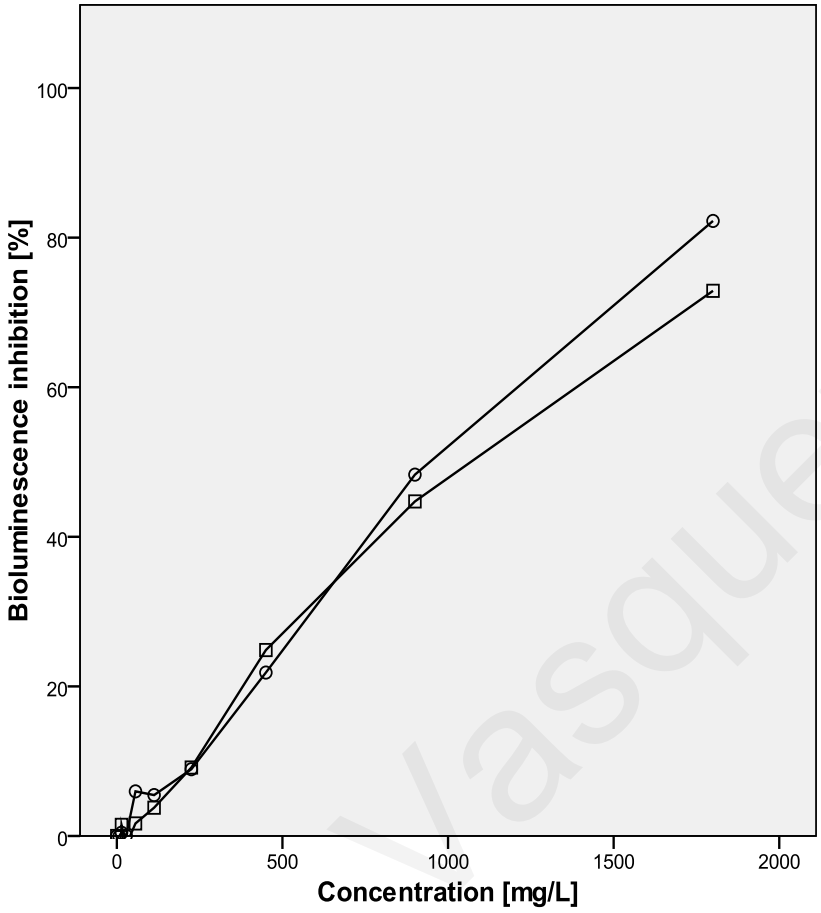


Figure 5.9 Inhibition percentage of the bioluminescence of *Vibrio fischeri* after an exposure time of 5 (circle) and 15 (square) min to ofloxacin

The EC_1 values were 50.54 and 30.71 mg/L and the EC_{50} values were 970.3 mg/L (95% confidence range: 835 to 1128 mg/L) and 792.6 mg/L (95% confidence range: 404.8 to 1552 mg/L) for the 5 and 15 min exposure time, respectively.

The dose-response curves for sulfamethoxazole are presented in Figure 5.10. The slopes for sulfamethoxazole were 1.898 and 2.094 for the 5 and 15 min exposure times, respectively. The corresponding estimating equations were $\log C = 0.5228 \times \log \Gamma + 2.131$ and $\log C = 0.4746 \times \log \Gamma + 2.189$. For both exposure times, the R^2 were 1 and the f_k 0.8583.

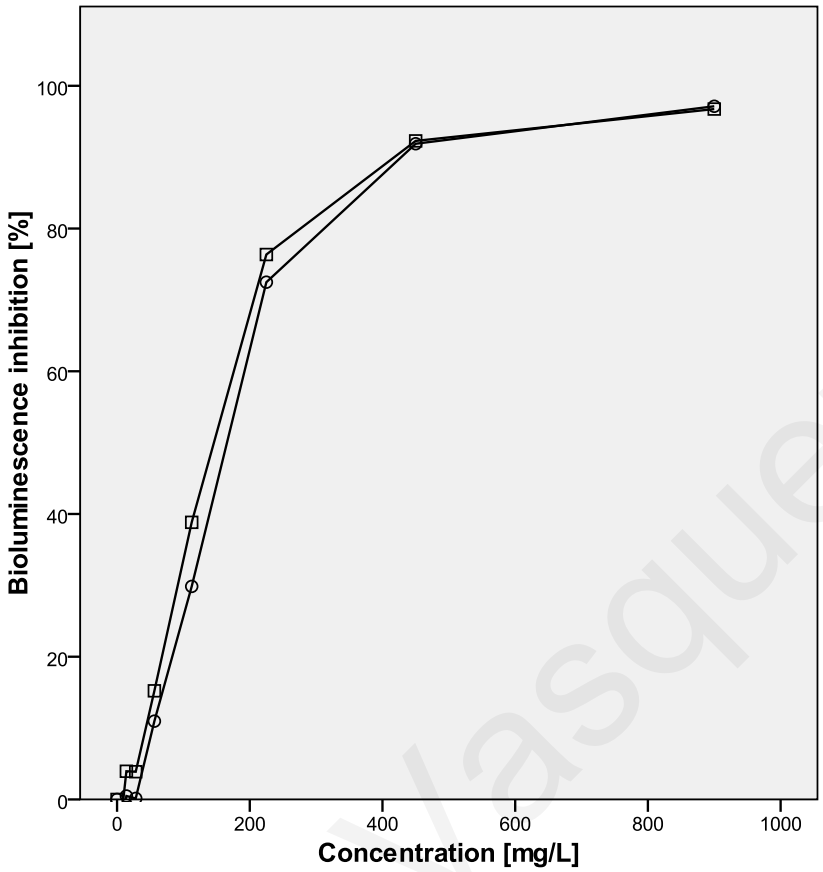


Figure 5.10 Inhibition percentage of the bioluminescence of *Vibrio fischeri* after an exposure time of 5 (circle) and 15 (square) min to sulfamethoxazole

The EC_1 values were 12.24 and 17.45 mg/L for the 5 and 15 min exposure time, respectively. The EC_{50} values were 135.2 mg/L (95% confidence range: 112.8 to 162.0 mg/L) and 154 (95% confidence range 132.5 to 179.8 mg/L) for the 5 and 15 min exposure time, respectively.

The antibiotics studied demonstrated dose-response curves presented in Figure 5.11. Sulfamethoxazole was found to be clearly more toxic, followed by erythromycin and then ofloxacin.

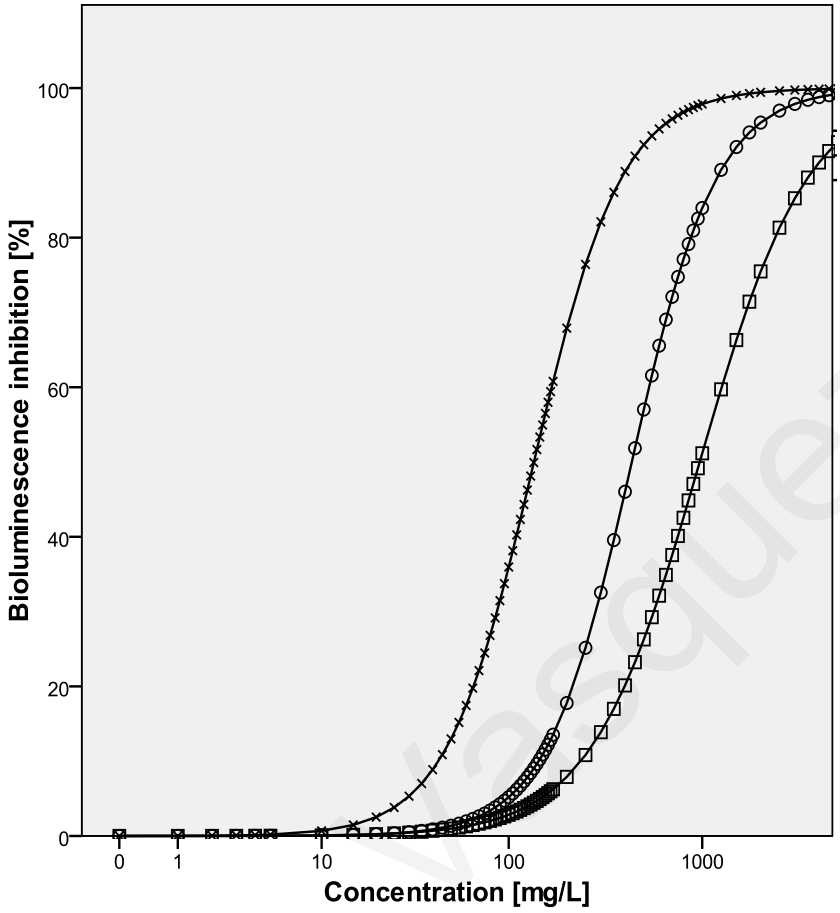


Figure 5.11 Dose-response curves for the percentage of the bioluminescence inhibition of *Vibrio fischeri* for erythromycin (circle), ofloxacin (square) and sulfamethoxazole (cross) at 5 min exposure time

5.2.2 Acute toxicity assessment using the crustacean *Daphnia magna*

At least four independent experiments were performed for each API. Each experiment was performed in quadruplicates. For each concentration tested at least 60 neonates were tested. Immobilization at 24 and 48 h were the endpoints investigated to evaluate the effects.

Immobilization is considered to be a binary observation and the data acquired quantal-response. For a group of n_x individuals receiving a particular dose x, the number of individuals surviving is assumed to follow a binomial distribution with mean $n_x p_x$, where the probability of survival p_x, is related to the level of the dose, usually by a S-shaped curve [320].

The data was elaborated and a Probit analysis was performed using the NCSS statistical system, whereas the graphs were prepared using the IBM SPSS Statistics 19 Software. The Probit analysis is a method of analysing the relationship between a stimulus and a quantal (all-or-none) effect. The idea of Probit analysis was originally proposed Bliss [321]. The Probit analysis assumes that the percentage effect is related to the log dose as the cumulative normal distribution. The effect of transformation from percentage effects to probits are illustrated below Figure 5.12 [322].

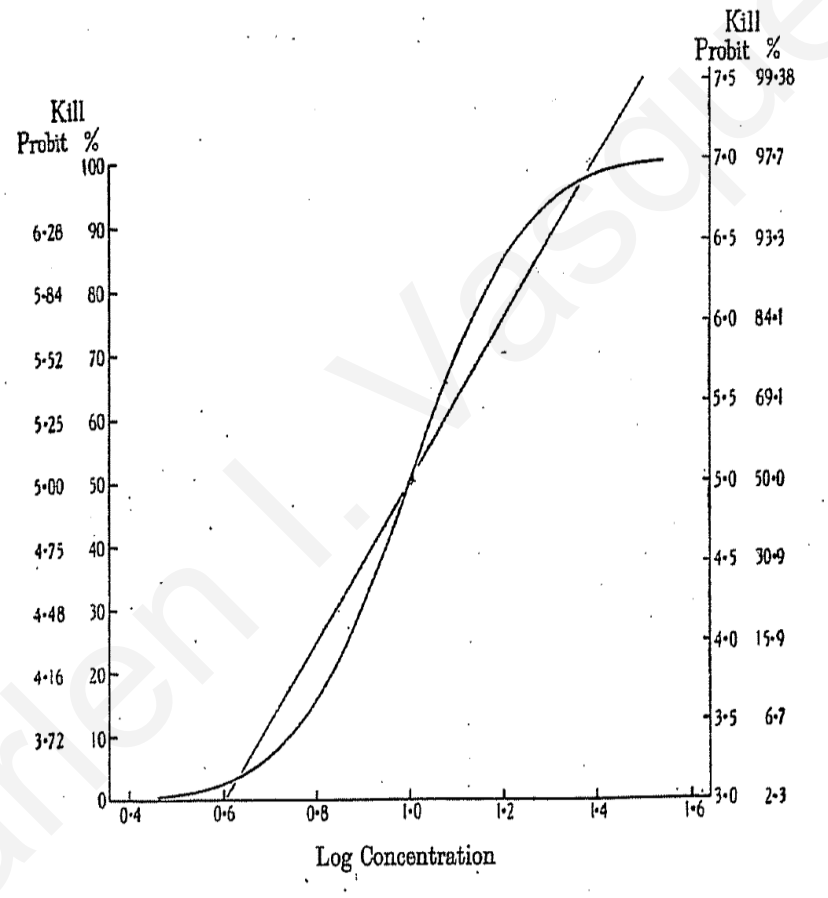


Figure 5.12 Probit transformation

The Probit model may be expressed mathematically as follows:

$$P = \alpha + \beta[\log(Dose)]$$

Equation 5.4 Mathematical expression of the Probit model

Where *P* is the inverse normal transform of the response rate (the Probit) plus five. Five is added to reduce the possibility of negative probits. The α and β are the estimated values of the intercept and the slope of the straight line, respectively.

A χ^2 statistic for testing the significance of the difference between the calculated values and the expected ones was applied. A total of the χ^2 was used to test the overall significance of the differences from the model. The degrees of freedom (*df*) of the χ^2 test and the probability to the right of the total χ^2 test were calculated.

5.2.2.1 β-blockers

The dose-response curves for atenolol, metoprolol and propranolol, as calculated by the Probit analysis are presented in Figure 5.13-Figure 5.16.

Atenolol demonstrated a sharper dose-response curve when the immobilization period was 24 h compared with the 48 h, as presented in Figure 5.13. The α values with the standard error were -0.28 (±0.78) and -0.30 (±0.67) for the 24 and 48 h, respectively. The β values with the standard error were 1.79 (±0.33) and 1.94 (±0.29), respectively. The α and β values are not statistically different for the two exposure times examined. The total χ^2 were 9.36 and 5.33 with *df* of 5, corresponding to a probability level of 0.1 and 0.38.

A selection of seven EC values for atenolol was made presented in Table 5.1. The minimum values expected to cause an effect to *D. magna* are 44.09 mg/L (±14.05) and 34.30 mg/L (±9.71) for acute exposure times of 24 and 48 h, respectively. The EC_{50} values were 872 mg/L (±244.64) and 544.43 mg/L (±94.75) for 24 and 48 h, respectively. Both the EC_1 and EC_{50} values were not statistically different for the exposure times investigated.

The same set of EC values for metoprolol is presented in Table 5.2. The EC_1 values were 14.21 mg/L (±4.26) and 13.15 mg/L (±3.24) after 24 and 48 h exposure times; whereas the EC_{50} values were 190.94 mg/L (±17.38) and 114.83 mg/L (±9.24). The EC_1 values are not statistically different; in contrast to the EC_{50} values at 24 and 48 h.

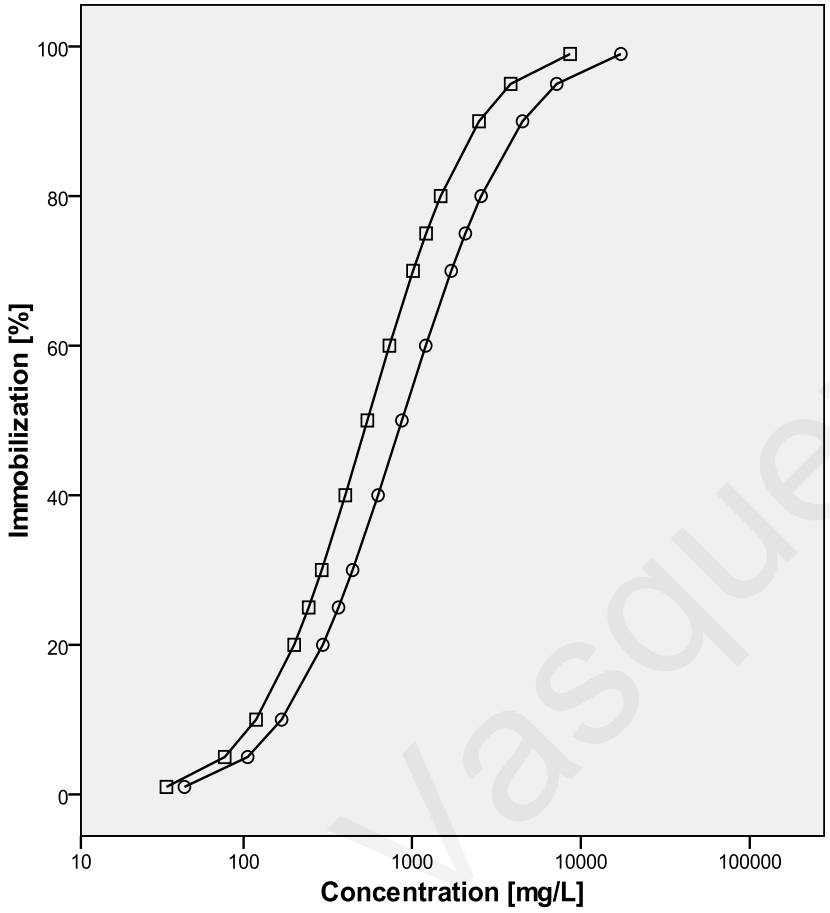


Figure 5.13 Inhibition percentage of the immobilization of *Daphnia magna* after an exposure time of 24 (circle) and 48 (square) h to atenolol

Table 5.1 Effective concentrations for atenolol towards *Daphnia magna*

EC	1	5	25	50	75	95	99
24 h	44.09 (14.05)	105.71 (19.00)	367.17 (55.82)	872.41 (244.64)	2072.88 (891.42)	7199.49 (4704.09)	17260.72 (14019.35)
48 h	34.30 (9.71)	77.09 (13.58)	244.24 (24.98)	544.43 (94.75)	1213.57 (339.15)	3844.99 (1702.19)	8642.35 (4838.23)

Concentrations are expressed in mg/L; in parenthesis is the calculated standard error.

Table 5.2 Effective concentrations for metoprolol towards *Daphnia magna*

EC	1	5	25	50	75	95	99
24 h	14.21 (4.26)	30.42 (6.61)	89.91 (10.39)	190.94 (17.38)	405.52 (54.98)	1198.38 (292.28)	2565.02 (839.44)
48 h	13.15 (3.24)	24.81 (4.64)	61.27 (6.85)	114.83 (9.24)	215.25 (19.66)	531.48 (83.83)	1002.73 (215.65)

Concentrations are expressed in mg/L; in parenthesis is the calculated standard error.

The dose-response curves for metoprolol after an exposure time of 24 and 48 h are presented in Figure 5.14. The values for α were 0.30 (±0.54) and -9.23 (±0.53) for 24 and 48 h, respectively. The slopes (β) were to be 2.06 (±0.24) and 2.47 (±0.25) for the 24 and 48 h, respectively. The α values are statistically different; whereas the β values are not statistically different. The total χ^2 were 6.69 and 7.20 with *df* of 3, corresponding to a probability level of 0.08 and 0.07 for the 24 and 48 h of exposure time.

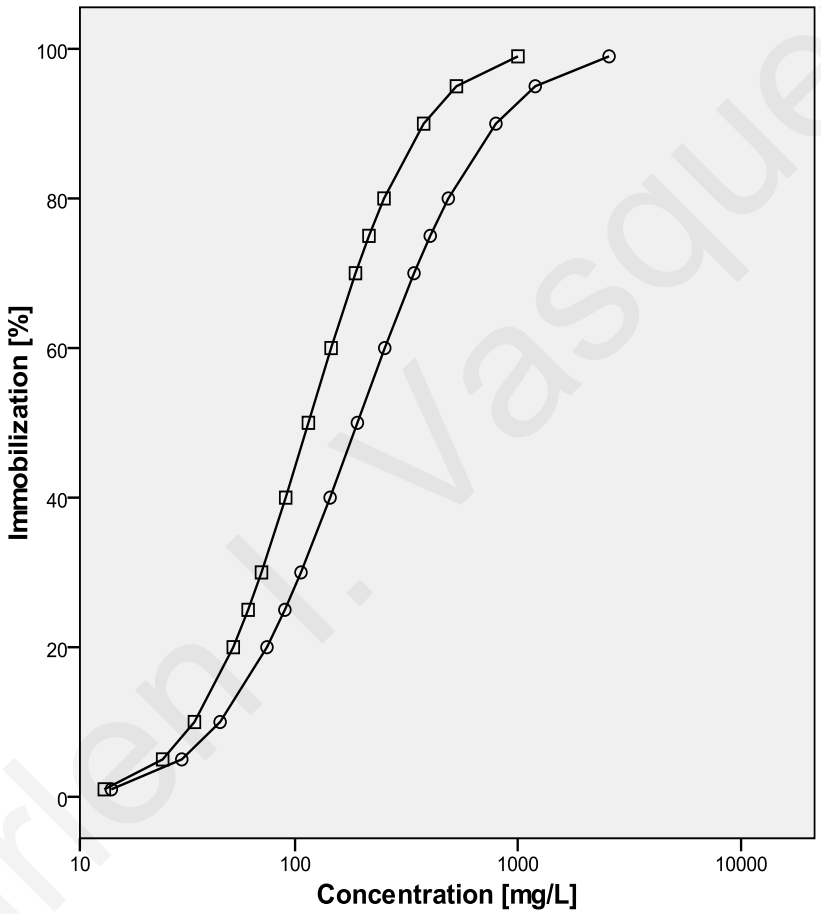


Figure 5.14 Inhibition percentage of the immobilization of *Daphnia magna* after an exposure time of 24 (circle) and 48 (square) h to metoprolol

The dose-response curves for propranolol are presented in Figure 5.15. The α values for 24 and 48 h were 5.38 mg/L (±0.08) and 6.44 mg/L (±0.12), which are statistically different; whereas the β values were 3.06 (±0.26) and 3.05 (±0.30), which are not statistically different. The total χ^2 were 4.18 and 7.67 with *df* of 3 and a probability level of 0.24 and 0.05 for the 24 and 48 h, respectively.

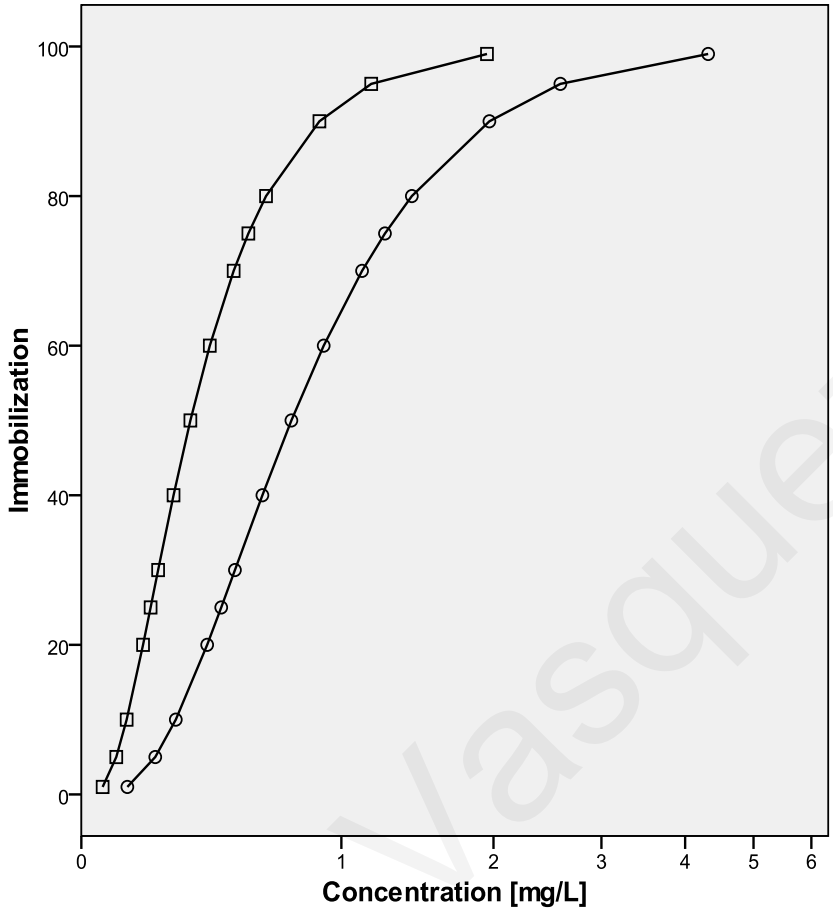


Figure 5.15 Inhibition percentage of the immobilization of *Daphnia magna* after an exposure time of 24 (circle) and 48 (square) h to propranolol

Seven EC values for propranolol are presented in Table 5.3. The EC_1 values for 24 and 48 h exposure time are statistically different with the values of 0.13 mg/L (±0.02) and 0.06 mg/L (±0.01) mg/L, respectively. Similarly, the EC_{50} values were 0.75 mg/L (±0.04) and 0.34 mg/L (±0.03) with statistically significant difference.

Table 5.3 Effective concentrations for propranolol towards *Daphnia magna*

EC	1	5	25	50	75	95	99
24 h	0.13 (0.02)	0.22 (0.03)	0.45 (0.03)	0.75 (0.04)	1.25 (0.08)	2.59 (0.28)	4.32 (0.64)
48 h	0.06 (0.01)	0.10 (0.02)	0.20 (0.02)	0.34 (0.03)	0.56 (0.04)	1.17 (0.12)	1.95 (0.29)

Concentrations are expressed in mg/L; in parenthesis is the calculated standard error.

A comparison of the toxicity effects of β-blockers was performed and is illustrated in Figure 5.16. The EC_1 values for atenolol and metoprolol are in the range of 10-100 mg/L, with

the latter being more toxic; whereas for propranolol is in the range of 0.1-1 mg/L. This was observed for both exposure times 24 and 48 h.

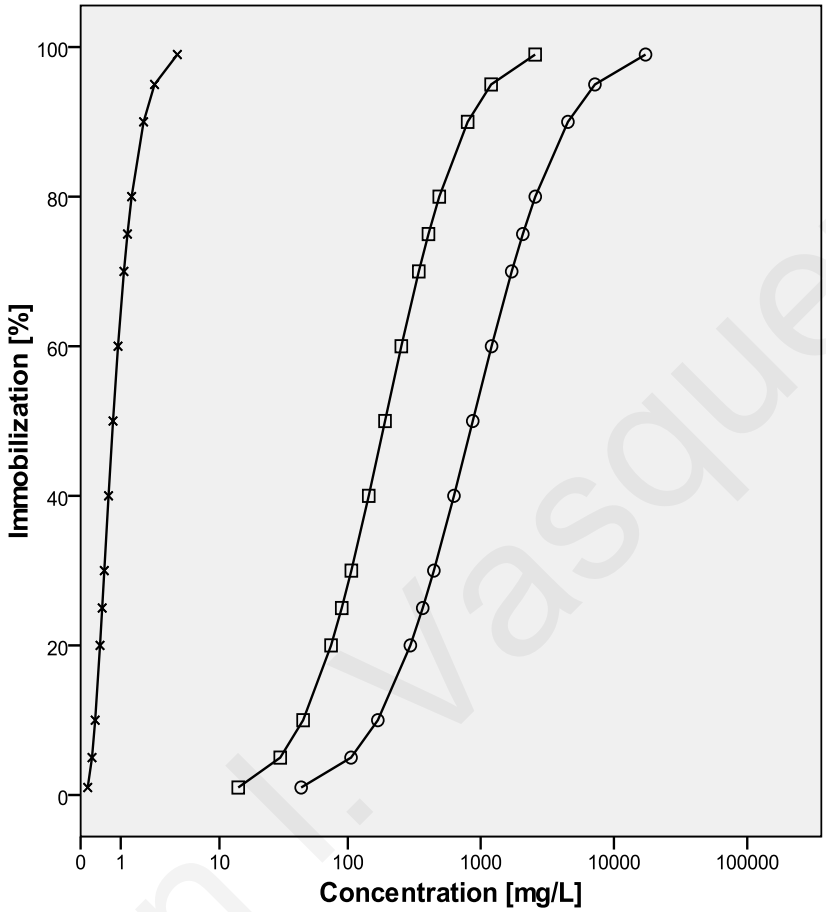


Figure 5.16 Inhibition percentage of the immobilization of *Daphnia magna* after an exposure time of 24 h to atenolol (circle), metoprolol (square) and propranolol (cross)

5.2.2.2 Non-steroidal anti-inflammatory APIs

Diclofenac and ibuprofen were evaluated. The results are presented in Figures 5.17-5.18. Regarding diclofenac the α values were 0.51 (±0.51) and 0.35 (±0.47) and the β values were 2.52 (±0.29) and 3.20 (±0.30) for 24 and 48 h respectively. The α values are not to statistically different; in contrast with the β values. The total χ^2 were 1.87 and 3.08 with *df* 7 and a probability level of 0.97 and 0.88, respectively.

The EC values for diclofenac are presented in Table 5.4. The EC_1 values for 24 and 48 h exposure times were not statistically different with values of 7.20 mg/L (±1.73) and 5.30 mg/L (±0.99), respectively. The EC_{50} values were 60.16 mg/L (±4.72) and 28.21 mg/L (±1.92), being statistically different.

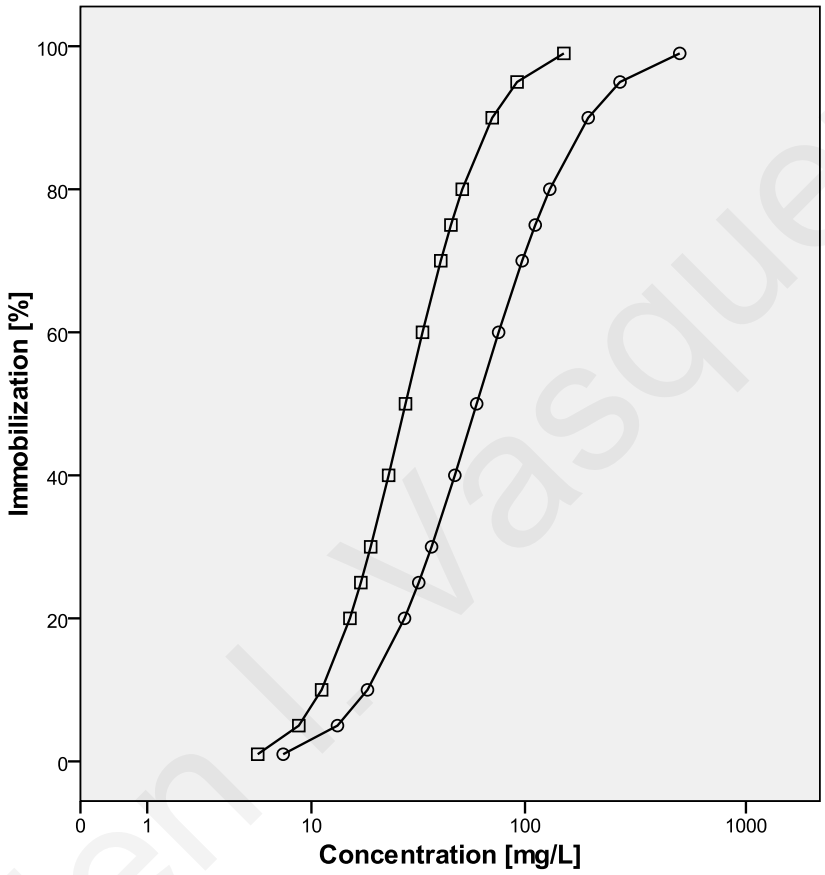


Figure 5.17. Inhibition percentage of the immobilization of *Daphnia magna* after an exposure time of 24 (circle) and 48 (square) h to diclofenac

Table 5.4 Effective concentrations for diclofenac towards *Daphnia magna*

EC	1	5	25	50	75	95	99
24 h	7.20 (1.73)	13.41 (2.31)	32.51 (2.99)	60.16 (4.72)	111.31 (13.25)	269.77 (56.42)	502.37 (139.61)
48 h	5.30 (0.99)	8.65 (1.25)	17.37 (1.58)	28.21 (1.92)	45.81 (3.27)	92.01 (10.49)	150.17 (23.11)

Concentrations are expressed in mg/L; in parenthesis is the calculated standard error.

The dose-response curves for ibuprofen are presented in Figure 5.18. The α values were 0.34 (±0.63) and 3.50 (±0.27) for the exposure time of 24 and 48 h, respectively. The

respective β values were 2.70 (±0.41) and 1.45 (±0.21). The total χ^2 were 3.88 and 8.32 with *df* 3 and a probability level of 0.27 and 0.04 for the exposure time of 24 and 48 h, respectively.

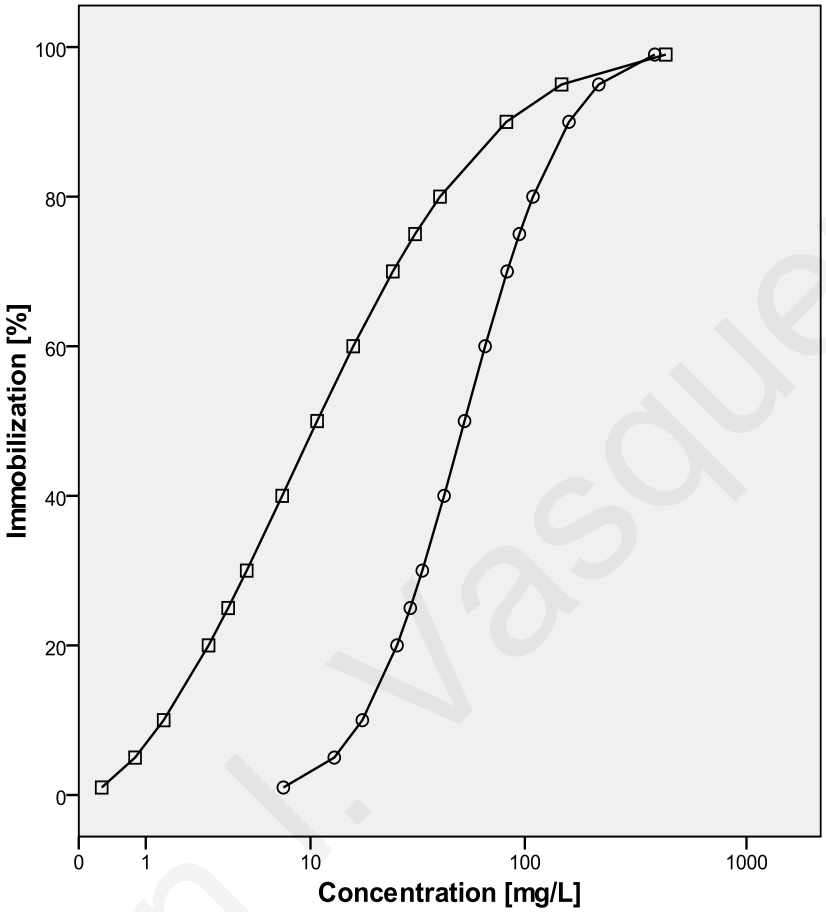


Figure 5.18 Inhibition percentage of the immobilization of *Daphnia magna* after an exposure time of 24 (circle) and 48 (square) h to ibuprofen

The EC values for ibuprofen are presented in Table 5.5. Both EC_1 and EC_{50} values are statistically different with values of 7.31 mg/L (±1.76) and 0.27 mg/L (±0.17) for the EC_1 values and 53.14 mg/L (±5.91) and 10.78 mg/L (±1.50) for the EC_{50} values, for the exposure time of 24 and 48 h, respectively.

Table 5.5 Effective concentrations for ibuprofen towards *Daphnia magna*

EC	1	5	25	50	75	95	99
24 h	7.31 (1.76)	13.07 (2.10)	29.89 (2.48)	53.14 (5.91)	94.45 (17.20)	216.10 (64.82)	386.44 (149.07)
48 h	0.27 (0.17)	0.79 (0.37)	3.69 (0.94)	10.78 (1.50)	31.45 (4.85)	146.81 (50.14)	433.24 (213.52)

Concentrations are expressed in mg/L; in parenthesis is the calculated standard error.

The toxicity values for the anti-inflammatory APIs studied are shown in Figure 5.19 demonstrating that the EC_1 and EC_{50} values between diclofenac and ibuprofen are not statistically different when *D. magna* was exposed for 24 h; whereas this was not the case when the exposure time increased to 48 h with ibuprofen being more toxic with statistical significance at the 0.05 probability level.

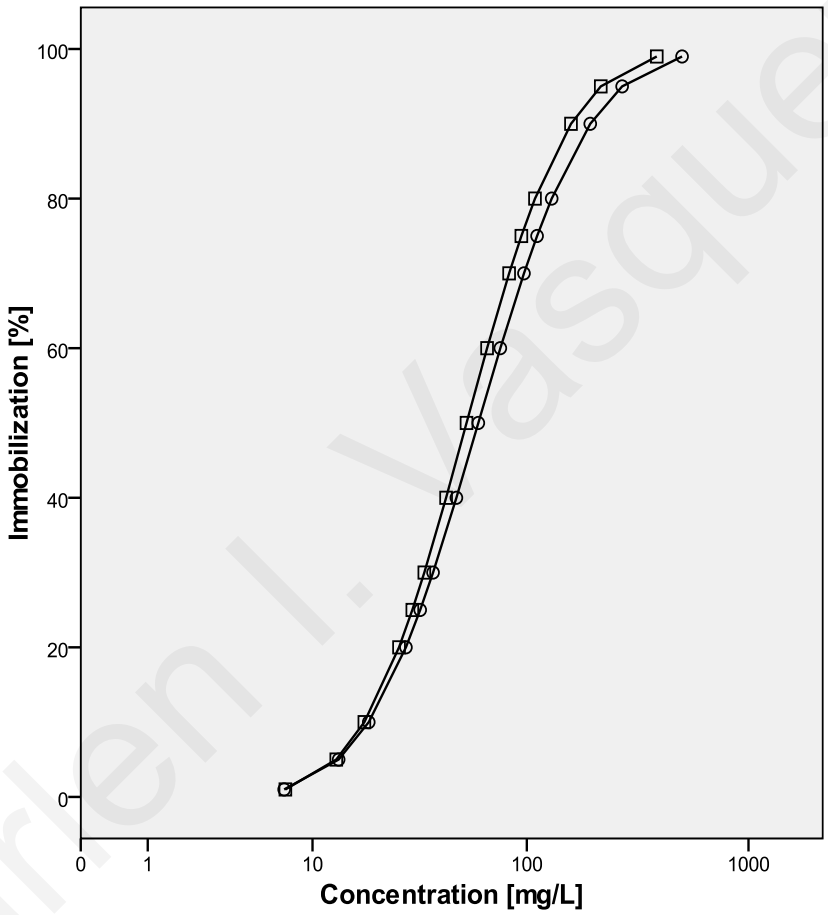


Figure 5.19 Inhibition percentage of the immobilization of *Daphnia magna* after an exposure time of 24 h to diclofenac (circle) and ibuprofen (square)

5.2.2.3 Antibiotics

Erythromycin, ofloxacin and sulfamethoxazole were evaluated. The dose-response curves for erythromycin after an exposure time of 24 and 48 h are presented in Figure 5.20. The α and β values for the 24 h exposure time were 1.50 (±0.45) and 1.60 (±0.24), respectively. For the 48 h exposure time the values were 1.95 (±0.39) and 1.70 (±0.22), respectively; hence no

statistical significant difference between the two exposure times was observed. The total χ^2 were 5.29 and 8.17 with *df* 4 with a probability level of 0.09 and 0.26, respectively.

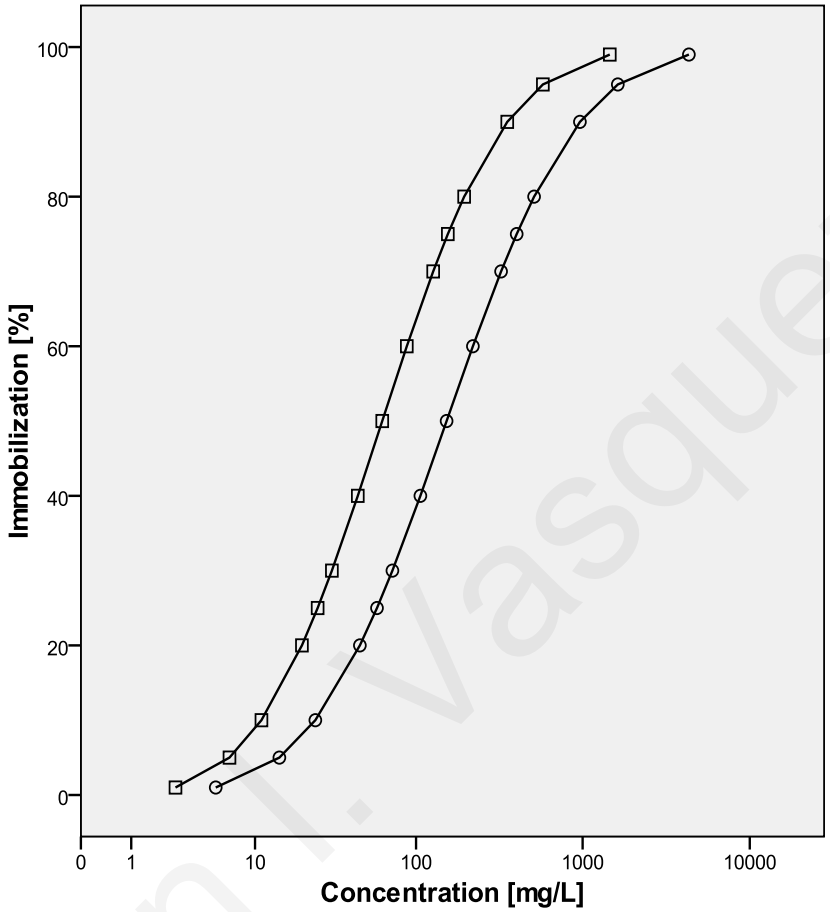


Figure 5.20 Inhibition percentage of the immobilization of *Daphnia magna* after an exposure time of 24 (circle) and 48 (square) h to erythromycin

Seven EC values for erythromycin are shown in Table 5.6. The EC_1 values for the 24 and 48 h exposure times were not statistically different from each other with values of 5.40 mg/L (±2.21) and 2.68 mg/L (±1.09). In contrast, the EC_{50} values were statistically different with values of 152.81 mg/L (±24.98) and 62.40 mg/L (±6.89).

Table 5.6 Effective concentrations for erythromycin towards *Daphnia magna*

EC	1	5	25	50	75	95	99
24 h	5.40 (2.21)	14.38 (3.94)	57.98 (7.50)	152.81 (24.98)	402.74 (112.61)	1623.78 (770.05)	4322.89 (2662.05)
48 h	2.68 (1.09)	6.74 (1.99)	25.05 (3.86)	62.40 (6.89)	155.45 (25.77)	577.99 (179.66)	1453.65 (614.05)

Concentrations are expressed in mg/L; in parenthesis is the calculated standard error.

Ofloxacin had similar dose-response curves when *D. magna* was exposed for 24 and 48 h, as shown in Figure 5.21. The α values for the exposure time of 24 and 48 h were 0.90 (±0.42) and 0.74 (±0.40). The β values were 1.98 (±0.22) and 2.23 (±0.22). Both α and β values were not statistically different between the two exposure times evaluated. The total χ^2 were 2.69 and 10.55 with a *df* 5. This corresponded to a probability level of 0.75 and 0.06, respectively.

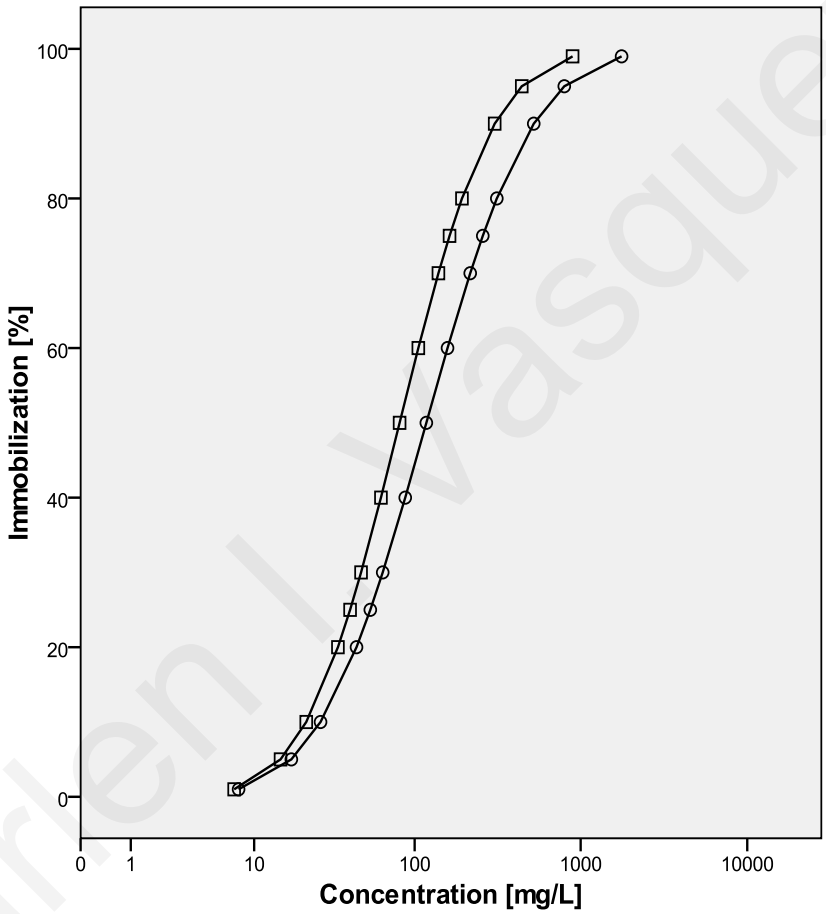


Figure 5.21 Inhibition percentage of the immobilization of *Daphnia magna* after an exposure time of 24 (circle) and 48 (square) h to ofloxacin

In Table 5.7 the selected EC values for ofloxacin are presented. The EC_1 for the 24 and 48 h exposure time were not statistically different with values of 7.87 mg/L (±2.07) and 7.34 mg/L (±1.67), respectively. On the other hand, the EC_{50} values of 117.72 mg/L (±11.97) and 81.04 mg/L (±6.29) for the 24 and 48 h exposure time, respectively, were statistically different from each other.

Table 5.7 Effective concentrations for ofloxacin towards *Daphnia magna*

EC	1	5	25	50	75	95	99
24 h	7.87 (2.07)	17.38 (3.15)	53.73 (4.93)	117.72 (11.97)	257.93 (42.96)	797.22 (225.44)	1761.00 (648.99)
48 h	7.34 (1.67)	14.84 (2.43)	40.40 (3.59)	81.04 (6.29)	162.57 (18.83)	442.62 (88.84)	894.37 (237.68)

Concentrations are expressed in mg/L; in parenthesis is the calculated standard error.

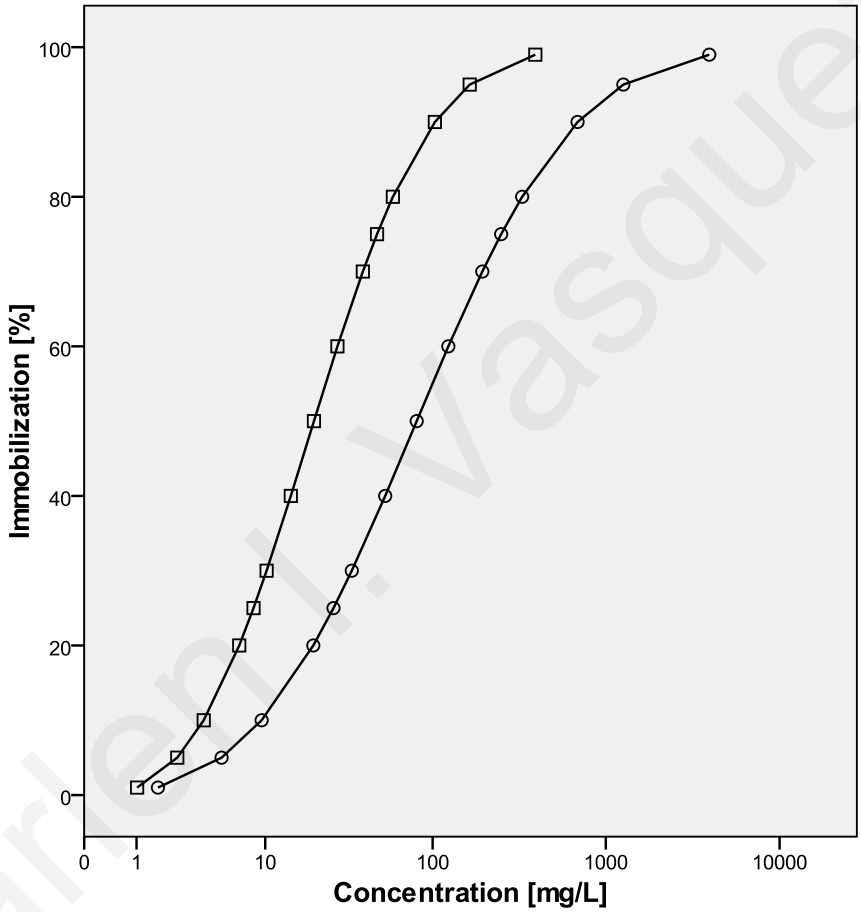


Figure 5.22 Inhibition percentage of the immobilization of *Daphnia magna* after an exposure time of 24 (circle) and 48 (square) h to sulfamethoxazole

Finally, the dose-response curves for sulfamethoxazole are presented Figure 5.22. The α values were 2.37 (±0.30) and 2.66 (±0.24) for the 24 and 48 h exposure times, being not statistically different, respectively. The β values were 1.38 (±0.23) and 1.80 (±0.20), respectively. Therefore, the slopes were statistically different at 24 and 48 h with the latter provoking a steeper curve.

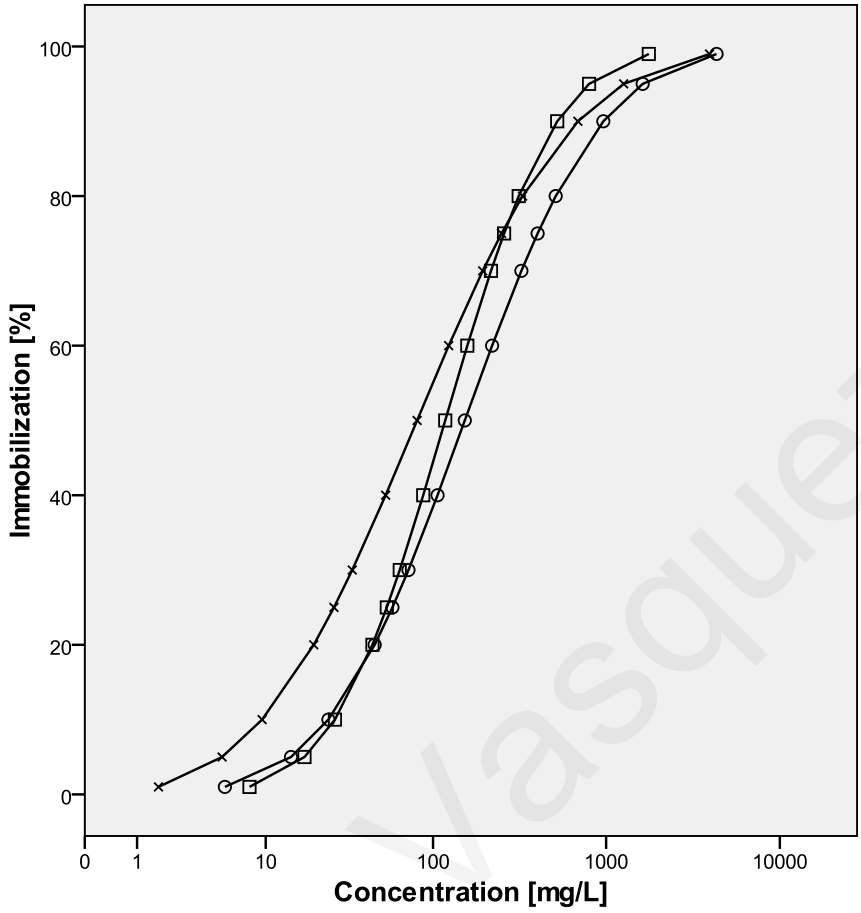


Figure 5.23 Inhibition percentage of the immobilization of *Daphnia magna* after an exposure time of 24 h to erythromycin (circle), ofloxacin (square) and sulfamethoxazole (cross)

Seven selected EC values for sulfamethoxazole for the exposure times evaluated are presented in Table 5.8. The EC_1 values were again not statistically different with each other with values of 1.66 mg/L (±0.69) and 1.02 mg/L (±0.31). On the other hand, the EC_{50} values were statistically different with values of 80.76 mg/L (±23.79) and 19.97 mg/L (±1.95) for the 24 and 48 h exposure time, respectively.

Table 5.8 Effective concentrations for sulfamethoxazole towards *Daphnia magna*

EC	1	5	25	50	75	95	99
24 h	1.66 (0.69)	5.17 (1.25)	26.17 (4.00)	80.76 (23.79)	249.29 (117.59)	1261.55 (930.89)	3939.80 (3653.68)
48 h	1.02 (0.31)	2.43 (0.52)	8.42 (0.89)	19.97 (1.95)	47.35 (7.76)	164.03 (47.74)	392.54 (151.28)

Concentrations are expressed in mg/L; in parenthesis is the calculated standard error.

The toxic effects of the antibiotics examined are compared in Figure 5.23. Sulfamethoxazole is considered to be the most toxic antibiotic as lower values of EC_1 and EC_{50} values were for both exposure times of 24 and 48 h. The toxicity of ofloxacin and erythromycin is not statistically different.

5.2.3 Chronic toxicity assessment using the green algae *Pseudokirchneriella subcapitata*

At least four independent experiments were performed for each API solution tested. One or two range-finding experiments were performed before selecting the definite range of concentrations, so as to obtain an effect of 2-98% inhibition on the growth of *P. subcapitata*.

The EC_x for *P. subcapitata* was calculated using a linear interpolation method for sublethal toxicity as proposed by Norberg-King [323]. This point estimate method has been widely used for the assessment of chronic toxicity tests [181,324,325]. The main reason for applying this method is that the dose-response curve of growth tests usually varies widely from straight line to curvilinear relationships such as, quadratic or cubic functions. The advantage of the selected approach relative to standard parametric regression methods is that the particular form of the model is not needed to be specified. The approach is sufficiently general to accommodate a wide range of data with a single, unified formulation [323].

The only assumption made concerning the distribution was that the data within a group being resampled was independent and identically distributed. Moreover, the method was applied if the data obtained from the test met the following criteria: (i) being monotonically non-increasing, in which the mean response for each higher concentration was less than or equal to the mean response for the previous concentration; (ii) followed a piecewise linear response function; and (iii) being random, independent, and representative.

A bootstrapping method was applied to calculate the standard error and consequently the confidence intervals. As briefly described by Norberg-King [323], the control mean (Y_1) and the observed means at each concentration were considered in order of increasing concentration. If the observed mean of the lowest API concentration (Y_2) was less or equal

than Y_1 mean, the Y_1 mean was used as the response. If Y_2 mean was greater than Y_1 mean then the average of Y_1 and Y_2 means are used as response (M_1 and M_2). The new mean was compared to the next higher API concentration (Y_3) and the process was repeated for the remaining concentrations.

The EC_x was calculated using the following equation:

$$EC_x = C_j + [M_1\left(1 - \frac{x}{100}\right) - M_j]\frac{(C_{j+1} - C_j)}{(M_{j+1} - M_j)}$$

Equation 5.5 Linear interpolation estimate calculation

C_j: tested concentration with observed mean response > than M_1 (1-x/100)
C_{j+1}: tested concentration with observed mean response < than M_1 (1-x/100)
M_1: smoothed mean response for the control
M_j: smoothed mean response for concentration j
M_{j+1}: smoothed mean response for concentration j+1
x: percent reduction in response relative to control response
EC_x: estimated concentration at which there is a percent reduction from the smoothed mean control response

5.2.3.1 β-blockers

The EC_1 values with their 95% confidence intervals in parenthesis for atenolol, metoprolol and propranolol were 11.41 mg/L (5.38-12.21 mg/L), 0.034 mg/L (0.023-1.05 mg/L) and 0.0002 mg/L (0.0002-0.0002 mg/L), respectively (Figure 5.24). Propranolol had the lowest EC_1 value, suggesting higher toxicity. Atenolol, on the contrary, had the highest EC_1 value, hence lower toxicity.

The EC_{50} values and their 95% confidence intervals in parenthesis for the β-blockers tested, as shown in Figure 5.25, were 59.85 mg/L (56.01-62.41 mg/L) for atenolol, 7.23 mg/L (0.9863-8.29 mg/L) for metoprolol and 0.0081 mg/L (0.0077-0.0086 mg/L) for propranolol.

Propranolol was found to have the lowest EC_{50} value, indicating the higher toxicity. The EC_{50} values for metoprolol and atenolol were higher, demonstrating lower toxicity.

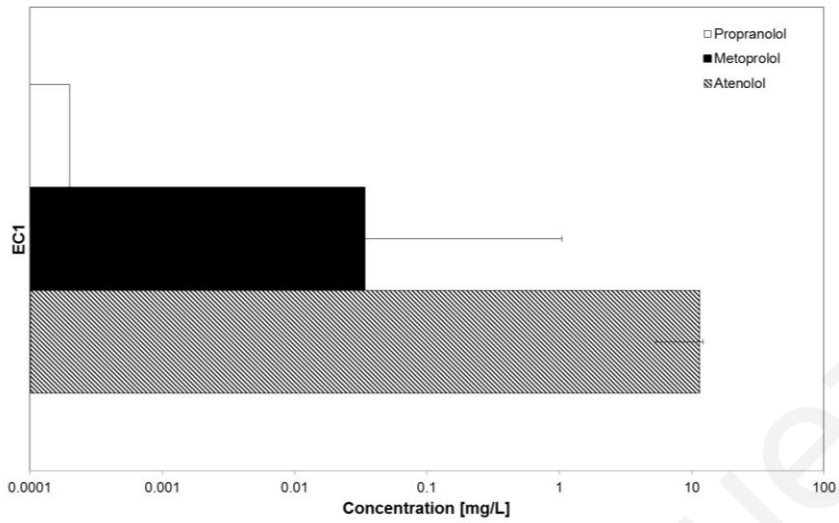


Figure 5.24 EC_1 value for atenolol, metoprolol and propranolol for the growth inhibition of *Pseudokirchneriella subcapitata* after an exposure time of 72 h

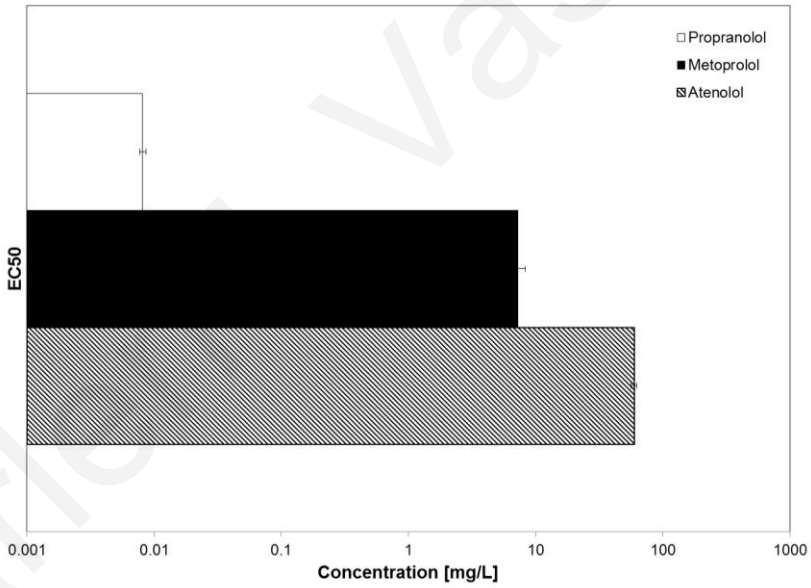


Figure 5.25 EC_{50} values for atenolol, metoprolol and propranolol for the growth inhibition of *Pseudokirchneriella subcapitata* after an exposure time of 72 h

5.2.3.2 Non-steroidal anti-inflammatory APIs

Diclofenac and ibuprofen were evaluated. The EC_1 value for diclofenac was 0.11 mg/L (0.04-3.47 mg/L) and for ibuprofen was 0.0109 mg/L (0.0107-0.011 mg/L), indicating the higher toxicity of ibuprofen compared to diclofenac (Figure 5.26).

The EC_{50} values for non-steroidal anti-inflammatory APIs are presented in Figure 5.27 with diclofenac being again less toxic than ibuprofen with values and their 95% confidence intervals in parenthesis of 39.26 mg/L (15.09-65.13 mg/L) and 0.544 mg/L (0.537-0.556 mg/L), respectively.

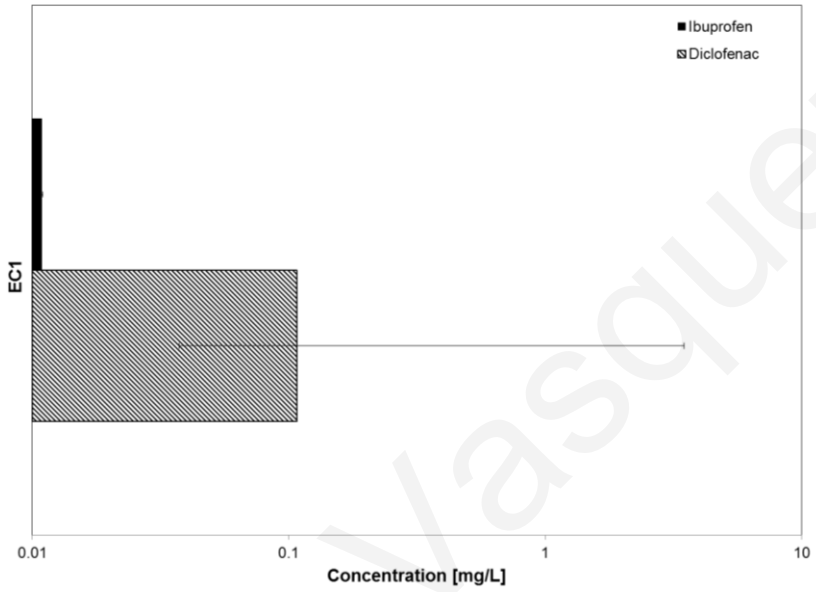


Figure 5.26 EC_1 value for diclofenac and ibuprofen for the growth inhibition of *Pseudokirchneriella subcapitata* after an exposure time of 72 h

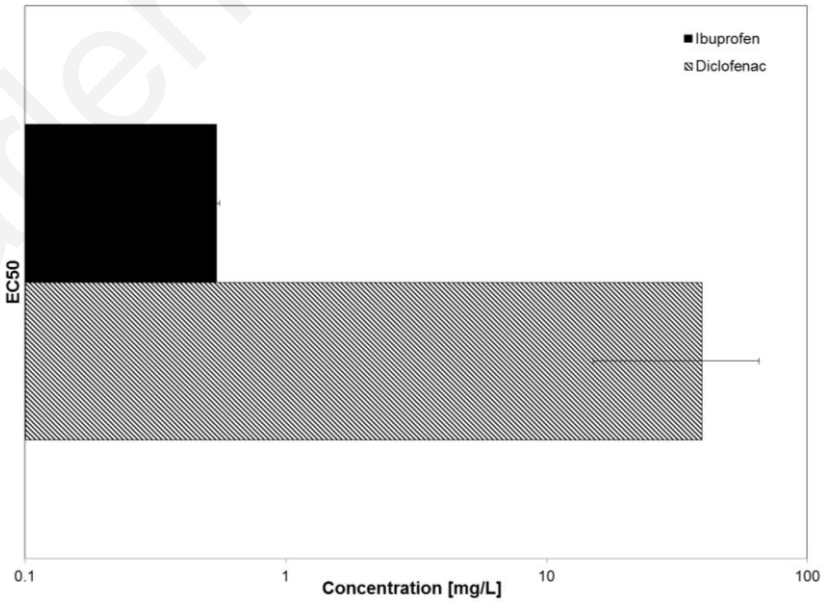


Figure 5.27 EC_{50} values for diclofenac and ibuprofen for the growth inhibition of *Pseudokirchneriella subcapitata* after an exposure time of 72 h

5.2.3.3 Antibiotics

Erythromycin, ofloxacin and sulfamethoxazole had very similar EC_1 values, as shown in Figure 5.28. The values and their 95% confidence intervals in parenthesis were 0.0066 mg/L (0.0006-0.0102 mg/L), 0.0053 mg/L (0.0025-0.3388 mg/L) and 0.005 mg/L (0.0029-0.1177 mg/L), respectively.

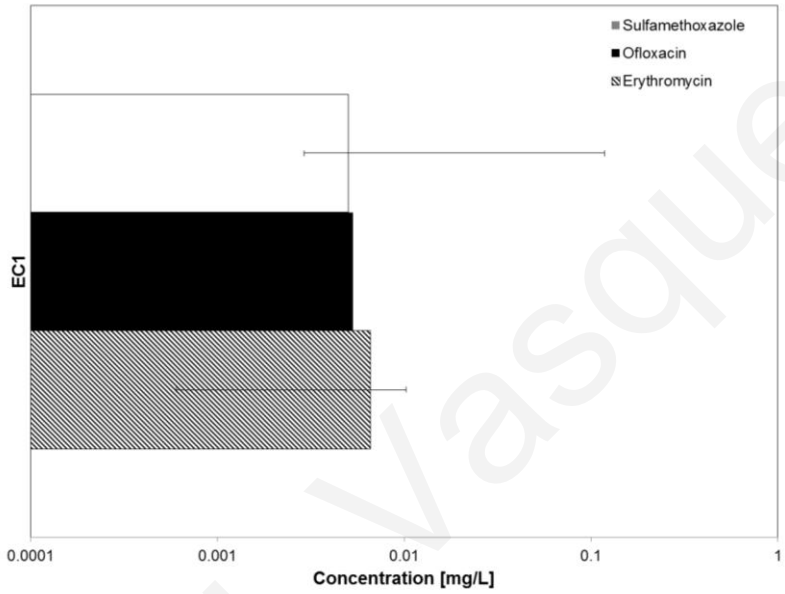


Figure 5.28 EC_1 for erythromycin, ofloxacin and sulfamethoxazole for the growth inhibition of *Pseudokirchneriella subcapitata* after an exposure time of 72 h

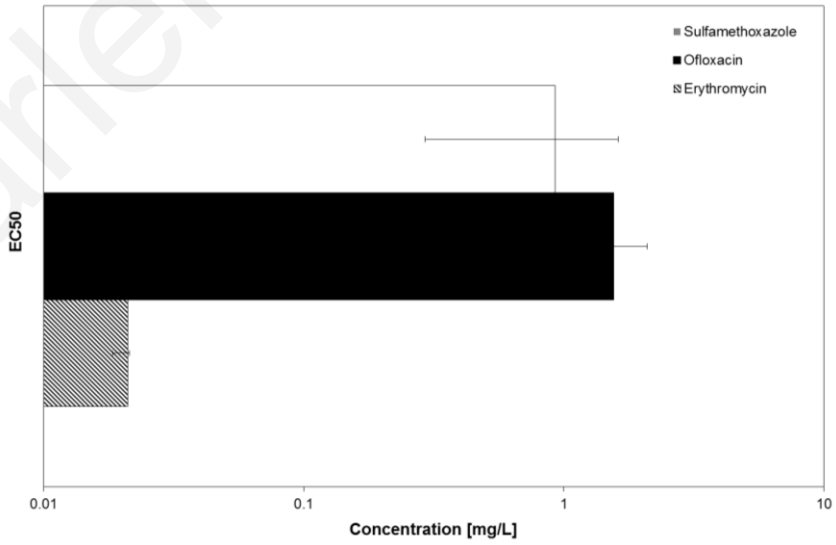


Figure 5.29 EC_{50} values for erythromycin, ofloxacin and sulfamethoxazole for the growth inhibition of *Pseudokirchneriella subcapitata* after an exposure time of 72 h

The EC_{50} values for the antibiotics were not as similar as the EC_1 values, with erythromycin being the more toxic, followed by sulfamethoxazole and ofloxacin, as presented in Figure 5.29.

The EC_{50} values and their 95% confidence intervals in parenthesis were 0.0211 mg/L (0.0184-0.0214 mg/L), 1.559 mg/L (0.303-2.094 mg/L) and 0.929 mg/L (0.294-1.617 mg/L) for erythromycin, ofloxacin and sulfamethoxazole respectively.

5.2.4 Initial risk assessment of the active pharmaceutical ingredients evaluated

From the EC_{50} calculated in this work a categorization of the APIs according the European Directive 93/67/EEC was made (Table 5.9), so as to better compare the results obtained for the APIs studied. *V. fischeri* was found to have the highest robustness of the endpoints evaluated. The same stands true for *D. magna,* with the exception of propranolol, which was categorized as very toxic to *D. magna.* The toxicity to *P. subcapitata* on the other hand was in the range of extremely toxic to harmful, indicating a greater sensitivity of the chronic testing.

Table 5.9 Categorization of active pharmaceutical ingredients according to the European Directive 93/67/EEC

Active Pharmaceutical Ingredient	**Extremely toxic EC_{50} <0.1 mg/L**			**Very toxic EC_{50} 0.1-1 mg/L**			**Toxic EC_{50} 1-10 mg/L**			**Harmful EC_{50} 10-100 mg/L**			**Non-Toxic EC_{50} >100 mg/L**		
	V	*D*	*P*	*V*	*D*	*P*	*V*	*D*	*P*	*V*	*D*	*P*	*V*	*D*	*P*
Atenolol										+		+		+	
Metoprolol									+				+	+	
Propranolol			+		+					+					
Diclofenac										+	+	+			
Ibuprofen						+				+	+				
Erythromycin			+										+	+	
Ofloxacin									+				+	+	
Sulfamethoxazole						+					+		+		

V: V. fischeri, D: D. magna, P: P. subcapitata

Propranolol and erythromycin were extremely toxic to *P. subcapitata*, ibuprofen and sulfamethoxazole very toxic and metoprolol and ofloxacin toxic. Atenolol and diclofenac were in the category of harmful to *P. subcapitata.*

The Predicted Environmental Concentrations (PECs) of the APIs examined were calculated by Hapeshi [3]; whereas the Measured Environmental Concentrations (MECs) for Cyprus and for other countries were compiled from the literature, as already reviewed in Chapter 1. The PEC or the MEC value depending on which had the highest value was used in order to assess the worst case scenario. The ratios of the PEC or the MEC value to the EC_1 value for *V. fischeri*, *D. magna* and *P. subcapitata* were then computed (Table 5.10).

Table 5.10 Comparison of the environmental concentrations and the lowest effects calculated

	PEC (μg/L)	MEC (μg/L)	MEC (μg/L)	PEC/EC_1 or MEC/EC_1		
				V. fischeri	*D. magna*	*P. subcapitata*
Atenolol	0.61	**0.94**	0.7	0.00002	0.00003	0.00008
Metoprolol	0.086	**9.59**	1.4	0.00007	0.00005	0.02765
Propranolol	0.054	0.28	**1.9**	0.07231	0.00044	4.70000
Diclofenac	0.64	**5.51**	5.5	0.00013	0.00104	0.00855
Ibuprofen	0.35	3.46	**7.1**	0.00013	0.00192	0.08545
Erythromycin	0.005	0.4	**6**	0.00017	0.00002	0.14030
Ofloxacin	0.015	**4.82**	0.6	0.00012	0.00002	0.17736
Sulfamethoxazole	0.098	**0.46**	0.24	0.00057	0.00008	0.18800

[1]Maximum Measured Environmental Concentration in Cyprus found in outlet (OUTs) samples
[2]Maximum Measured Environmental Concentration in the literature, as reviewed in Chapter 1
Bold values were used for the calculation of the ratio; underlined values demonstrate values greater than 1, hence indicating a significant risk

When the MEC/EC_1 exceeds 1, it can be suggested that risk mitigation measures should be addressed, as proposed by the EMEA [13]. The results indicate that detrimental effects at the environmental concentrations may be expected only for propranolol to *P. subcapitata*; whereas for *V. fischeri* and *D. magna* no risks are expected.

In order to investigate the relationship between the endpoints investigated, as suggested by Webb [326], a correlation among the EC_{50} values for each species was performed. Pearson's correlations coefficients were calculated with the use of the SPSS software and the results are presented in Table 5.11.

Only the response of *P. subcapitata* and *D. magna* were positively (0.781) correlated with significance at the 0.05 probability level. Logarithmic transformation of the data did not provide a better correlation between the endpoints examined.

Table 5.11 Pearson's correlation coefficients for the species evaluated

		Pseudokirchneriella	*Daphnia*	*Vibrio*
Pseudokirchneriella	Pearson Correlation	1	.781*	-.279
	Sig. (2-tailed)		.022	.504
	N	8	8	8
Daphnia	Pearson Correlation	.781*	1	-.061
	Sig. (2-tailed)	.022		.886
	N	8	8	8
Vibrio	Pearson Correlation	-.279	-.061	1
	Sig. (2-tailed)	.504	.886	
	N	8	8	8

*. Correlation is significant at the 0.05 probability level (2-tailed).

A linear regression was applied to the response pair *P. subcapitata* and *D. magna* which is presented in Figure 5.30.

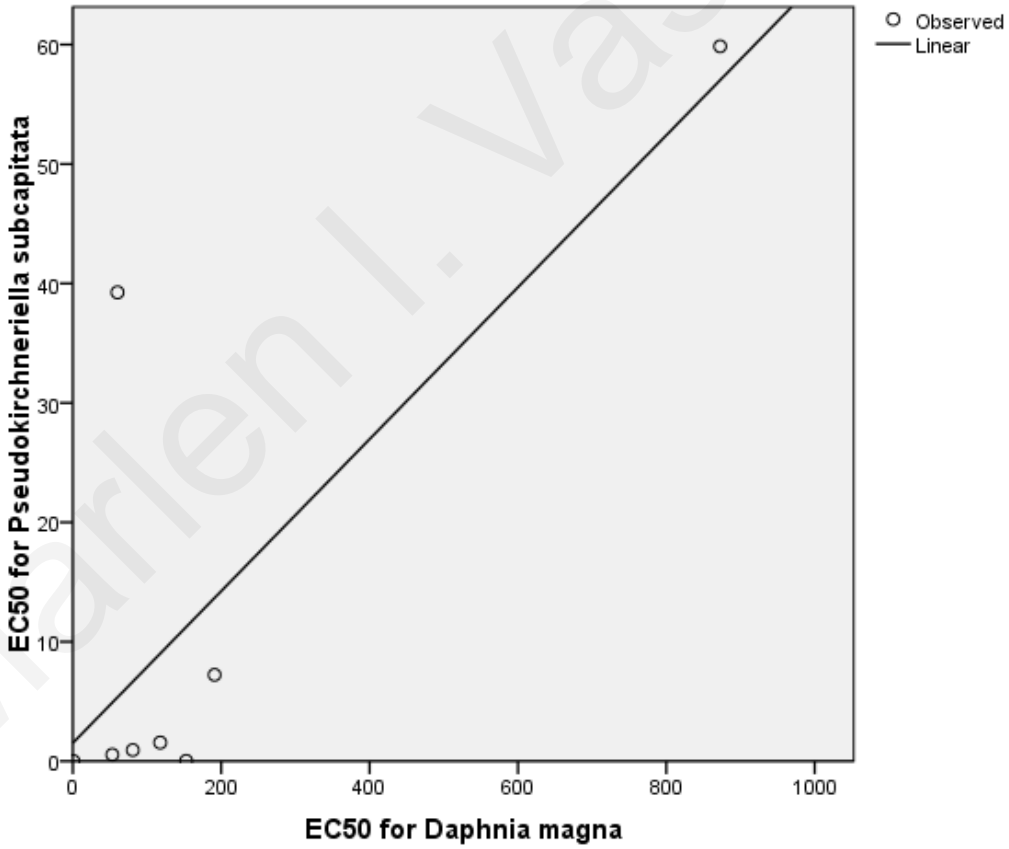


Figure 5.30 Paired comparison of the EC_{50} values for the active pharmaceuticals ingredients

The equation that describes the relationship between the responses was $y = 0.063x + 1.519$ (R^2=0.61), where *y*: EC_{50} value for the APIs towards *P. subcapitata* and *x*: EC_{50} value

for the APIs towards *D. magna*. The R^2 in this case indicates whether the EC_{50} values for APIs towards *D. magna* can be used for the prediction of the EC_{50} values for APIs towards *P. subcapitata*. Approximately 61% of the variation in the EC_{50} values for APIs towards *P. subcapitata* can be correlated with the EC_{50} values for APIs towards *D. magna*. The remaining 39% percent can be explained by unknown, confound variables and/or inherent variability.

5.3 Discussion

As it is known adverse pharmaceutical reactions on humans can be caused by previously unrecognized drug-receptor interactions, previously unidentified receptors and by a broad diversity in drug-metabolizing phenotypes (genetic polymorphisms). These variables are poorly characterized in aquatic biota. Additionally, just as animal models are frequently called into question for their relevance to human health, likewise, human and other mammalian toxicity data could also not necessarily be transferable to aquatic organisms [148].

Narcosis or baseline toxicity was observed for both *V. fischeri* and *D. magna* when exposed to the APIs, with the exception of the high toxicity of propranolol for *D. magna* that may be attributed to a specific mode of action. The baseline toxicity model suggests that the mode of action is not reactive when considering overall acute effects, and no interaction with specific receptors in the organism is present. Regarding propranolol, the membrane disturbance caused by its accumulation into membranes may be the main reason for its toxicity. Such a partitioning may cause membrane expansion or swelling, increase in fluidity, lowering of the phase transition temperature, and ion permeability of the membrane [35,148,211].

Even though at the starting phase of this thesis, the studies related to the assessment of APIs was scarce, a significant number of publications have been produced during the past years, as presented in detail in Chapter 1. Some studies have included *V. fischeri*, *D. magna* and *P. subcapitata*. In this work, compounds with potential low-level effects were identified, with *P. subcapitata* being the most sensitive organism and having the most sensitive endpoint studied.

Regarding the acute effects observed towards *V. fischeri*, the results are in accordance with previous studies [43,107,166,207,241,256,262], which have already been reviewed in Chapter 1. The EC_{50} values for the acute exposure time of *D. magna* calculated in this thesis were in the range of the EC_{50} values reported in the literature [131,132,148,160,205]. Finally, the findings of this work regarding the chronic ecotoxicity of APIs towards *P. subcapitata* were in the range found in previous studies [60,107,166,226,239,260]

Effects on *P. subcapitata* were observed at concentrations 0 to 10000 orders of magnitude lower than the ones calculated for *V. fischeri* and *D. magna*, indicating that the use of a 100-factor to extrapolate chronic toxicity could over or underestimate the effects. This is in accordance with Fent [327], who reported that on average the chronic toxicity is 1000 times lower than the acute toxicity data. Some even more extreme cases have been reported in fish for ethinylestradiol and propranolol, in which the acute/chronic ratios were 150000 and 49000, respectively [328]. Acute data is not considered in the EMEA guidelines, since continuous exposure time of the aquatic environment via treated wastewater is assumed.

The presence of the pharmacological targets similar as the ones present in humans may explain if a mode of action is expected, as proposed by Gunnarsson et al [329]. APIs typically have the following protein targets: receptors, ion channels, enzymes and transporters [330]. For instance, it has been shown that orthologs of the estrogen receptor are not present in some bacteria, algae, fungi and invertebrates meaning that effects are less probable at levels of the pharmacological dose. For *V. fischeri*, a greater exposure time is needed to better estimate effects of antibiotics as already mentioned by Backhaus et al. [331] and addressed in Chapter 7.

Specific modes of action for the investigated microorganisms are still not well documented. A specific mode of action in photosynthesis was suspected for β-blockers in algae [43] and then questioned by the same research group [332]. The indications derived from the application of a toxicokinetic model which assumed that the APIs with aliphatic amines are bioavailable molecules when neutral and are charged in the cell according to the cytoplasm's pH (ion-trapping model). Regarding the antibiotics, the photosynthetic apparatus of *P. subcapitata* was recently found to be affected by erythromycin, ciprofloxacin and sulfamethoxazole which may explain their high toxicity [232].

The exposure time of *Daphnia* to ibuprofen has been found to cause an alteration in expression pattern of genes in the eicosanoid pathway [333] a precursor of prostaglandin. Taking into consideration that ibuprofen is known to inhibit the prostaglandin synthesizing enzyme cyclooxygenase, it can be suggested that specific mode of action may be present even in relatively distant from human species, such as *D. magna*. However, the limitations of the development of the mode of action approach as a routine analysis at this point were recognized by Scholz et al. [334] as being expensive, time-consuming; require advanced technologies, appropriate technical skills and statistical analysis.

A key element in understanding the possible adverse effects of the APIs present in wastewater in Cyprus is its dilution factor when released into the environment. This factor is expected to be variable since the release to the environment depends on the seasonality and on the demand of treated wastewater for irrigation purposes. The common practice is the release into the groundwater, surface water used for irrigation purposed and into the sea during winter period, in which fortunately the river flows are higher and consequently the dilution is expected to be higher. Future studies in which the actual environmental concentrations are investigated, apart from the concentrations present in treated wastewater, are necessary to completely understand the exposure time scenarios of organisms.

The devastating effects to the vulture population caused by diclofenac poisoning [199] is an example that APIs have the potential of causing irreversible environmental consequences. As Sumpter stated [335] reducing inputs of chemicals into the freshwater environments is the best strategy to minimize the chances of a similar event occurring in the aquatic environment and the improvement in wastewater treatment processes is a good step in this direction. The prevention and minimization of adverse alterations to ecosystems are still the principal goal of environmental management [336].

5.4 Conclusions

Biological assessments should both estimate the condition of a biological resource (magnitude of alteration) and provide environmental managers with a diagnosis of the potential causes of impairment [336]. A first step for environmental risk assessment was

applied in this work in which worst-case scenarios, a relatively simple estimation for the PECs and standard acute and chronic tests were considered. The risk assessment identified that only propranolol may pose a risk to algae, whereas no significant risk was found for the acute endpoints investigated in *V. fischeri* and *D. magna* for the concentration levels examined. The assessment of the effects of single APIs however, was a prerequisite for the assessment of the effects of mixtures of APIs.

CHAPTER 6. ASSESSMENT OF EFFECTS OF MIXTURES OF ACTIVE PHARMACEUTICAL INGREDIENTS

6.1 Background information

The pollution of the environment as a result of APIs is an increasing area of concern with still unanswered questions regarding their ecotoxicological effects [337]. As already mentioned, most studies on the effects of APIs on biological systems are conducted using one API at a time. However, APIs do not occur as isolated, pure substances in the environmental compartments. Therefore, there is an obvious need to study the effect of mixtures rather than single APIs, as mixtures may have different mechanisms or modes of action and consequently they may direct their effect against other targets than single APIs [338]. The behaviour of each component in a mixture may vary depending on the mixture composition, concentration and the bioassay used to evaluate the effects. Furthermore, the duration and frequency of exposure could change the toxicological effects of such mixtures.

The assessment of toxicity of chemical mixtures is a great challenge nowadays. Uniform guidelines are difficult to be created due to the variation in the constituents of the mixtures and the high number of potential adverse effects to human health and the environment. Regulation is a stringent necessity, since in environmental compartments isolated substances are not found, but they are found as components of a mixture. Some efforts are made by the European Union to establish regulations for the risk assessment of mixtures emitted in the environment. Guidelines developed by the WHO and US EPA are already available, but they are more focused on the possible adverse effects on human health. In the publication "State of the Art Report on Mixture toxicity" by Kortenkamp and Backhaus [339] the scientific and regulatory status for toxicity of chemical mixtures are discussed. Recently, an opinion on the toxicity and assessment of chemical mixtures in general, in which a decision tree for evaluating their risks was proposed by a Scientific Committee (SCCS, SCER and SCENHR) of the European Commission [340].

Single APIs can be present in concentrations that individually can provoke only low, non-significant effects. However, API combinations can have unfavourable joint outcomes that

may be enhancive, synergistic, antagonistic or additive. Potentiation or enhancement occurs when the effects of one API are motivated by the presence of another API. Synergism is observed when the mixture effects of APIs are significantly greater than the single effects of each API; whereas antagonism is observed in the opposite situation than synergism. At this point it should be mentioned that a standardized definition for synergism or antagonism with global consensus has not yet been established [341]. Synergistic effects may be present at the community organizational level due to the combined effects of toxicants belonging to different taxonomic groups and the indirect adverse effects on the structure and functioning of the community [342].

API mixtures may interact at a common site such as a receptor or an enzyme, hence activating the same specific target in an additive way. It may not be possible to predict and fully characterise these effects in a model as they can be altered depending on the components of the mixtures and their individual concentration. Many effects are more complex than simply binding the compound to a receptor or enzyme and act through a combination of actions such as altering gene expression, changing levels of intracellular concentrations of ions, altering cellular metabolism or expression of cellular regulators. Each of these mechanisms can be affected at different levels depending on the mixtures involved [343].

In the present work, two designs were applied to *V. fischeri* and *D. magna* so as to understand how APIs behave in aqueous and wastewater matrices and what their effects are to non-target organisms, (i) as mixtures at their environmental concentrations (MEC) and 100×MEC and (ii) as equitoxic mixtures. The experimental design of using constant-ratio mixtures has been widely used [35,148,211,341]. The so-called "reference mixtures" as the ones used in this chapter are mainly designed for the exploration of conceptual mixture toxicity questions [342]. A computerized simulation performed by the CompuSyn software was used to describe the behaviour of the mixtures, as suggested by Chou [341,344].

6.2 Results

6.2.1 Computerized simulation of active pharmaceutical ingredients mixture effects

As stated by Chou [344] the basis for the median-effect equation, derived mathematically, can be considered as the unified theory for (i) the Michaelis-Menten equation of enzyme kinetics, (ii) the Hill equation for higher-order ligand binding saturation, (iii) the Henderson-Hasselbalch equation for pH ionization, and (iv) the Scatchard equation for receptor binding. The method is quantitative, mechanism- and unit-independent and can be applied also to a small number of data. The median equation is calculated as follows:

$$\frac{F_a}{F_u} = \left(\frac{D}{D_m}\right)^m$$

F_a: fraction affected by D

F_u: fraction unaffected, *i.e.*, $F_u = 1 - F_a$

D: Concentration of the API

D_m: median EC_{50} of the API

m: slope of the dose-response curve

Equation 6.1 Median equation calculation

The following equation is used to plot the dose-response curve:

$$\log\left(\frac{F_a}{F_u}\right) = m(\log D - \log D_m)$$

Equation 6.2 Equation for the linearization of dose-response curves

The equations are ratios; hence dimensionless quantities. The R^2 value is the linear correlation coefficient of the median-effect plot and also the conformity of the data with the mass-law. When m equals 1 indicates a hyperbolic, m greater than 1 a sigmoidal and m lower than 1 a flat sigmoidal dose-response curve.

The equations used for the mixture are presented in Equation 6.3-6.4:

$$\left(\frac{F_{a_1}}{F_{u_1}}\right)^{1/m} + \left(\frac{F_{a_2}}{F_{u_2}}\right)^{1/m} = \frac{D_1}{D_{m_1}} + \frac{D_2}{D_{m_2}}$$

Equation 6.3 Median-effect calculation used for a two-active pharmaceutical ingredient mixture

The equations used for the n-mixtures median-effect calculations are described in detail by Chou [344].

The Chou-Talalay method introduces the "Combination Index" (CI), in which synergism has CI<1, additive effect CI=1 and antagonism CI>1. It should be mentioned that the range of values of synergism are from 0 to 1; whereas of antagonism from 1 to ∞.

$$CI_x = \sum_{j=1}^{n} \frac{D_{x_{1-n}}\left(D_j \Big/ \sum_1^n D\right)}{D_{m_j}\left(F_{a_{x_j}} \Big/ 1 - F_{a_{x_j}}\right)^{1/m_j}}$$

Equation 6.4 Calculation of the Combination Index

An example of the application of the equation for a mixture of two APIs is shown below.

$$CI = \frac{D_1}{D_{m_1}\left(F_a / 1 - F_a\right)^{1/m_1}} + \frac{D_2}{D_{m_2}\left(F_a / 1 - F_a\right)^{1/m_2}}$$

Table 6.1 Description and symbols of synergism or antagonism [344]

Range of Combination Index	Description	Graded symbols	Graphic symbols
<0.1	Very strong synergism	+++++	
0.1-0.3	Strong synergism	++++	
0.3-0.7	Synergism	+++	
0.7-0.85	Moderate synergism	++	
0.85-0.90	Slight synergism	+	
0.90-1.10	Nearly additive	±	
1.10-1.20	Slight antagonism	-	
1.20-1.45	Moderate antagonism	--	
1.45-3.3	Antagonism	---	
3.3-10	Strong antagonism	----	
>10	Very strong antagonism	-----	

The Combination Index was categorized from very strong synergism (five plus) (+++++) to very strong antagonism (five minus) (-----), as presented in Table 6.1. Isobolograms and polygonograms were used to describe the relationships in binary mixtures, as proposed by Chou [344]. The following abbreviations were used for the mixtures M_1: β-blockers, M_2: non-steroidal anti-inflammatory APIs, M_3: antibiotics, WW: wastewater.

6.2.2 Evaluation of mixture toxicity effects to *Vibrio fischeri*

All the independent experiments were performed at least three times. Each independent experiment was performed in duplicate. All quality criteria were met, as described in Chapter 3.

Preliminary experiments were performed in which *Vibrio fischeri* was exposed to a mixture of the eight APIs at (i) their MECs and (ii) one hundred higher concentration of their MECs (100×MECs). Both concentrations of the mixtures indicated very low toxicity which was no statistically different from the toxicity of the control (data not shown). Therefore, in the next step, equitoxic solutions of the EC_{50} values for the eight APIs were investigated and the results are presented in the following sections. The EC_{50} values deriving from Chapter 5 were used for the preparation of the equitoxic solutions.

6.2.2.1 Mixtures of β-blockers

The dose-response curves of the β-blockers applied for single compounds and for mixtures are presented in Figure 6.1. The EC_{50} values for atenolol (ATL), metoprolol (MTL) and propranolol (PRL) were 91.89, 1610 and 91.48 mg/L, respectively. Their slopes were 4.83, 1.04 and 1.56, indicating sigmoidal curves, and the R^2 were 1, 1 and 0.94, respectively. The dose-response curve for the mixture of the β-blockers had an EC_{50} value of 474.9 mg/L, a slope of 1.57 and a R^2 of 0.99.

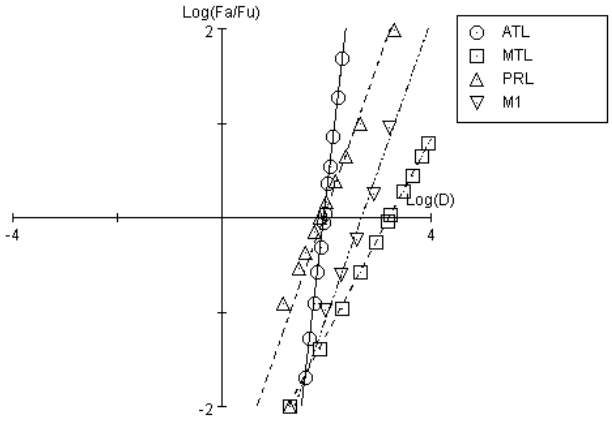


Figure 6.1 Dose-response curves after an exposure time of 5 min for β-blockers as single compounds for atenolol (ATL), metoprolol (MTL) and propranolol (PRL) and as mixtures (M_1)

The CI of the β-blockers (M_1) is shown in Figure 6.2. The effects ranged from additive (±) at the EC_5 value (72.57 mg/L) to slightly synergistic (+) from the EC_{10} (116.87 mg/L) to EC_{15} values (157.02 mg/L), to moderate synergistic (++) from the EC_{20} (196.10 mg/L) to EC_{75} values (957.27 mg/L). Higher concentrations make the mixture behave as slightly synergistic (+) and nearly additive (±) from the EC_{80} (1436.35 mg/L) to EC_{90} values (1929.53 mg/L). Higher concentrations had moderate antagonistic (--) effects.

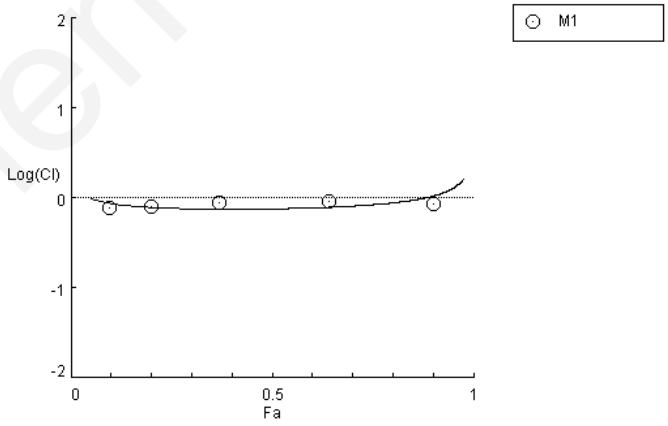


Figure 6.2 Combination index (CI) for the β-blockers mixtures

6.2.2.2 Mixtures of non-steroidal anti-inflammatory APIs

Diclofenac (DCF) and ibuprofen (IBF) had similar dose-response curves slopes of 1.09 and 1.05, respectively, indicating hyperbolic curves (Figure 6.3). Ibuprofen (EC_{50} 41.23 mg/L) was more toxic than diclofenac (EC_{50} 62.06 mg/L) and for both the R^2 was greater than 0.99. The mixture (M_2) had an EC_{50} value of 21.21 mg/Ldemonstrating a higher toxicity. The slope of the mixture was 1.97 (R^2=0.99).

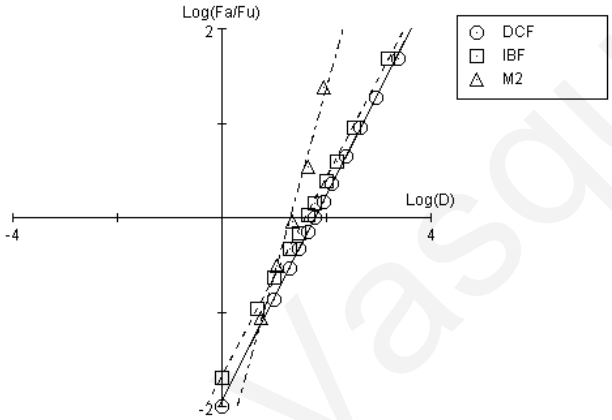


Figure 6.3 Dose-response curves after an exposure time of 5 min for non-steroidal anti-inflammatory APIs as single compounds for diclofenac (DCF) and ibuprofen (IBF) and as mixtures (M_2)

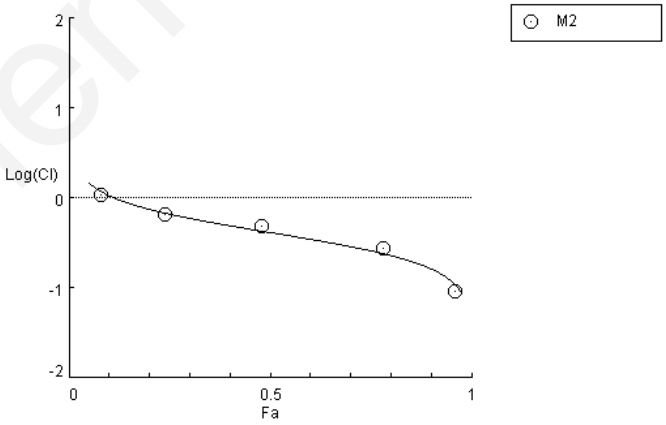


Figure 6.4 Combination index (CI) for the non-steroidal anti-inflammatory APIs mixtures

The effects of the β-blocker mixture (M_2) are shown in Figure 6.4, in which a clear synergistic effect was observed. The effects were additive (±) at concentrations up to the EC_{10} value (6.97 mg/L), slightly synergistic (+) at concentration around the EC_{15} value (8.80 mg/L)

and moderate synergistic (++) at the EC_{20} level (10.50 mg/L). Synergistic effects (+++) were found to range from the EC_{25} (12.15 mg/L) to the EC_{65} values (29.05 mg/L). Strong synergism (++++) was observed for concentrations up to the EC_{95} value (94.54 mg/L). Finally, very strong synergism (+++++) was observed for concentrations greater than the EC_{97} (123.83 mg/L).

The isobologram that could be derived by the non-steroidal anti-inflammatory APIs mixture is presented in Figure 6.5, in which the synergistic effects of the mixture are obvious.

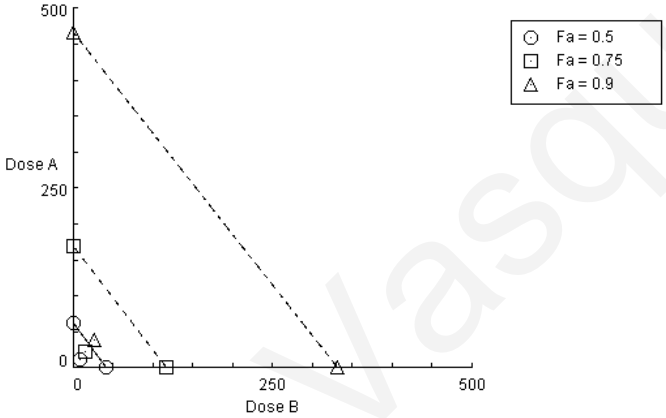


Figure 6.5 Isobologram for the non-steroidal anti-inflammatory APIs mixtures

This relationship can be presented schematically, as shown in Figure 6.6.

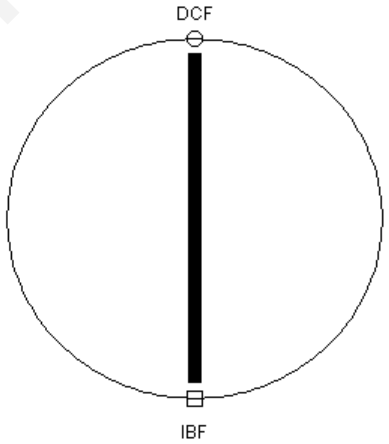


Figure 6.6 Polygonogram demonstrating the relationship between non-steroidal anti-inflammatory APIs

6.2.2.3 Mixtures of antibiotics

The effects of the antibiotics as single compounds (erythromycin: ERY, ofloxacin: OFL and sulfamethoxazole: SMX) and mixture (M_3) are presented in Figure 6.7. The EC_{50} values of 428.16, 432.55 and 114.65 mg/L for erythromycin, ofloxacin and sulfamethoxazole, respectively, indicate that sulfamethoxazole is the most toxic antibiotic. The slopes were 2.03, 1.97 and 1.58; hence describing sigmoidal curves and the R^2 were 1, 1 and 0.95, respectively. The mixture of antibiotics (M_3) had an EC_{50} value of 1081.05 mg/L and was described by a sigmoidal curve with a slope of 1.82 and a R^2 of 0.99.

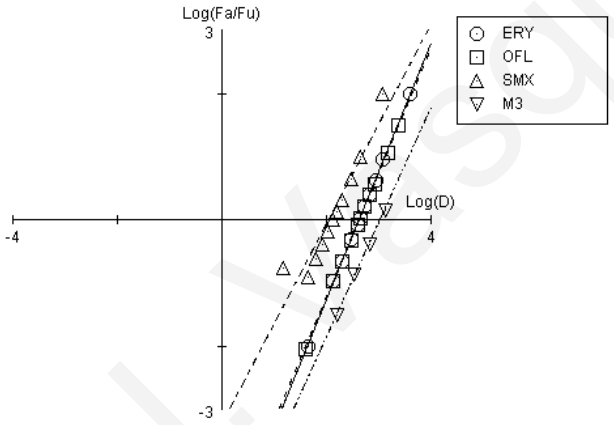


Figure 6.7 Dose-response curves after an exposure time of 5 min for antibiotics as single compounds for erythromycin (ERY), ofloxacin (OFL) and sulfamethoxazole (SMX) and as mixtures (M_3)

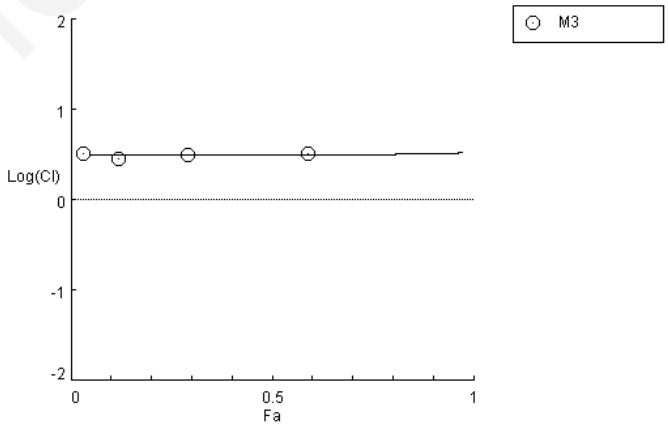


Figure 6.8 Combination index (CI) for antibiotic mixtures

The CI of the mixture (M_3) is illustrated in Figure 6.8 and an antagonistic behaviour can easily be recognized. The values indicated antagonism (---) at concentrations up to the EC_{95} value (5449.37 mg/L). At the EC_{97} value (7297.82 mg/L) the mixture had a strongly antagonistic (----) behaviour.

6.2.2.4 Multi-component mixtures of β-blockers and non-steroidal anti-inflammatory APIs

The dose-response curves of the β-blockers (M_1) and non-steroidal anti-inflammatory APIs (M_2) and their mixture (M_1M_2) are shown in Figure 6.9. The mixture had an EC_{50} value of 561.44 mg/L and a curve with a sigmoidal slope of 1.28 and a R^2 of 0.99.

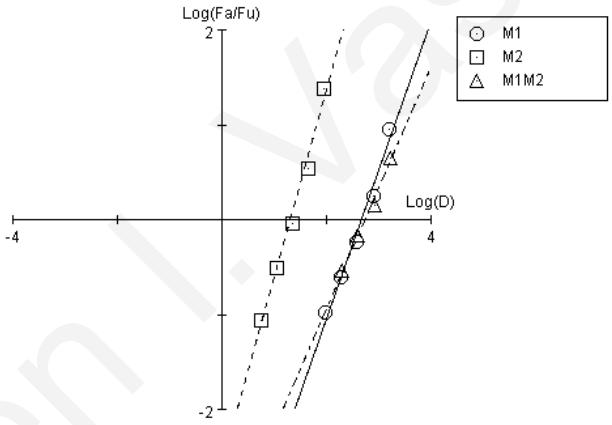


Figure 6.9 Dose-response curves after an exposure time of 5 min for β-blockers (M_1), non-steroidal anti-inflammatory APIs (M_2) and mixture (M_1M_2)

The CI of the mixtures is presented in Figure 6.10 and it can be seen that even though the β-blockers (M_1) and the non-steroidal anti-inflammatory APIs (M_2) had a synergistic or additive effect, their mixture (M_1M_2) had an antagonistic (---) behaviour.

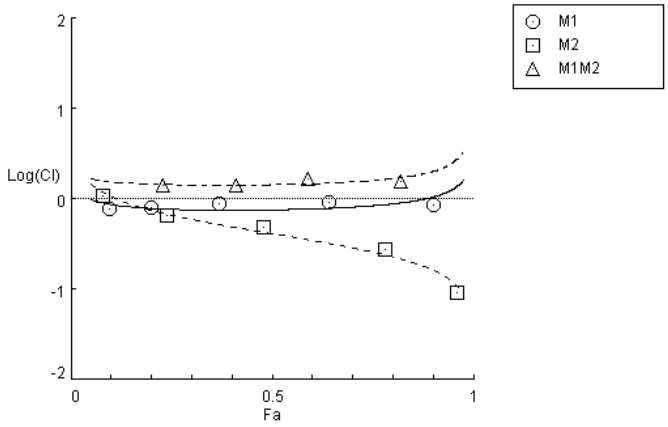


Figure 6.10 Combination index (CI) for β-blockers (M_1), non-steroidal anti-inflammatory APIs (M_2) and mixture (M_1M_2)

6.2.2.5 Multi-component mixtures of non-steroidal anti-inflammatory APIs and antibiotics

The effects of the non-steroidal anti-inflammatory APIs (M_2) and the antibiotics (M_3) and their mixture (M_2M_3) are presented in Figure 6.11. They had an EC_{50} value of 21.22 and 1081.05 mg/L, whereas their mixture had an EC_{50} value of 533.82 mg/L. The slopes were 1.97, 1.82 and 1.3 demonstrating sigmoidal curves. The R^2 were 0.99, 1 and 0.99, respectively.

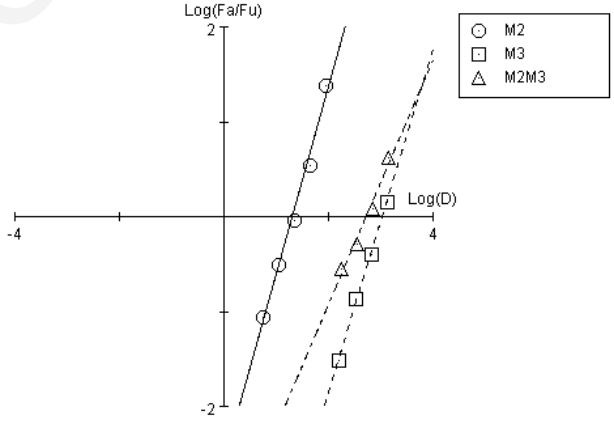


Figure 6.11 Dose-response curves after an exposure time of 5 min for non-steroidal anti-inflammatory APIs (M_2), antibiotics (M_3) and mixture (M_2M_3)

The CI of the mixtures is shown in Figure 6.12. The non-steroidal anti-inflammatory APIs behaved from synergistically to strong synergistically, the antibiotics antagonistically and their mixture (M_2M_3) antagonistically (---) for concentrations up to their EC_{95} value (5140.91 mg/L). At higher concentrations were strongly antagonistic (----).

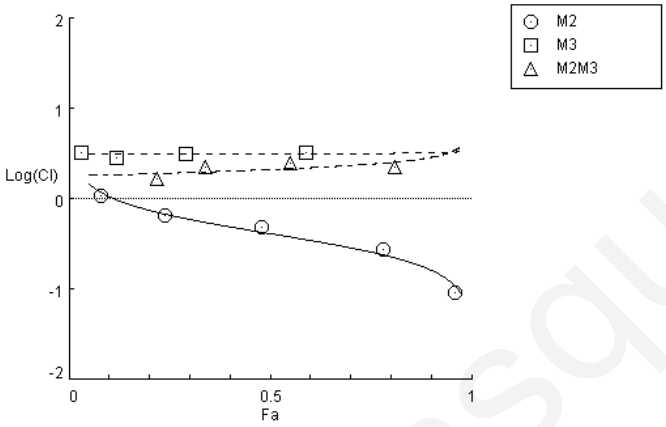


Figure 6.12 Combination index (CI) for non-steroidal anti-inflammatory APIs (M_2), antibiotics (M_3) and mixture (M_2M_3)

6.2.2.6 Multi-component mixtures of β-blockers and antibiotics

The dose-response curves for the β-blockers (M_1), antibiotics (M_3) and their mixtures (M_1M_3), as shown in Figure 6.13, had EC_{50} values of 474.91, 1081.05 and 1960.54 mg/L, respectively. The slopes were 1.57, 1.82 and 1.17 indicating sigmoidal curves. The R^2 in all cases were 0.99.

The CI for the β-blockers (M_1), the antibiotics (M_3) and their mixture (M_1M_3) are illustrated in Figure 6.14. It can be seen that the β-blockers had a slight synergistic or additive behaviour; the antibiotics had an antagonistic one. Their mixture (M_1M_3) had an antagonistic (---) behaviour at concentrations up to the EC_{25} value (765.96 mg/L) and strongly antagonistic (----) behaviour up to the EC_{90} value (12844.50 mg/L) and very strongly antagonistic (-----) for higher concentrations.

To sum up, the mixtures of APIs with similar mode of action in humans like the β-blockers and non-steroidal anti-inflammatory APIs demonstrate synergistic or additive effect; whereas

the antibiotics having different modes of action had an antagonistic effect. The more complex mixtures had always antagonistic behaviour, suggesting that most probably the bioavailability of the substances is affected when present in complex mixtures.

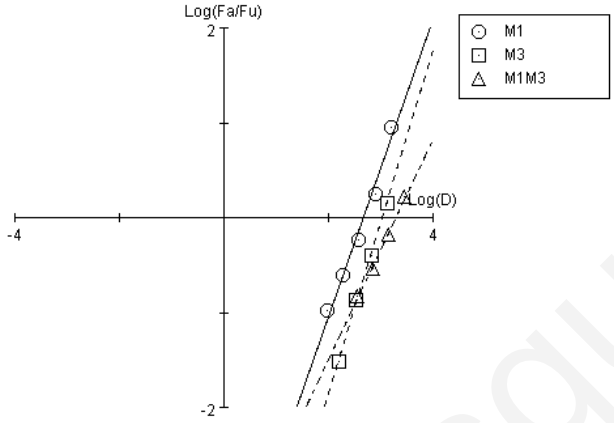


Figure 6.13 Dose-response curves after an exposure time of 5 min for β-blockers (M_1), antibiotics (M_3) and mixture (M_1M_3)

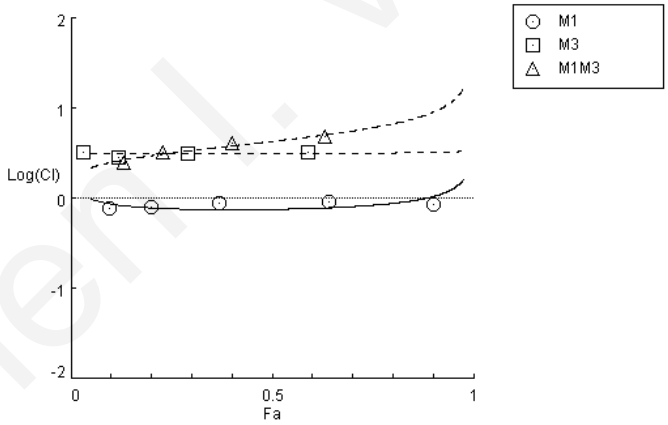


Figure 6.14 Combination index (CI) for β-blockers (M_1), antibiotics (M_3) and mixture (M_1M_3)

6.2.2.7 Multi-component mixtures of β-blockers in wastewater

The effects of the β-blockers in water and wastewater, shown in Figure 6.15, were quite similar. The EC_{50} values were 474.91 and 497.13 mg/L and the slopes were 1.57 and 1.66, respectively. The R^2 were in both cases 0.99.

The CI values for the β-blockers in water and wastewater were comparable, as seen in Figure 6.16. The β-blockers in wastewater (WWM_1) were slightly antagonistic (-) at the EC_5 (86.69 mg/L), nearly additive (±) between EC_{10} and EC_{15} (135.04-177.68 mg/L), slightly synergistic (+) up to the EC_{20} (218.40 mg/L), moderate synergistic (++) at the EC_{25} (259.10 mg/L), synergistic (+++) up to the EC_{75} (953.86 mg/L), moderate synergistic (++) at the EC_{80} (1131.34 mg/L), nearly additive (±) from the EC_{85} to the EC_{90} (1390.98-1830.19 mg/L), slightly antagonistic (-) at the EC_{95} (2850.92 mg/L) and moderate antagonistic (--) at the EC_{97} (3907.91 mg/L).

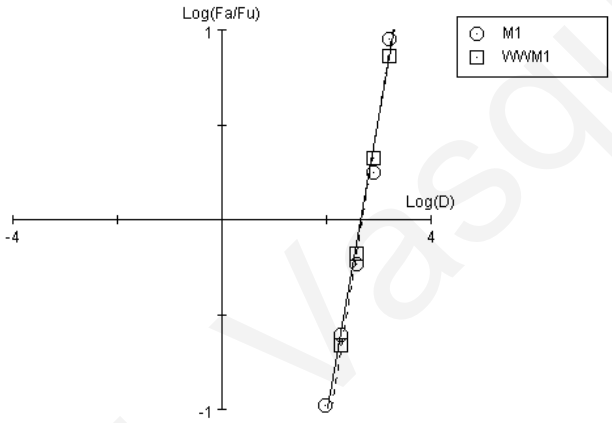


Figure 6.15 Dose-response curves after an exposure time of 5 min for β-blockers in water (M_1) and in wastewater (WWM_1)

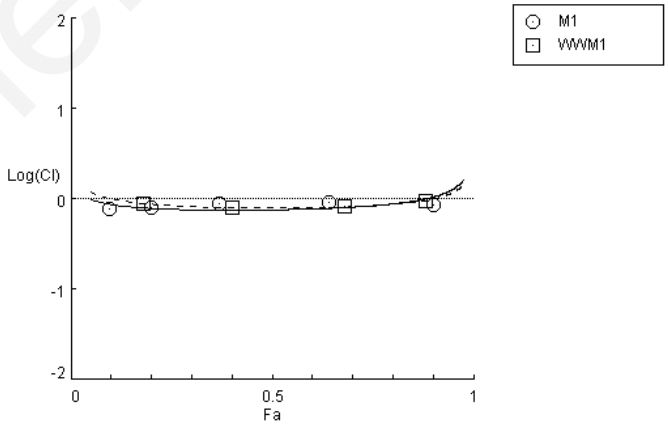


Figure 6.16 Combination index (CI) for β-blockers in water (M_1) and wastewater (WWM_1)

6.2.2.8 Multi-component mixtures of non-steroidal anti-inflammatory APIs in wastewater

The non-steroidal anti-inflammatory APIs in water (M_2) and wastewater (WWM_2) had different behaviour, as illustrated in Figure 6.17. The EC_{50} values for the non-steroidal anti-inflammatory APIs in water (M_2) and in wastewater (WWM_2) were 21.22 and 66.61 mg/L, respectively. The slopes were 1.97 and 0.89, respectively, indicating a flat sigmoidal shape. The R^2 for both curves were 0.99.

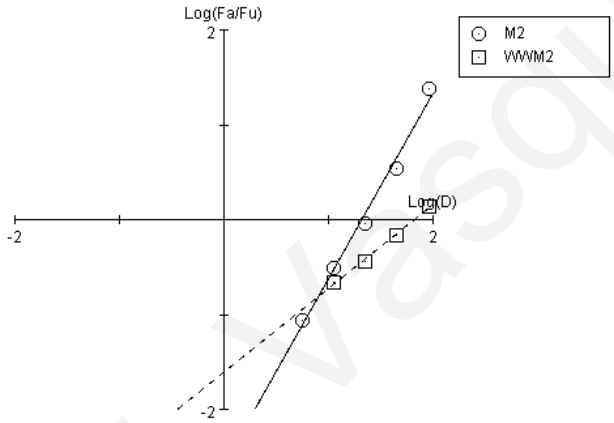


Figure 6.17 Dose-response curves after an exposure time of 5 min for non-steroidal anti-inflammatory APIs in water (M_2) and in wastewater (WWM_2)

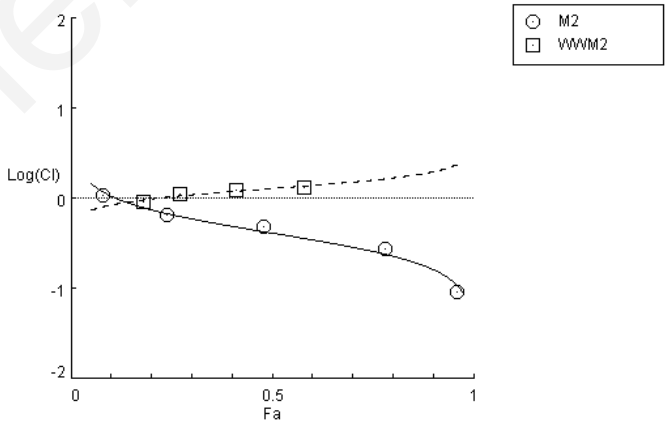


Figure 6.18 Combination index (CI) for non-steroidal anti-inflammatory APIs in water (M_2) and wastewater (WWM_2)

The CI values deviated severely when the wastewater was used as the matrix for the non-steroidal anti-inflammatory APIs (WWM_2), as presented in Figure 6.18. The wastewater mixture (WWM_2) was moderate synergistic (++) up to the EC_{10} value (5.59 mg/L), nearly additive (±) up to the EC_{30} value (25.62 mg/L), slightly antagonistic (-) from the EC_{35} to the EC_{40} values (33.15-42.17 mg/L), moderate antagonistic (--) up to the EC_{60} values (105.21 mg/L) and antagonistic (---) at higher concentrations.

The different behaviour between the non-steroidal anti-inflammatory APIs in water (M_2) and in wastewater (WWM_2) is demonstrated in the isobolograms shown in Figure 6.19. It can be easily recognized that the synergistic effect of the non-steroidal anti-inflammatory APIs was reversed when the matrix was wastewater.

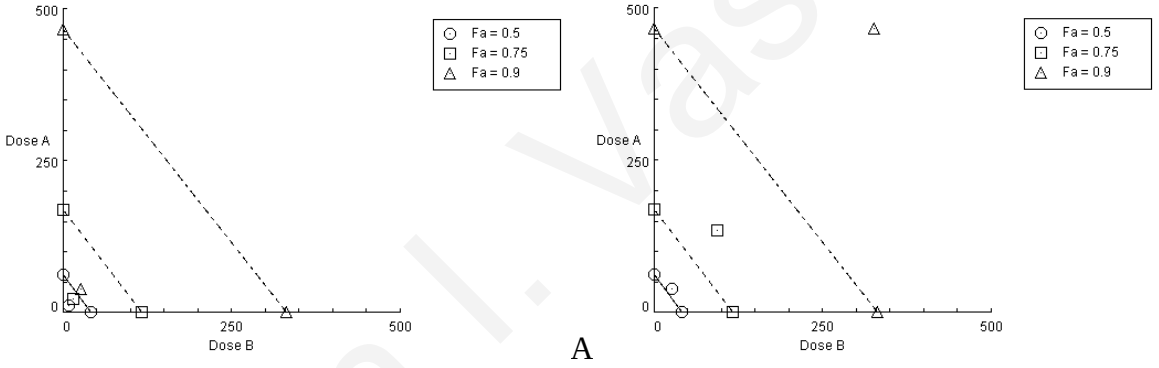


Figure 6.19 Isobologram for the non-steroidal anti-inflammatory APIs mixtures in water (A) and in wastewater (B)

6.2.2.9 Multi-component mixtures of antibiotics in wastewater

The dose-response curves of the antibiotics in water (M_3) and in wastewater (WWM_3) indicate different behaviour (Figure 6.20). The first had an EC_{50} value of 1081.05 mg/L and a slope of 1.82 and the latter an EC_{50} value of 3191.85 mg/L and a slope of 1.46. The R^2 were 0.99 and 1, respectively.

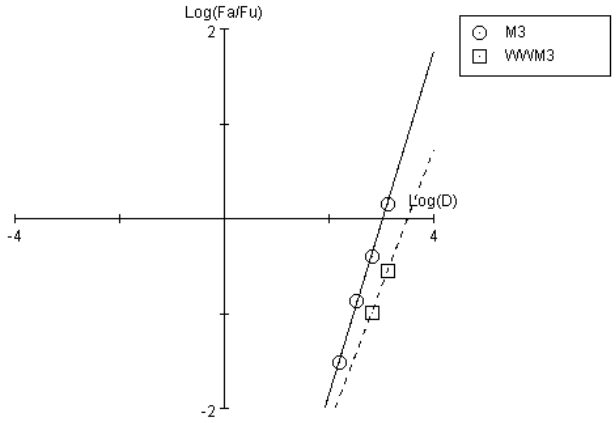


Figure 6.20 Dose-response curves after an exposure time of 5 min for antibiotics in water (M_3) and in wastewater (WWM_3)

The CI values were different when the antibiotics were in wastewater (Figure 6.21). For concentrations between the EC_5 and the EC_{60} values (424.70-4213.75 mg/L) the mixture in wastewater (WWM_3) was strongly antagonistic (----) and at higher concentrations were very strongly antagonistic (-----).

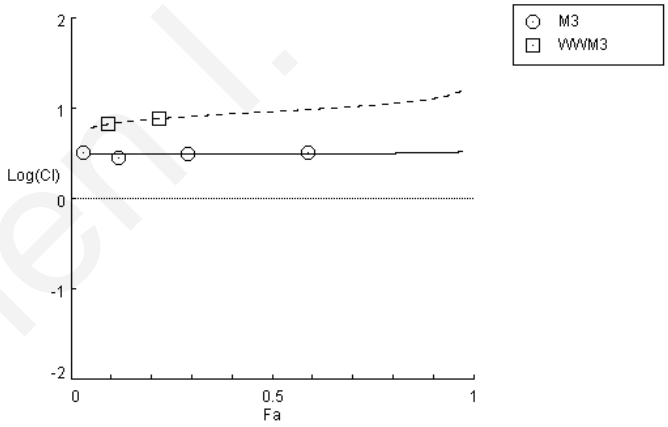


Figure 6.21 Combination index (CI) for antibiotics in water (M_3) and wastewater (WWM_3)

6.2.2.10 Multi-component mixtures of β-blockers and non-steroidal anti-inflammatory APIs in wastewater

The β-blockers and non-steroidal anti-inflammatory APIs in water (M_1M_2) and in wastewater (WWM_1M_2) had similar dose-response curves (Figure 6.22), with EC_{50} values of

561.44 and 671.28 mg/L and slopes of 1.28 and 1.33, respectively. The R^2 for both curves was 0.99.

The CI values for both mixtures were similar, as presented in Figure 6.23. The mixture in wastewater behaved primarily antagonistically (---) for concentrations up to the EC_{95} (6121.10 mg/L). At the EC_{97} (9123.43 mg/L) the effects of the mixture were strongly antagonistic (----).

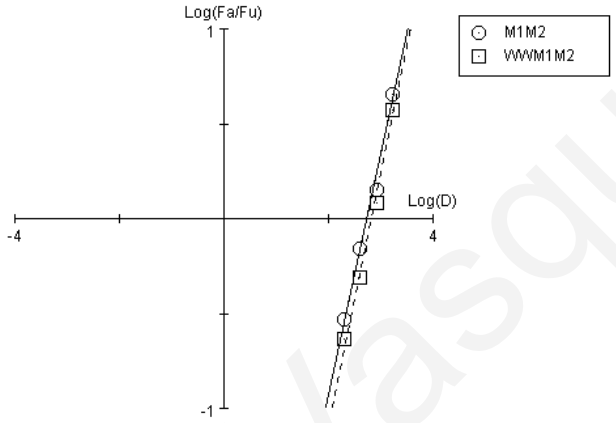


Figure 6.22 Dose-response curves after an exposure time of 5 min for β-blockers (M_1) and non-steroidal anti-inflammatory APIs (M_2) in water (M_1M_2) and in wastewater (WWM_1M_2)

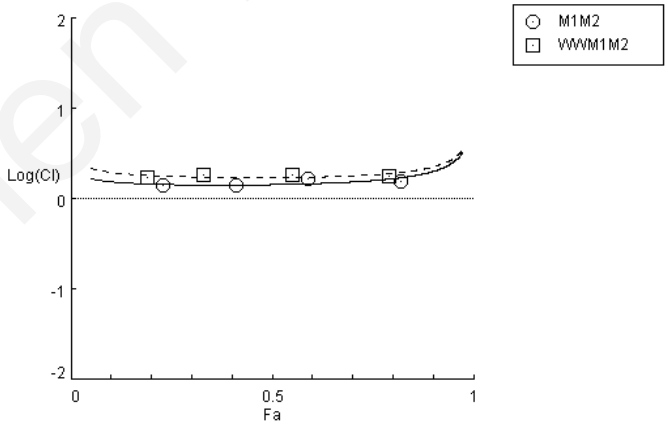


Figure 6.23 Combination index (CI) for β-blockers (M_1) and non-steroidal anti-inflammatory APIs (M_2) in water (M_1M_2) and in wastewater (WWM_1M_2)

6.2.2.11 Multi-component mixtures of non-steroidal anti-inflammatory APIs and antibiotics in wastewater

Figure 6.24 illustrates the dose-response curves for the non-steroidal anti-inflammatory APIs and antibiotics in water (M_2M_3) and in wastewater (WWM_2M_3). The EC_{50} value and the slope of the mixture in water were 533.82 mg/L and 1.3. Respectively, the EC_{50} value and the slope of the mixture in wastewater were 1518.73 mg/L and 1.5, suggesting a lower toxic effect.

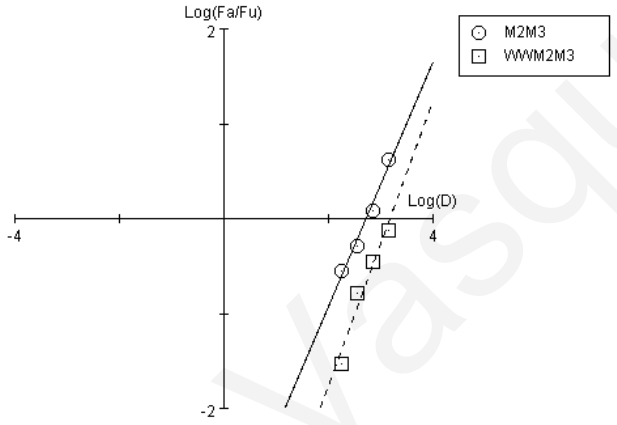


Figure 6.24 Dose-response curves after an exposure time of 5 min for non-steroidal anti-inflammatory APIs (M_2) and antibiotics (M_3) in water (M_2M_3) and in wastewater (WWM_2M_3)

All the CI values of the mixture in wastewater indicated a strongly antagonistic (----) behaviour at all the concentrations evaluated, as shown in Figure 6.25.

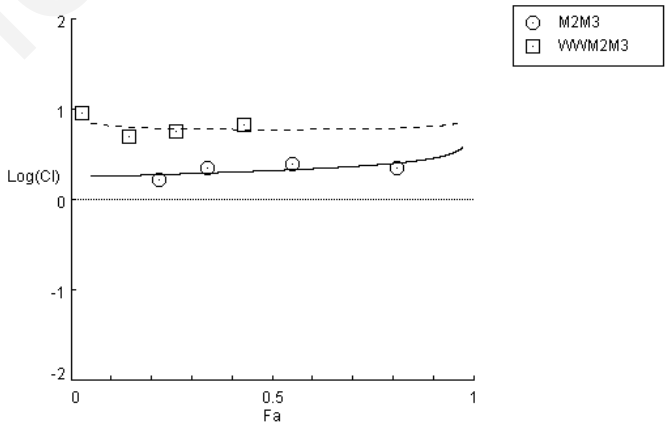


Figure 6.25 Combination index (CI) for non-steroidal anti-inflammatory APIs (M_2) and antibiotics (M_3) in water (M_2M_3) and in wastewater (WWM_2M_3)

6.2.2.12 Multi-component mixtures of β-blockers and antibiotics in wastewater

Figure 6.26 presents the dose-response curves for the β-blockers and antibiotics in water (M_1M_3) and in wastewater (WWM_1M_3). The EC_{50} values were 1960.45 and 1687.53 mg/L and the slopes 1.17 and 1.35, respectively. The slopes indicated a shift from hyperbolic to sigmoidal shape. The R^2 for both curves was greater than 0.99.

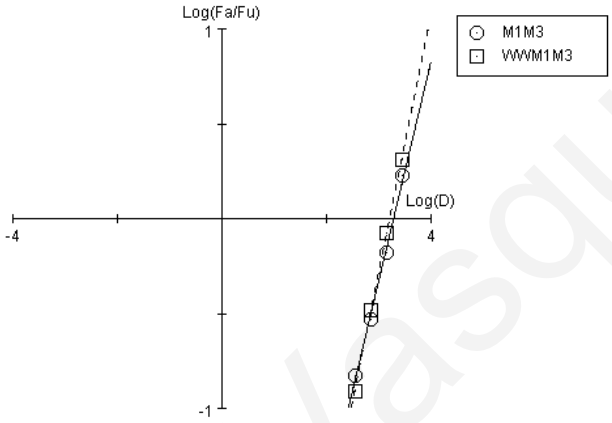


Figure 6.26 Dose-response curves after an exposure time of 5 min for β-blockers (M_1) and antibiotics (M_3) in water (M_2M_3) and in wastewater (WWM_1M_3)

The CI values for the mixture of β-blockers and antibiotics in wastewater (WWM_1M_3) categorized the behaviour as antagonistic (---) for concentrations up to the EC_{30} (901.28 mg/L). Higher concentrations led to a strongly antagonistic behaviour (----).

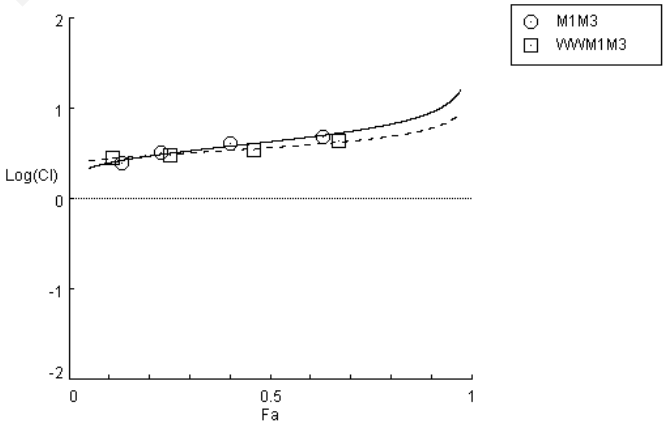


Figure 6.27 Combination index (CI) for β-blockers (M_1) and antibiotics (M_3) in water (M_1M_3) and in wastewater (WWM_1M_3)

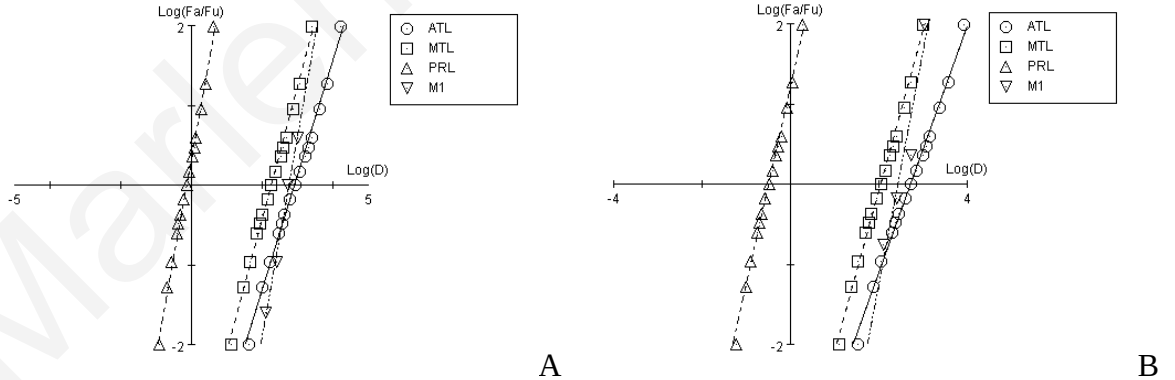
To conclude, two types of effects were observed when the mixtures were in wastewater. The APIs mixture in wastewater may behave in a similar manner (shape) to the mixture in water, although it exhibits lower toxicity (concentration), or the APIs mixture in wastewater may demonstrate an antagonistic or strongly antagonistic behaviour, compared to the effects of the mixture when present in water.

6.2.3 Evaluation of mixture toxicity effects to *Daphnia magna*

Preliminary experiments using the eight APIs at their MECs and 100×MECs were not able to produce acute effects to *D. magna* (data not shown). Consequently, the EC_{50} values for the APIs as computed in Chapter 5 were used for the preparation of equitoxic solutions.

6.2.3.1 Mixtures of β-blockers

The dose-response curves for atenolol (ATL), metoprolol (MTL) propranolol (PRL) and their mixture (M_3) for the immobilization of *D. magna* after 24 (A) and 48 (B) h of exposure time are shown in Figure 6.28.



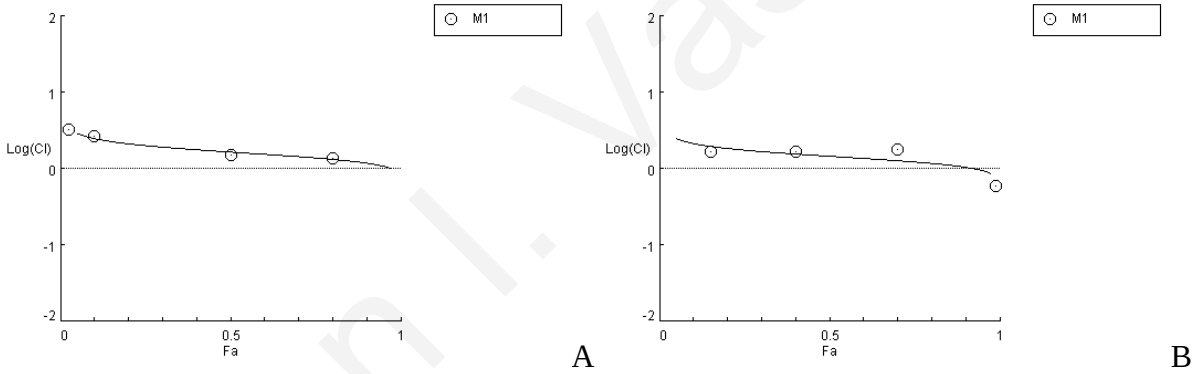
A B

Figure 6.28 Dose-response curves after an exposure time of 24 (A) and 48 (B) h for β-blockers as single compounds for atenolol (ATL), metoprolol (MTL) and propranolol (PRL) and as mixtures (M_1)

The EC_{50} values were 872.40, 190.94 and 0.75 mg/L for the 24 h exposure time and 544.43, 114.83 and 0.34 mg/L for the 48 h exposure time, respectively. The slopes were 1.44, 1.65 and 2.46 after an exposure time of 24 h and 1.55, 1.98 and 2.46 after an exposure time of

48 h, respectively. When the β-blockers were tested as a mixture (M_1) the EC_{50} value was 587.91 mg/L and the slope was 2.5. The R^2 for the abovementioned curves was greater than 0.99.

The CI of the β-blockers mixture (M_1) after 24 (A) and 48 (B) h exposure time are shown in Figure 6.29. The values indicated antagonism (---) up to the EC_{65} (752.86 mg/L) when exposed for 24 h and up to the EC_{50} value (283.57 mg/L) when exposed for 48 h. Moderate antagonism (--) was observed at concentrations up to the EC_{85} (1175.61 mg/L) for the 24 h exposure time and up to the EC_{75} (413.08 mg/L) for the 48 h. Slight antagonism (-) occurred at the EC_{90} (1414.36 and 601.76 mg/L for 24 and 48 h exposure time, respectively). Additive effects explained the behaviour at higher concentrations for the 24 h exposure time; whereas for the 48 h exposure time the behaviour was additive (±) to slightly synergistic (+).



A B

Figure 6.29 Combination index (CI) after an exposure time of 24 (A) and 48 (B) h for the β-blockers mixtures

6.2.3.2 Mixtures of non-steroidal anti-inflammatory APIs

The non-steroidal anti-inflammatory APIs diclofenac (DCF) and ibuprofen (IBF) and their mixture (M_2) presented dose-response curves as illustrated in Figure 6.30.

The EC_{50} values were 60.15, 28.21 and 53.14, 10.78 mg/L for diclofenac and ibuprofen after 24 and 48 h exposure time, respectively. The slopes of diclofenac and ibuprofen curves were 2.02 and 2.17 for 24 h exposure time and 2.57 and 1.16 for 48 h exposure time. The

mixture (M_2) had EC_{50} values of 35.75 and 26.51 mg/L and slopes of 3.40 and 2.83 after 24 and 48 h exposure time, respectively.

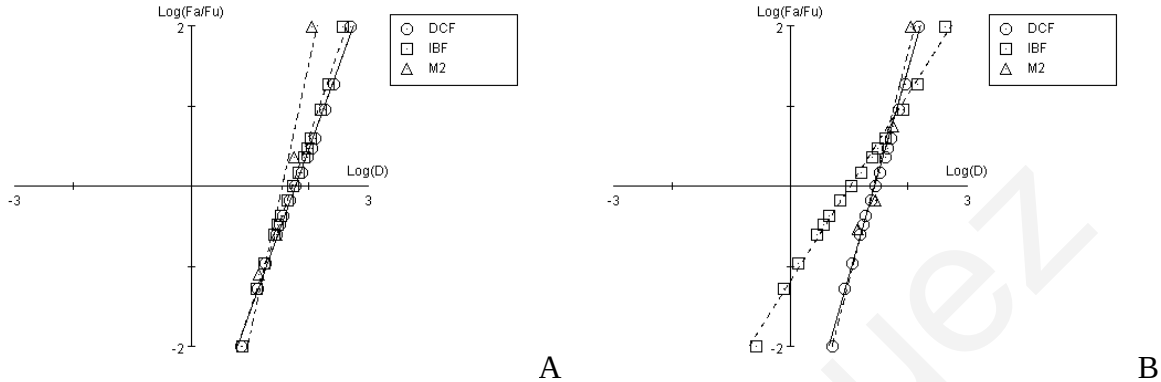


Figure 6.30 Dose-response curves after an exposure time of 24 (A) and 48 (B) h for β-blockers as single compounds for diclofenac (DCF) and ibuprofen (IBF) and as mixtures (M_2)

The CI values demonstrate different behaviour of the mixture for 24 and 48 h exposure time (Figure 6.31). At 24 h exposure time (A) the effects were additive (±) up to the EC_{10} (18.73 mg/L), slightly synergistic (+) at the EC_{15} (21.46 mg/L), moderate synergistic (++) up to the EC_{30} (27.86 mg/L) and synergistic (+++) for higher concentrations. On the contrary, at 48 h of exposure time (B), the effects were strongly antagonistic (----) for concentrations up to the EC_{15} (14.37 mg/L), antagonistic (---) from EC_{20} to EC_{55} (16.25-28.45 mg/L), moderate antagonistic (--) up to the EC_{70} (35.75 mg/L), slightly antagonistic (-) at the EC_{75} (39.07 mg/L), additive (±) up to the EC_{85} (48.90 mg/L), moderate synergistic (++) up to the EC_{95} (74.96 mg/L) and higher concentrations were synergistic (+++).

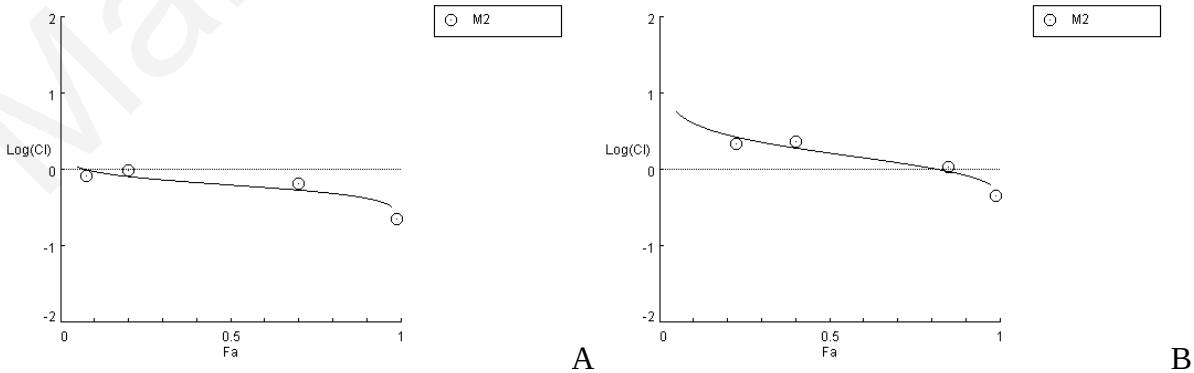


Figure 6.31 Combination index (CI) after an exposure time of 24 (A) and 48 (B) h for the non-steroidal anti-inflammatory APIs mixtures

The spreading of the values in the isobolograms after 24 and 48 h exposure time can be seen in Figure 6.32 (A) and (B), in which the above mentioned behaviour can also be visualized.

The polygonograms that illustrate the relationship among the non-steroidal anti-inflammatory APIs are presented in Figure 6.33. During the 24 h exposure time the synergism developed was much stronger, than that observed after the 48 h exposure time.

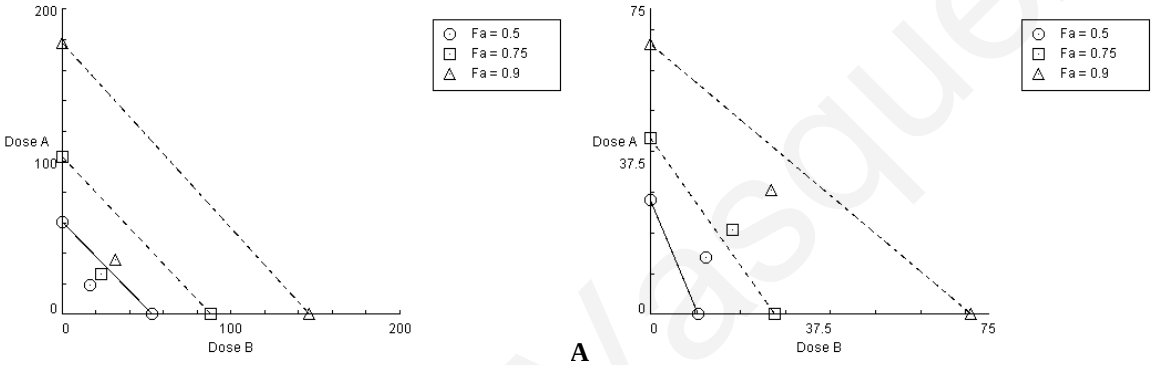


Figure 6.32 Isobologram after an exposure time of 24 (A) and 48 (B) h for the non-steroidal anti-inflammatory APIs mixtures

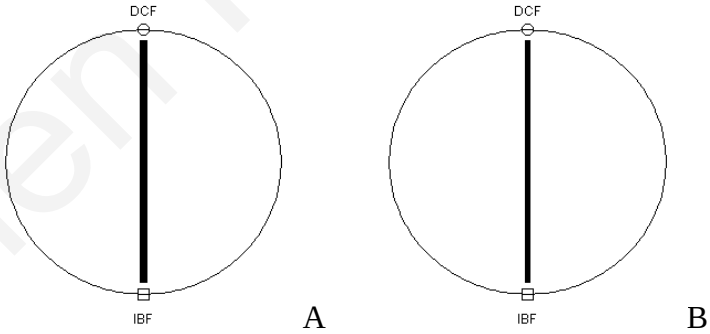


Figure 6.33 Polygonogram demonstrating the relationship between non-steroidal anti-inflammatory APIs after an exposure time of 24 (A) and 48 (B) h

6.2.3.3 Mixtures of antibiotics

The dose-response curves for the antibiotics erythromycin (ERY), ofloxacin (OFL), sulfamethoxazole (SMX) and their mixture (M_3) are illustrated in Figure 6.34. The EC_{50} values were 152.81, 117.21 and 80.78 mg/L for the 24 h exposure time and 62.38, 81.04 and

19.97 mg/L for the 48 h exposure time, respectively. The slopes were 1.28, 1.59 and 1.11 for the 24 h exposure time and 1.36, 1.79 and 1.44 for the 48 h exposure time. The R^2 was greater than 0.99 in all cases. The mixture of antibiotics (M_3) had an EC_{50} value of 256.54 and 140.82 mg/L for the 24 and 48 h exposure time, respectively. In the same order, the slopes were 1.14 and 1.44 and the R^2 0.84 and 0.92.

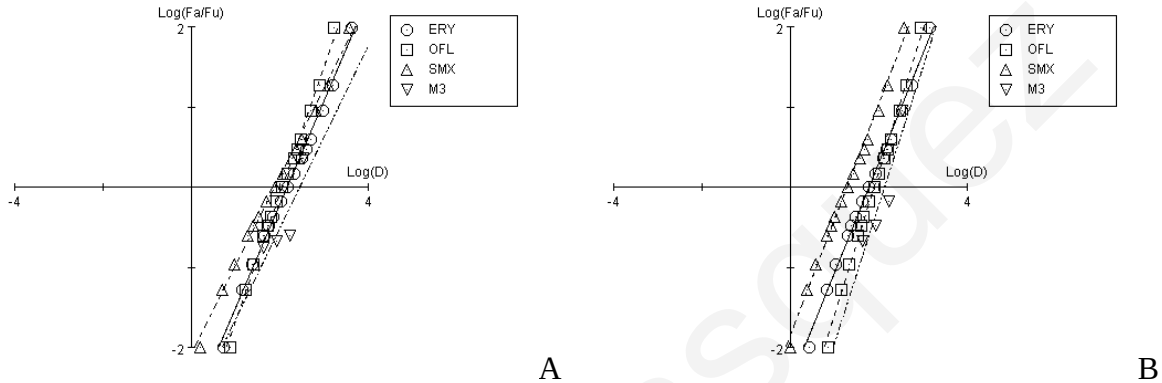


Figure 6.34 Dose-response curves after an exposure time of 24 (A) and 48 (B) h for β-blockers as single compounds for erythromycin (ERY), ofloxacin (OFL) and sulfamethoxazole (SMX) and as mixtures (M_3)

Antagonistic effects (---) were mainly observed for the antibiotics when exposed for 24 h at concentrations up to the EC_{90} (1965.23 mg/L) and strongly antagonistic (----) for higher concentrations. On the other hand, when exposed for 48 h, strong antagonism (----) was observed for concentrations up to the EC_{40} (111.28 mg/L) and antagonism (---) for higher concentrations.

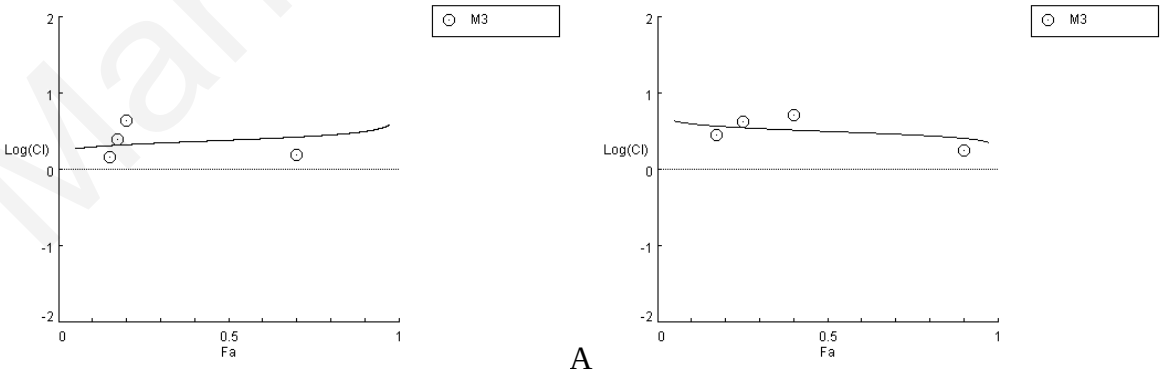
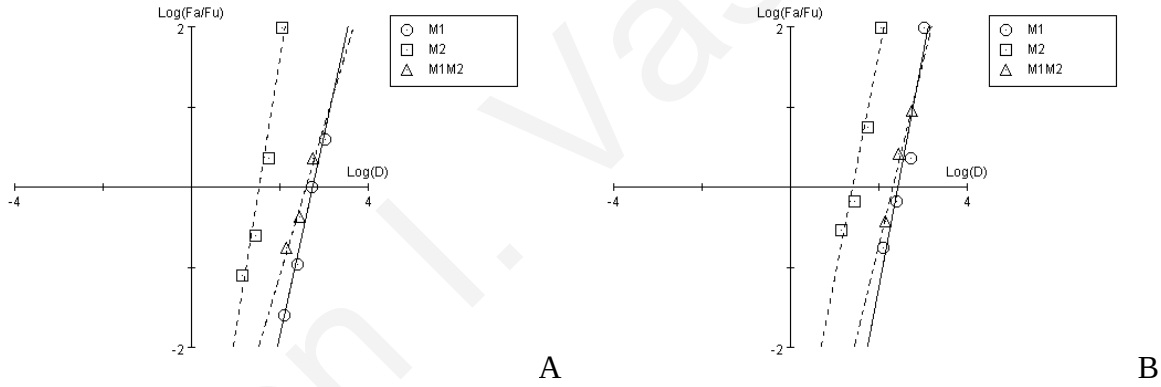


Figure 6.35 Combination index (CI) after an exposure time of 24 (A) and 48 (B) h for the antibiotic mixtures

6.2.3.4 Multi-component mixtures of β-blockers and non-steroidal anti-inflammatory APIs

β-blockers (M_1) and non-steroidal anti-inflammatory APIs (M_2) when mixed (M_1M_2) demonstrated dose-response curves as shown in Figure 6.36. When the exposure time was 24 h the EC_{50} values were 587.91 and 35.75 mg/L for the β-blockers (M_1) and the non-steroidal anti-inflammatory APIs (M_2), respectively. The slopes were 2.50 and 3.40 and the R^2 were 0.99 and 0.97, respectively. The mixture M_1M_2 had an EC_{50} value and slope of 401.5 mg/L and 1.86 (R^2=0.98). When the exposure time was increased to 48 h, the EC_{50} values were 283.57 and 26.51 mg/L for the β-blockers (M_1) and the non-steroidal anti-inflammatory APIs (M_2), respectively. The slopes were 2.92 and 2.83 and the R^2 were 0.96 and 0.97, respectively. The mixture M_1M_2 had an EC_{50} value and a slope of 213.60 mg/L and 2.28 (R^2=0.99).



A B

Figure 6.36 Dose-response curves after an exposure time of 24 (A) and 48 (B) h for β-blockers (M_1) and non-steroidal anti-inflammatory APIs (M_2) mixtures

The corresponding CI values for the mixtures are shown in Figure 6.37. The mixture of β-blockers and non-steroidal anti-inflammatory APIs (M_1M_2) had antagonistic (---) behaviour at all concentrations evaluated when the exposure time was 24 h. In the same way, when the exposure time was 48 h, lower concentrations caused strong antagonism (----) up to the EC_{15} (99.96 mg/L). Higher concentrations acted antagonistically (---).

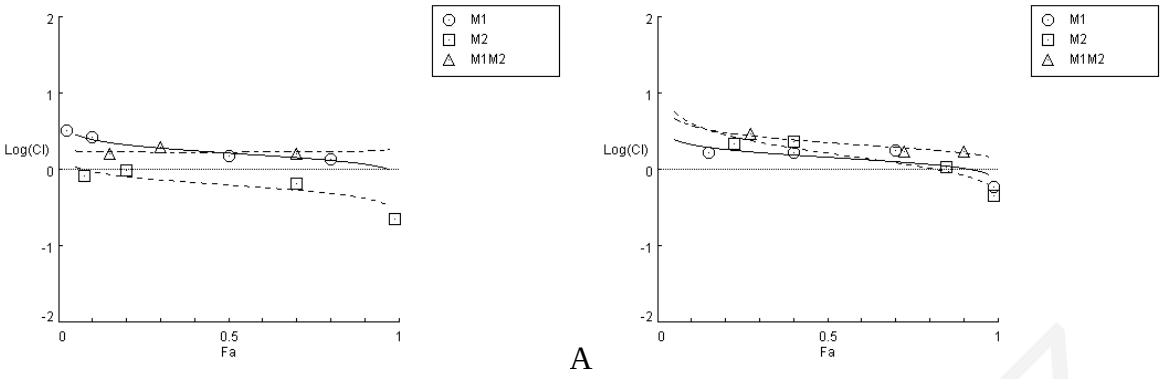


Figure 6.37 Combination index (CI) after an exposure time of 24 (A) and 48 (B) h for β-blockers (M_1) and non-steroidal anti-inflammatory APIs (M_2) mixtures

6.2.3.5 Multi-component mixtures of non-steroidal anti-inflammatory APIs and antibiotics

The non-steroidal anti-inflammatory APIs (M_2), the antibiotics (M_3) and their mixtures (M_2M_3) demonstrated dose-response curves, as shown in Figure 6.38. The EC_{50} values for the 24 h exposure times were 35.75, 286.54 and 245.60 mg/L for the non-steroidal anti-inflammatory APIs (M_2), the antibiotics (M_3) and their mixtures (M_2M_3), respectively. The corresponding slopes were 3.40, 1.14 and 1.81 with R^2 of 0.97, 0.84 and 0.99, respectively. The EC_{50} values for the 48 h exposure time decreased to 26.51, 140.82 and 99.34 mg/L for the non-steroidal anti-inflammatory APIs (M_2), the antibiotics (M_3) and their mixtures (M_2M_3), respectively. The slopes were 2.83, 1.72 and 2.20 and the R^2 0.97, 0.92 and 0.94, respectively.

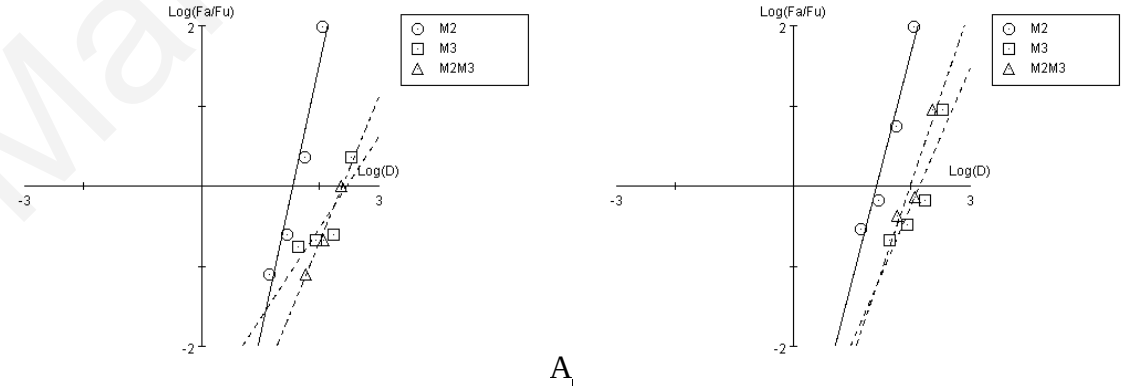


Figure 6.38 Dose-response curves after an exposure time of 24 (A) and 48 (B) h for non-steroidal anti-inflammatory APIs (M_2) and antibiotics (M_3) mixtures

The CI values, presented in Figure 6.39, for both 24 and 48 h exposure time had similar behaviour. Strongly antagonistic effects (----) were observed at lower concentrations that became antagonistic (---) as the concentrations increased. For the 24 h exposure time strong antagonistic (----) effects were observed up to the EC_{10} (73.06 mg/L) and for the 48 h exposure time up to the EC_{45} (90.66 mg/L). Higher concentrations had antagonistic (---) effects for both 24 and 48 exposure times.

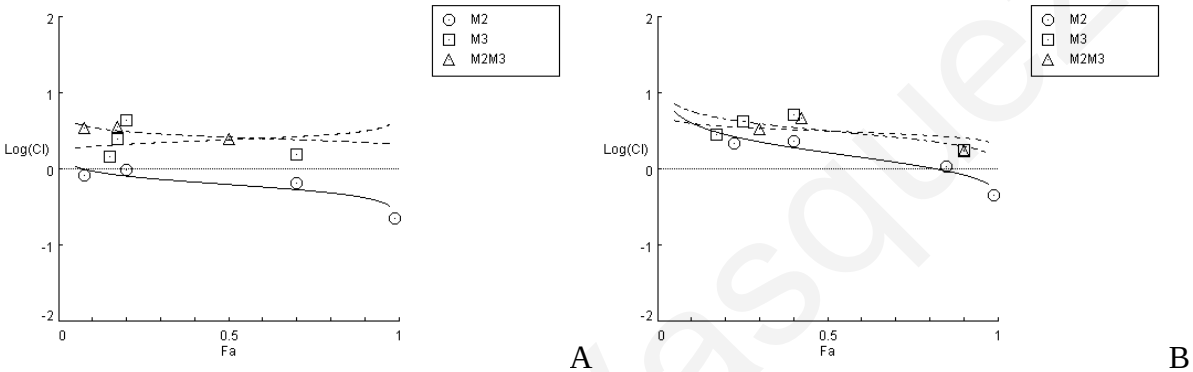


Figure 6.39 Combination index (CI) after an exposure time of 24 (A) and 48 (B) h for non-steroidal anti-inflammatory APIs (M_2) and antibiotics (M_3) mixtures

6.2.3.6 Multi-component mixtures of β-blockers and antibiotics

The β-blockers (M_1) and antibiotics (M_3) and their mixtures (M_1M_3) behaved as shown in Figure 6.40. The EC_{50} values for the β-blockers were 587.91 and 283.57 mg/L for the 24 and 48 h exposure time, respectively. The slopes were 2.50 and 2.92 and the R^2 0.99 and 0.96. The EC_{50} values for the antibiotics were 286.54 and 140.82 mg/L for the 24 and 48 h exposure time, respectively. The slopes were 1.14 and 1.72 and the R^2 0.84 and 0.92. The EC_{50} values for their mixture (M_1M_3) were 707.70 and 542.52 mg/L for the 24 and 48 h exposure time. The slopes were 2.24 and 1.38 and the R^2 1 and 0.90.

The CI values of the β-blockers and antibiotic mixtures (M_1M_3) are presented in Figure 6.41. It can be observed that a different antagonistic behaviour was observed at 24 and at 48 exposure times. The former was strongly antagonistic (----) at concentrations up to the EC_{35} (536.62 mg/L) and antagonistic (---) at higher concentrations. The latter was strongly antagonistic (----) at all concentrations evaluated.

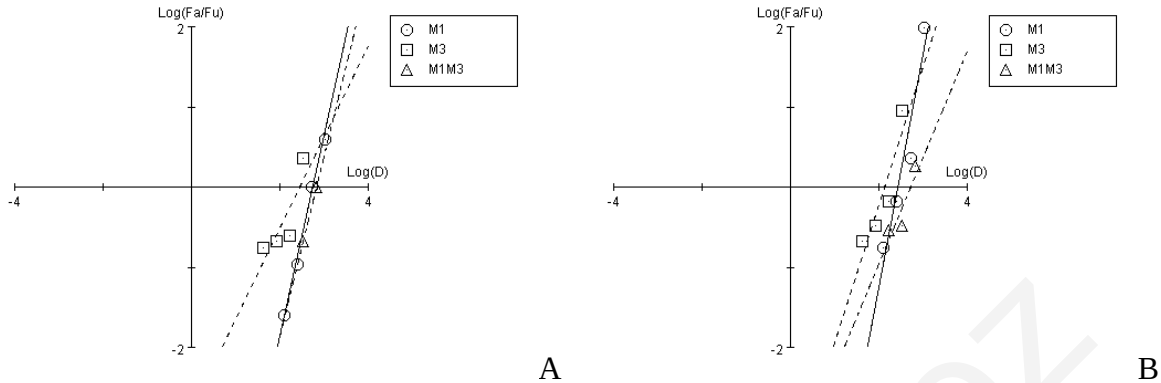


Figure 6.40 Dose-response curves after an exposure time of 24 (A) and 48 (B) h for β-blocker (M_1) and antibiotics (M_3) mixtures

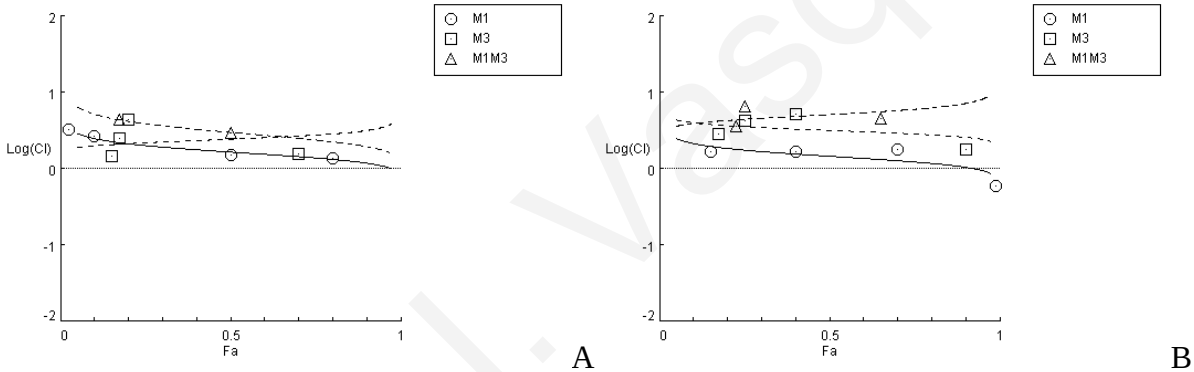


Figure 6.41 Combination index (CI) after an exposure time of 24 (A) and 48 (B) h for β-blockers (M_1) and antibiotics (M_3) mixtures

To conclude, it can be suggested that antagonistic behaviour was mainly observed for the mixtures evaluated. Antagonistic and strongly antagonistic effects were mainly observed in all combinations evaluated. An exception however, was the synergistic behaviour of non-steroidal anti-inflammatory APIs.

6.2.3.7 Multi-component mixtures of β-blockers in wastewater

Wastewater (WW), β-blockers in water (M_1) and in wastewater (WWM_1) were evaluated and the dose-response curves are shown in Figure 6.42. The mixture in wastewater (WWM_1) followed the behaviour of the curve of the mixture in water in a great extent in both 24 and 48 h exposure times.

The CI values illustrated in Figure 6.43 agree in that the trend of β-blockers in wastewater (WWM_1) follow the trend of β-blockers in water with an antagonistic (---) behaviour. However, the CI values at higher concentrations after the 48 h exposure time demonstrate moderate synergistic (++) and synergistic effects (+++) at the highest concentrations evaluated ($EC_{50}/2$ and EC_{50}).

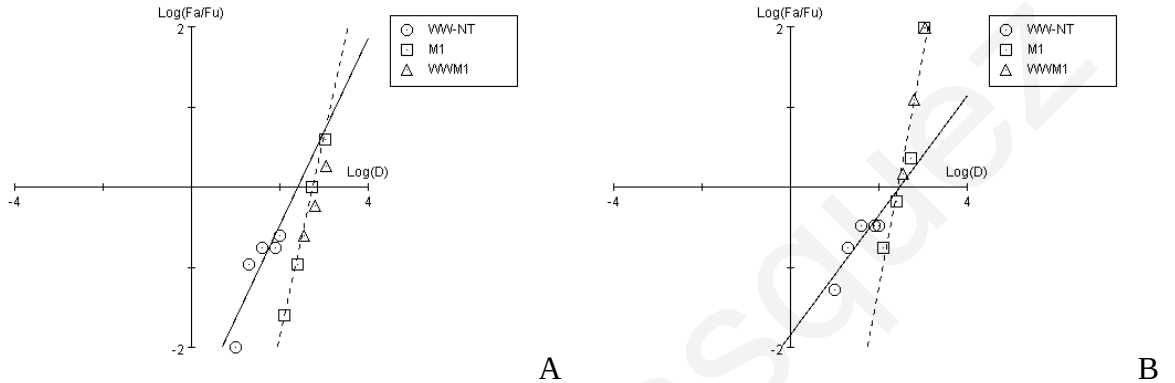


Figure 6.42 Dose-response curves after an exposure time of 24 (A) and 48 (B) h for β-blocker in water (M_1) and in wastewater (WWM_1) mixtures

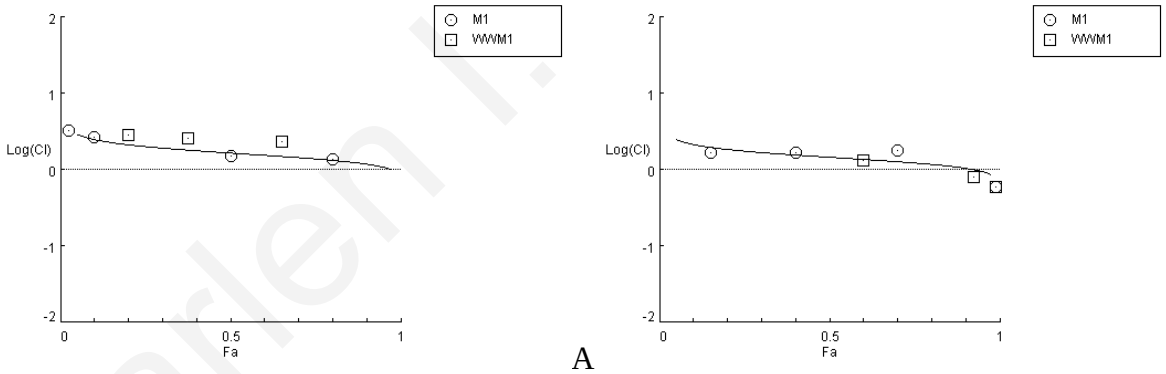
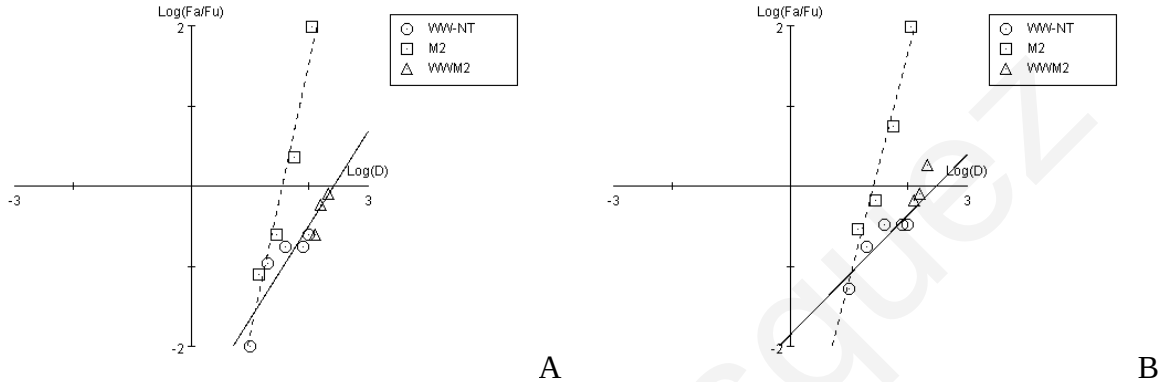


Figure 6.43 Combination index (CI) after an exposure time of 24 (A) and 48 (B) h for β-blocker in water (M_1) and in wastewater (WWM_1) mixtures

6.2.3.8 Multi-component mixtures of non-steroidal anti-inflammatory APIs in wastewater

A different behaviour was observed for the non-steroidal anti-inflammatory APIs when present in wastewater (WWM_2) compared to mixture in water (M_2) (Figure 6.44). The dose-response curve of the non-steroidal anti-inflammatory APIs in wastewater (WWM_2) followed

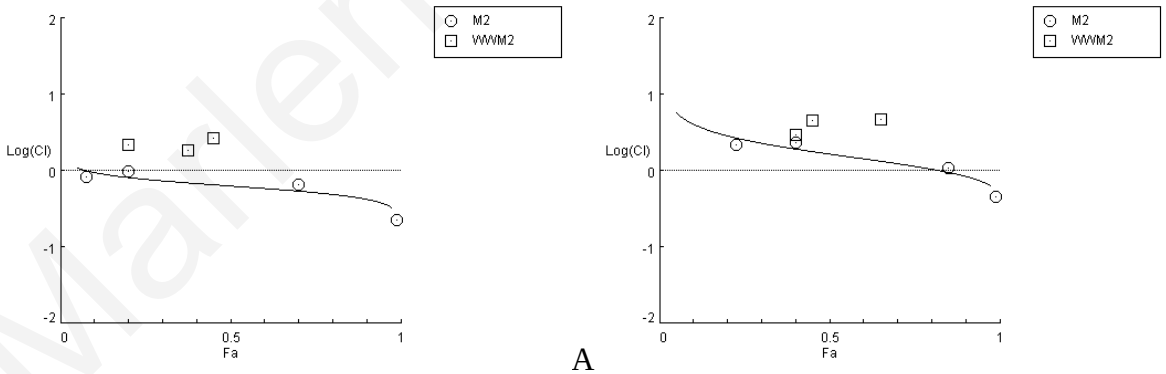
the dose-response curve of the wastewater (WW) and not the one of the non-steroidal anti-inflammatory APIs (M_2) in both 24 and 48 h exposure time. This finding suggests that the toxicity of the non-steroidal anti-inflammatory APIs in wastewater (WWM_2) is lower compared to the same mixture in water (M_2).



A B

Figure 6.44 Dose-response curves after an exposure time of 24 (A) and 48 (B) h for non-steroidal anti-inflammatory APIs in water (M_2) and in wastewater (WWM_2) mixtures

The CI values describe a different behaviour of the mixture depending on the matrix present. Antagonistic (---) and strongly antagonistic (---) effects were observed for the 24 and 48 h exposure time, respectively.

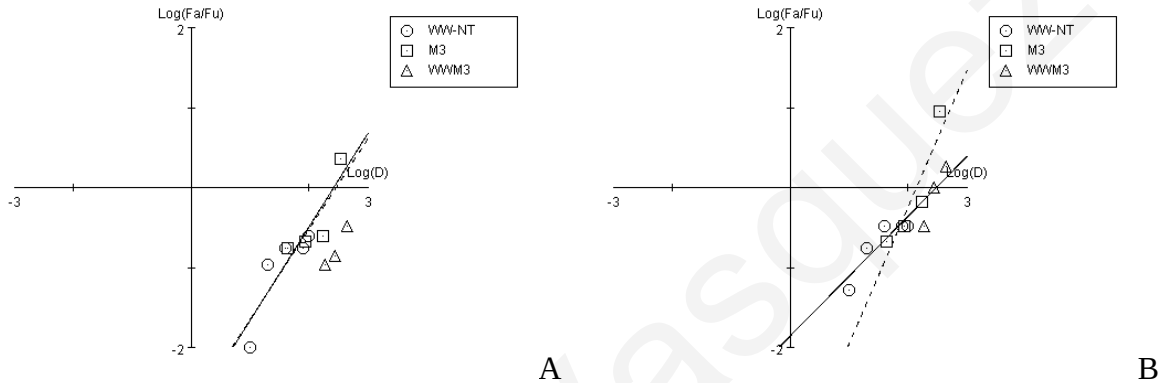


A B

Figure 6.45 Combination index (CI) after an exposure time of 24 (A) and 48 (B) h for non-steroidal anti-inflammatory APIs in water (M_1) and in wastewater (WWM_1) mixtures

6.2.3.9 Multi-component mixtures of antibiotics in wastewater

The antibiotics in wastewater (WWM_3) follow neither the dose-response curve of the wastewater (WW) nor the dose-response curve of the antibiotic mixture (M_3), as seen in Figure 6.46. The values of the mixture in wastewater (WWM_3) are placed more on the right, suggesting lower toxicity after a 24 and 48 h exposure time.

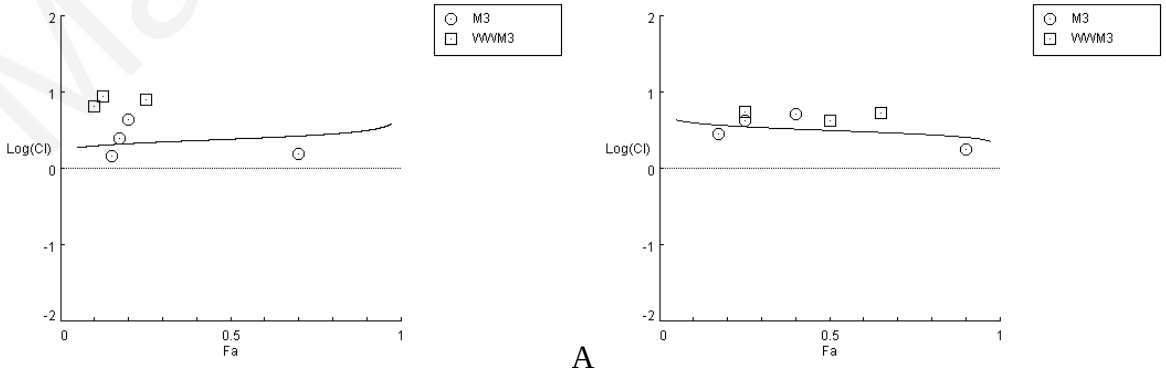


A B

Figure 6.46 Dose-response curves after an exposure time of 24 (A) and 48 (B) h for antibiotics in water (M_3) and in wastewater (WWM_3) mixtures

6.2.3.10 Multi-component mixtures of antibiotics in wastewater

As shown in Figure 6.47, the mixture of antibiotics in wastewater (WWM_3) was strongly antagonistic in both exposure times evaluated at all the concentrations tested. This is in agreement with the behaviour of the antibiotics when present in water (M_3).



A B

Figure 6.47 Combination index (CI) after an exposure time of 24 (A) and 48 (B) h for antibiotics in water (M_3) and in wastewater (WWM_3) mixtures

6.2.3.11 Multi-component mixtures of β-blockers, non-steroidal anti-inflammatory APIs and antibiotics in wastewater

The effects of the more complex mixtures and the CI values for each mixture are presented in Figure 6.48-Figure 6.53. The same trend was observed for all the mixtures, no matter the composition. Strongly antagonistic effects (----) were observed for both the 24 and 48 h exposure times. Antagonistic effects (---) were observed for the β-blocker and non-steroidal anti-inflammatory APIs mixture in wastewater (WWM_1M_2) after a 24 h exposure time at the $EC_{50}/2$ value. Antagonistic effects (---) were observed for the β-blockers and antibiotic mixture in wastewater (WWM_1M_3) after a 48 h exposure time at the EC_{50} value.

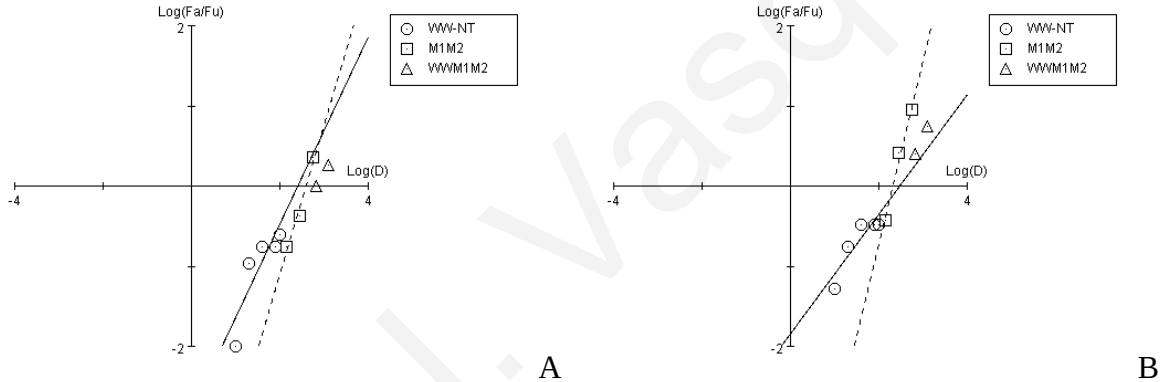


Figure 6.48 Dose-response curves after an exposure time of 24 (A) and 48 (B) h for β-blockers (M_1) and non-steroidal anti-inflammatory APIs (M_2) mixtures in water (M_1M_2) and in wastewater (WWM_1M_2)

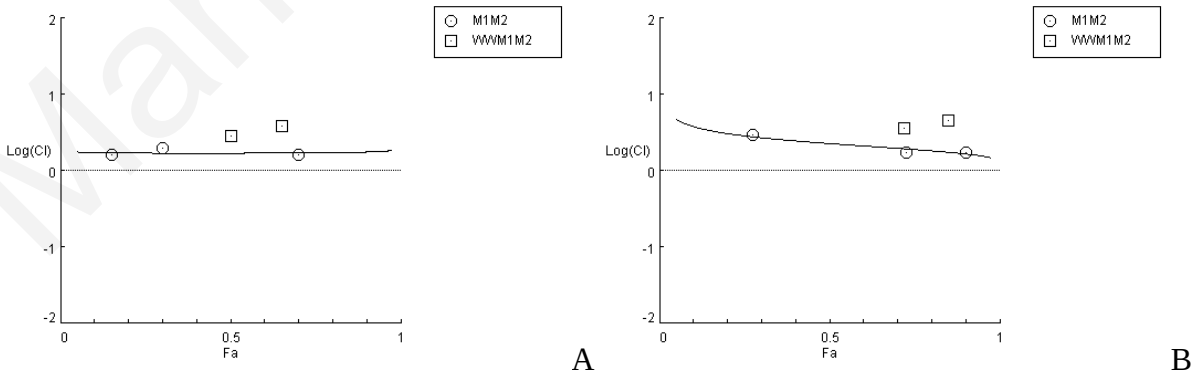


Figure 6.49 Combination index (CI) after an exposure time of 24 (A) and 48 (B) h for β-blockers (M_1) and non-steroidal anti-inflammatory APIs (M_2) mixtures in water (M_1M_2) and in wastewater (WWM_1M_2)

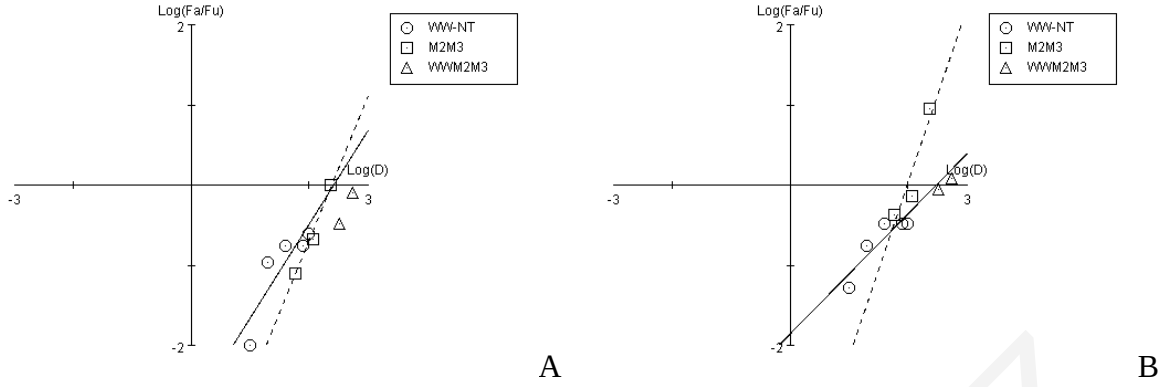


Figure 6.50 Dose-response curves after an exposure time of 24 (A) and 48 (B) h for non-steroidal anti-inflammatory APIs (M_2) and antibiotics (M_3) mixtures in water (M_2M_3) and in wastewater (WWM_2M_3)

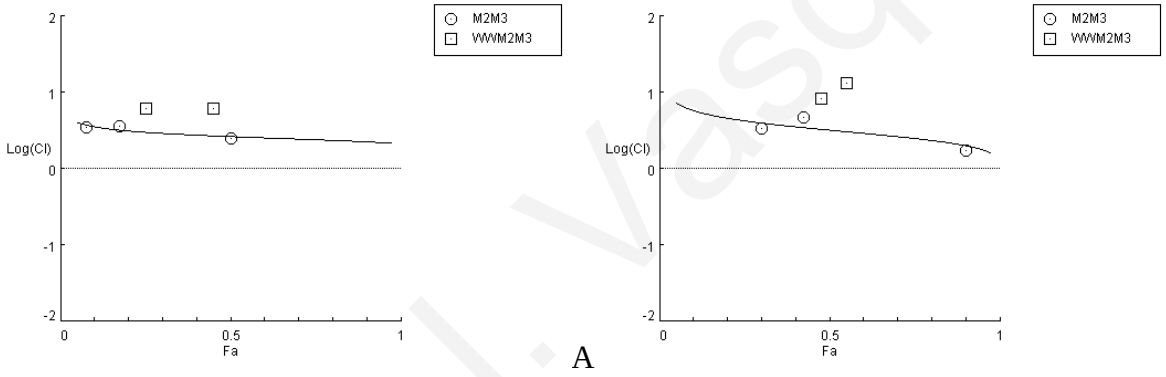


Figure 6.51 Combination index (CI) after an exposure time of 24 (A) and 48 (B) h for non-steroidal anti-inflammatory APIs (M_2) and antibiotics (M_3) mixtures in water (M_2M_3) and in wastewater (WWM_2M_3)

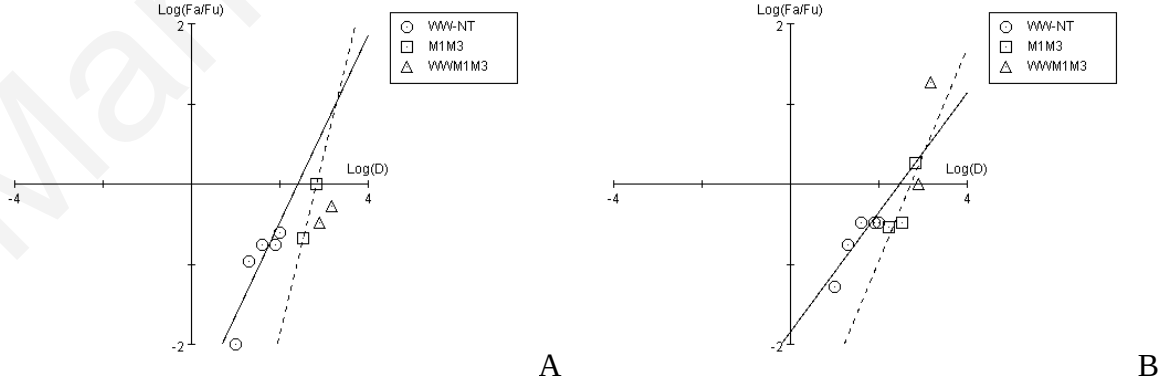


Figure 6.52 Dose-response curves after an exposure time of 24 (A) and 48 (B) h for β-blockers (M_1) and antibiotics (M_3) mixtures in water (M_1M_3) and in wastewater (WWM_1M_3)

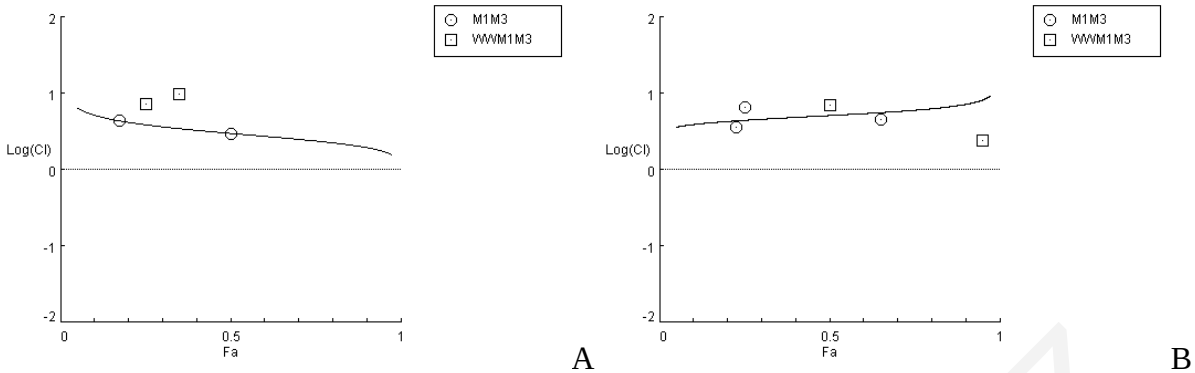


Figure 6.53 Combination index (CI) after an exposure time of 24 (A) and 48 (B) h for β-blockers (M_1) and antibiotics (M_3) mixtures in water (M_1M_3) and in wastewater (WWM_1M_3)

To summarize, it is noted that the effects of the mixtures in wastewater did not match the behaviour of the same mixtures in water. In all cases the mixtures in wastewater had an antagonistic behaviour. An exception however, was the behaviour of the β-blockers in wastewater (WWM_1) that had a synergistic effect at the highest concentrations evaluated (EC_{50} and $EC_{50}/2$ values).

6.3 Discussion

The selected APIs with different modes of action, when present at the MECs and up to 100× MECs were not able to produce acute effects to *V. fischeri* and *D. magna* (data not shown). Therefore the mixture concentrations were set to the sensitivity of the organism by taking into account the EC_{50} value for each API, as already calculated in Chapter 5. The effects of the mixtures differed depending on (i) the endpoint evaluated (growth inhibition of *V. fischeri* and immobilization of *D. magna* after 24 and 48 h exposure time), (ii) the composition of the mixtures (similar and dissimilar mode of action) and (iii) the matrix used (water and wastewater).

The interactions between the components of the mixtures varied according to the relative dose levels and the duration of the exposure time. As a general remark, the effects of the APIs in mixtures, regardless the mode of action, were antagonistic or additive at the low concentration levels (EC_5-EC_{10}) for both *V. fischeri* and *D. magna*. Potentiation or strong synergism was not observed at low concentrations. Toxicokinetic interactions, such as lower

absorption or uptake rates, metabolic interactions, such as metabolic modifications and toxicodynamic interactions that may be created by the biological responses resulting from exposure to the individual APIs, e.g., resulting from similar targets like the ligand-receptor interaction may be some of the reasons for which no strong synergism was observed [340]. It could be argued that the APIs evaluated, in some cases had a dissimilar mode of action and in others had a similar mode of action and behave antagonistically to each other.

The empirical models usually applied for fitting data points in dose-response experiments such as, the *power*, the *logit* and the *probit* have limitations when dealing with more complex mixtures, as explained by Chou [344]. The effects of mixtures can be predicted using the Concentration Addition (CA) and the Independent mode of Action (IA) models. Both concepts make the assumption that the components of a mixture do not interact among them and that the toxicity of the mixture is always higher than the effect of each component of the mixture because every toxicant is considered to contribute to the overall toxicity. They provide a "prediction window" as being the two extreme behaviours of mixture toxicities. The applicability of the CA model in the field of ecotoxicology, where APIs usually act as baseline toxicants was supported in previous studies [35,148,211]. However, as Chou argues it is better to determine synergism or antagonism in a quantitatively, rather than a qualitative way [341]. In the present thesis CA and IA were used as a first screening process (data not shown), which was complemented by the analysis using the Chou-Talalay method [344].

Binary mixtures (non-steroidal anti-inflammatory APIs) were the only ones demonstrating strong synergistic behaviour. This type of behaviour has also been reported in previous studies for other binary mixtures [211,241]. Strong synergism may indicate that (i) the classification of the mode of action of the APIs was erroneous and/or (ii) the interactions between mixture components may lead to mixture effects higher than those estimated by the conceptual models such as, CA and IA models. On the contrary, when a multi-component mixture (more than 2 components) is evaluated, the interactions occurring may weaken the effects of each component [36].

Antagonistic effects were observed for wastewater matrix when applied to *V. fischeri* in binary and quaternary mixtures of fenofibric acid, bezafibrate, gemfibrozil and wastewater [345]. The decrease of toxicity of bezafibrate, clofibric acid, fenofibric acid and gemfibrozil,

for *V. fischeri* and *D. magna* when present in wastewater was also observed by Rosal et al. [346]. This decrease was explained by reduced bioavailability due to interaction with other chemicals in the wastewater and/or with particulate matter. The decrease of the toxicity was higher for the compounds with higher hydrophobicity which is in agreement with the results obtained in this work.

Mixture effects for diclofenac and ibuprofen towards *D. magna* have been previously reported [211]. The same stands true for the effects of the mixtures of β-blockers towards *D. magna* [148]. These previous studies are in accordance with the results reported in this work. However, the previous studies use only the CA and IA models to describe the behaviour of the mixture. The effects of the mixtures, in a quantitative way, as assessed in this work have not been previously reported.

6.4 Conclusions

This work investigated conceptual questions regarding the effects of API mixtures. The hypothesis that the APIs may act additively or synergistically at very low concentrations when present in multi-component mixtures was rejected. Furthermore, in many of the evaluated mixtures antagonism was observed at the lowest concentrations. In general, the toxicity of mixtures decreased when the matrix used was wastewater, indicating less bioavailability and different behaviour when present in real matrices. Binary interactions demonstrated synergistic effects, which were masked or reduced in multi-component mixtures. None of the mixtures indicated any intrinsic risk for acute exposure times to *V. fischeri* and *D. magna*.

CHAPTER 7. ASSESSMENT OF EFFECTS OF TRANSFORMATION PRODUCTS OF ACTIVE PHARMACEUTICAL INGREDIENTS

7.1 Background information

The toxicity and genotoxicity of the mixture of transformation products of APIs formed after photolytic and photocatalytic treatment processes are addressed in this Chapter. A critical review was prepared in the framework of this thesis that identified the importance of transformation products of APIs resulting during photolysis and heterogeneous photocatalysis with regard to their toxicity. Furthermore, their degradation rates, elucidation and biological potency were thoroughly examined [347].

An assessment of the effects of the transformation products of atenolol, propranolol and ofloxacin was performed. These compounds were selected due to their photolability and toxicity effects. For simplicity reasons, the term "photo(cata)lytic" is used instead of "photolytic and photocatalytic" and (bio)transformation instead of "transformation and biotransformation".

7.2 Results

7.2.1 Evaluation of photo-transformation of active pharmaceutical ingredients by DOC monitoring and UPLC-MS/MS analysis

Photo-transformation was performed in a cylindrical immersion-type photoreactor containing ofloxacin solution (20 mg/L), as already described in Chapter 3. Photo(cata)lytic irradiation experiments were applied using a medium pressure mercury vapour lamp. The additional input of TiO_2 was done for the photocatalytic irradiation experiments. At least three photo(cata)lytic experiments were performed.

A first screening of the irradiated samples using UPLC-MS/MS analysis indicated reduced concentrations of ofloxacin as irradiation time increased. However, this was not followed by

an analogous DOC removal, suggesting that photo-transformed molecules were present in the irradiated samples, as shown in Figure 7.1. Experiments were performed in triplicate and average values are presented. Relative standard deviation was never higher than 5%.

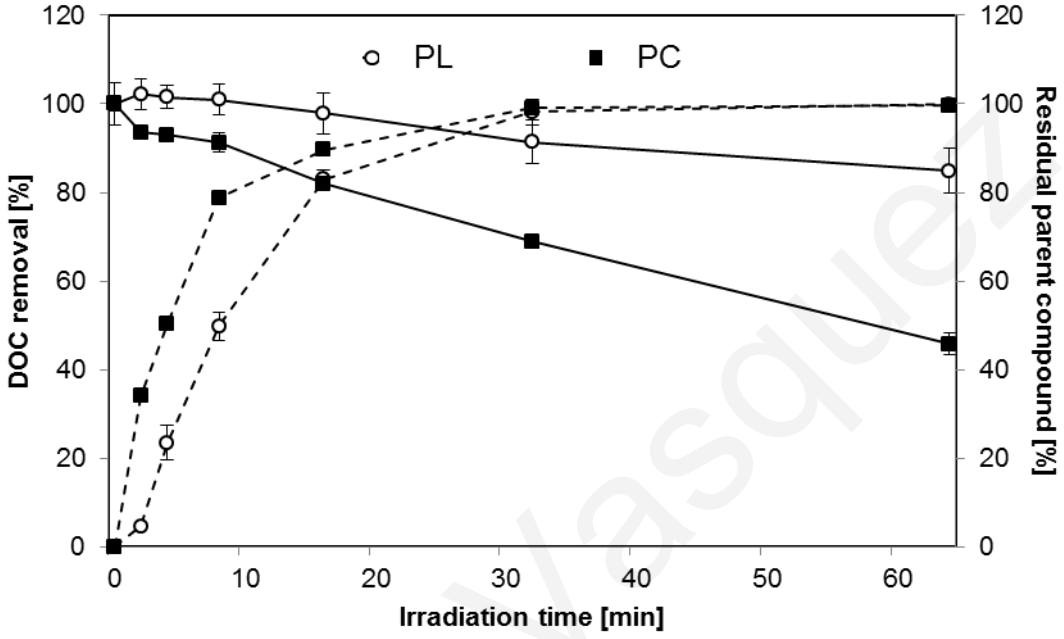


Figure 7.1 DOC removal (dashed line) after photolytic (PL, circle) and photocatalytic (PC, square) treatment versus the transformation percentage (straight line) of ofloxacin as quantified by UPLC-MS/MS analysis

The photo-transformation of ofloxacin by UPLC-MS/MS, both during photolytic and photocatalytic treatment, led almost to 100% transformation of the parent compound after 64 min irradiation. The photocatalytic treatment provoked the same transformation level but with a higher rate. Ofloxacin transformation percentages at 8 min of photolytic and photocatalytic treatment were 49±3% and 78±0% respectively. At 16 min, the values were 83±2% and 90±0%, respectively. Moreover, ofloxacin was not present after 32 min of irradiation during both treatment processes; this was accompanied with a 9±5% and 31±1% DOC removal for photolytic and photocatalytic treatment, respectively. In the same way, after 64 min of irradiation 15±5% and 54±3% of DOC removal was achieved.

Furthermore, linear regression analysis was used for describing the relationship between $\ln C/C_0$ (y) and irradiation time (x) during both treatment processes, as presented in Figure 7.2. Accordingly, the estimating equations were $y = -0.108x + 0.040$ ($R^2 = 0.988$) and $y = -0.087x$ -

0.330 (R^2 = 0.981) for the photolytic and photocatalytic treatment. The slope of the equations constitutes the reaction rate constants (k values), and the results indicate a first-order degradation rate during both processes.

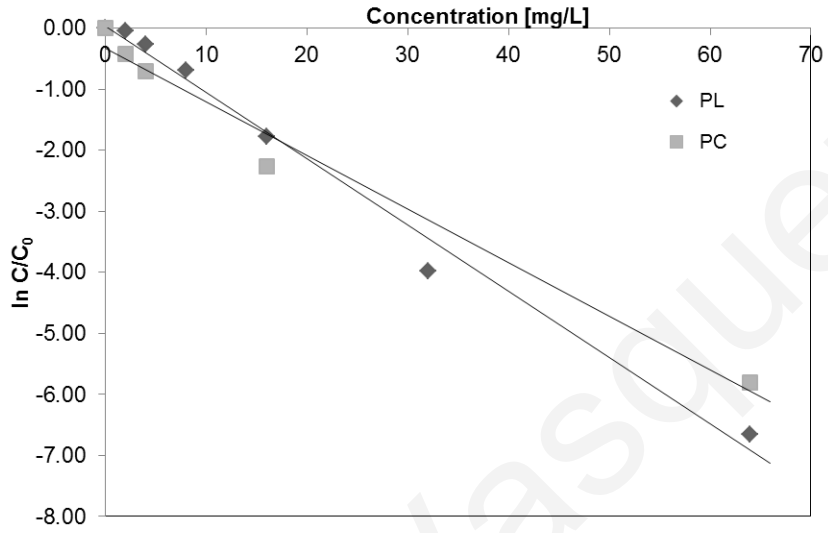


Figure 7.2 Linear regression of the concentration of ofloxacin versus ln C/C_0

During both photolytic and photocatalytic treatment processes the chromatographic peak area of ofloxacin was continuously decreasing as irradiation time increased. The data revealed that during both treatment processes, new peaks (photo-transformation products) appeared with retention times between 3 (PTP1 or PTP1(A)) and 5 (PTP2) min (Figure 7.3).

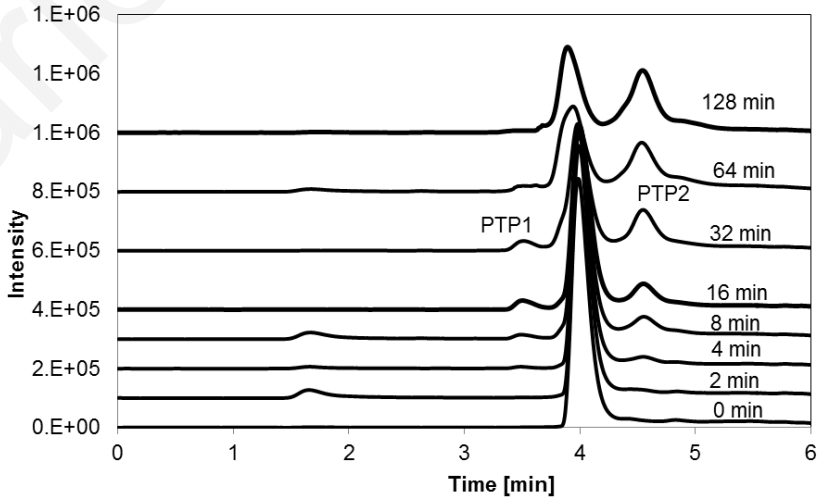


Figure 7.3 The area of the peaks of ofloxacin and the photo-transformation products 1 and 2

In line with the previous remark, the UV–Vis absorption signal of the solutions between 4 and 32 min irradiation presented 3 peaks centered at 3.4, 4 and 4.6 min. Since the analytical column was of reverse phase it can be assumed that PTP1 or PTP1(A) was the most polar (more polar than ofloxacin) as it was eluted first and the PTP2 the least polar as it was eluted after ofloxacin. P1 after 64 min irradiation was not present.

7.2.2 Elucidation of transformation products of active pharmaceutical ingredients

The photo-transformation products of ofloxacin after photo(cata)lytic treatment of two different initial concentrations of ofloxacin (20 and 2.77 mg/L) and the biotransformation products of ofloxacin and its photo(cata)lytically treated solutions were investigated in this Section.

The photo-transformation products formed when the initial concentration of ofloxacin was 20 mg/L, based on the MS peak intensities, are shown in Table 7.1. All detected peaks were higher than 2% of the highest peak intensity, allowing thus precise identification of by-products. Their relative intensities (RI) are provided below.

Table 7.1 Photo-transformation products of ofloxacin formed during photolytic and photocatalytic treatment expressed as m/z values

Photo-transformation products of ofloxacin [M+H$^+$] (m/z values)		
Irradiation time (min)	**Photolytic treatment**	**Photocatalytic treatment**
0	362	362
2	362	362
4	362, 278	362, 348
8	362, 348, 318, 260	362, 348, 318, 278, 260
16	362, 318, 304, 278, 260	362, 318, 277, 260
32	318, 260	318, 260
64	360, 358, 348, 318, 169, 153, <100	358, 318, 169, 153, <100

Underlined numbers indicate common m/z values between photolytic and photocatalytic treatment processes

Nine compounds were tentatively identified as photo-transformation products formed during the photolytic and photocatalytic treatment processes of ofloxacin (*m/z* 362). On the basis of the results presented herein two major pathways were suggested, in which piperazinyl

dealkylation and decarboxylation were described as major transformation mechanisms (Figure 7.4).

Two major analogues of ofloxacin were formed due to the oxidation of the N-piperazine ring (*i.e.*, PTP1 278 m/z, RI=15-40%; PTP1(A) 260 m/z, RI=45-60%). These MS-peaks may be attributed to PTP1. Regarding the PTP1, according to the UV-Vis absorption peak, it increased from 4 until 32 min irradiation, then decreased and finally disappeared.

The species with m/z 260 corresponded to the loss of the fluorine atom at position 6 after the break of piperazine ring. In parallel with the oxidation of the N-piperazine ring, decarboxylation reaction was also demonstrated by the presence of PTP2 (m/z=318, RI=30-100%).

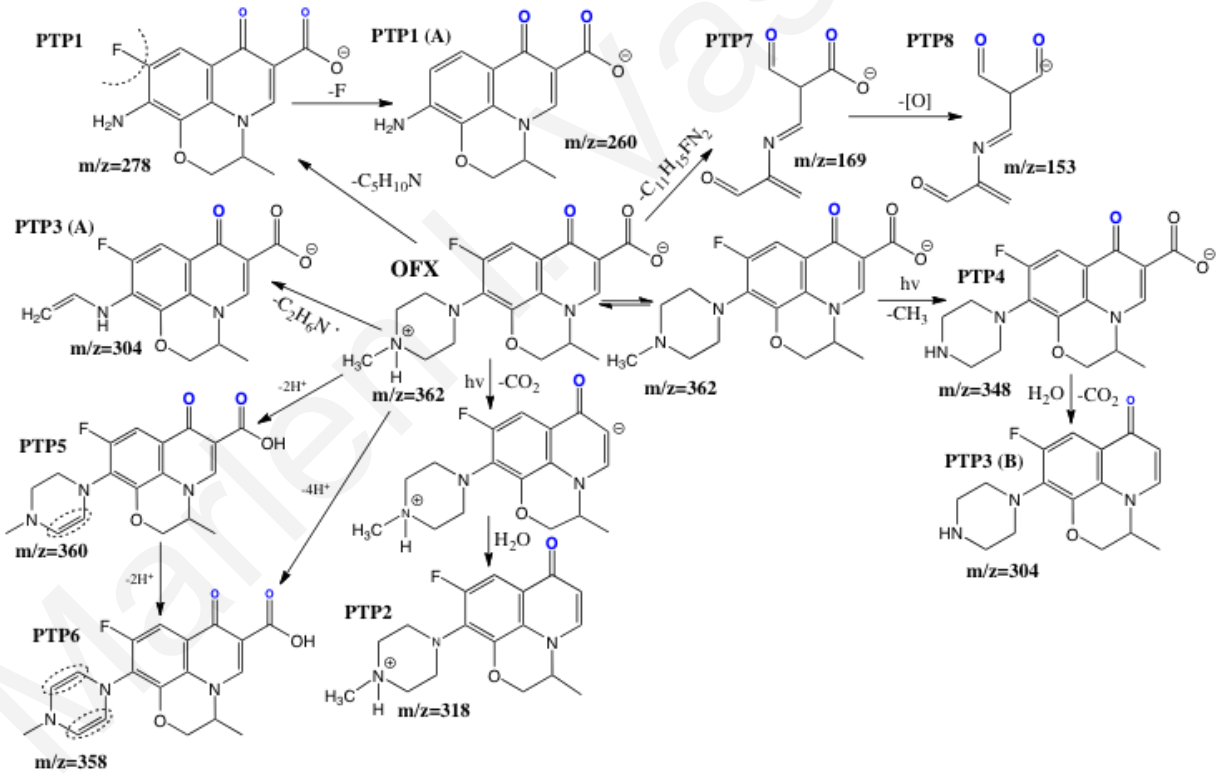


Figure 7.4 Proposed chemical structures of ofloxacin photo-transformation products formed during photo(cata)lytic treatment

Subsequent oxidation treatment could lead either to the formation of another photo-transformation product *i.e.*, PTP3(A) and PTP3(B) with m/z value 304. As shown, the

PTP3(A) and PTP3(B) may be formed after the oxidation of the N-piperazine ring and after CO_2 loss from PTP4, respectively.

The PTP4 with 348 m/z is one of the major transformation products, which may be generated after demethylation of piperazinyl ring. The PTP4 can be regarded as the promoter for the formation of PTP3(B).

PTP5 and PTP6 (*m/z* 360; RI=15-45% and 358; RI=20-70%, respectively) were also formed. These m/z values may indicate the possibility of loss of 2 and 4 hydrogen atoms leading to the formation of 1 and 2 double bonds in the piperazine ring, respectively.

Two additional PTPs with m/z values 169 (PTP7 RI=40-60%) and 153 (PTP8 RI=25-50%) were observed. The 169 m/z ion could be formed from the loss of $C_{11}H_{15}FN_2$, whereas the 153 m/z ion after the removal of an oxygen from 169 m/z (PTP7).

Table 7.2 Photo-transformation products of photolytically and photocatalytically treated ofloxacin solutions expressed as m/z values

	Photo-transformation products of ofloxacin [M+H$^+$] (m/z values)			
Irradiation time (min)	**Photolytic treatment**	**Photolytic treatment+ Closed Bottle test (28th day)**	**Photocatalytic treatment**	**Photocatalytic treatment+ Closed Bottle test (28th day)**
0	362	362, 318	362	362, 318
8	362, 348, 318, 260	n.d.	362, 348, 318, 278, 260	362, 348, 318, 153
16	362, 318, 278, 260, 304	362, 348, 304	362, 318, 277, 260 318, 260	n.d.
32	318, 260	391, 348, 318	358, 318, 169, 153,	<100
64	348, 360, 358, 318, 169, 153, <100	348, 304, 153	<100	<100
128	358, 153, <100	348	<100	<100

n.d.: not determined. The m/z values correspond to the following transformation products (TP): 348 (TP1), 318 (TP2), 304 (TP3(A)), 304 (TP3(B)), 391 (TP4), 278 (TP5), 260 (TP6), 360 (TP7), 358 (TP8), 169 (TP9) and 153 (TP10)

It should be noted that most of the proposed photo-transformation products generated during photolytic treatment maintained the major core of ofloxacin structure. After 64 min of irradiation, all the above described photo-transformation products were further transformed,

giving rise to the formation of species of lower molecular weight (<100 m/z) such as for example carboxylic acids.

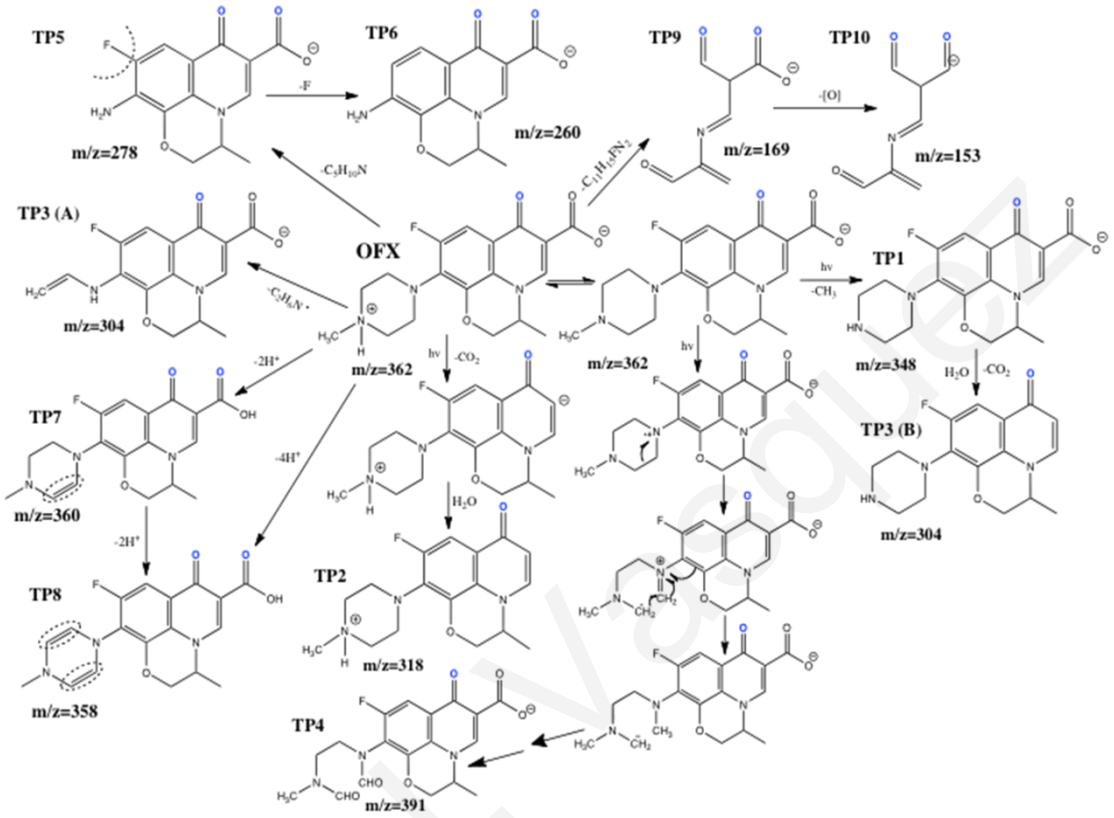


Figure 7.5 Proposed pathways for ofloxacin transformation based on the photo- and biotransformation products identified

The m/z values (Table 7.2) and chemical structures (Figure 7.5) of the photo-transformation products identified after photo(cata)lytic treatment of ofloxacin at 2.77 mg/L at various irradiation times, and the biotransformation products formed after the application of the Closed Bottle test, as identified by UPLC-MS/MS analysis, suggested ring cleavage and/or loss of methyl group of the secondary amine nitrogen of ofloxacin.

Based on the m/z values obtained by UPLC-MS/MS, three major transformation mechanisms are proposed to be followed during both the photo(cata)lytic and bio-transformation processes: demethylation, decarboxylation and opening of piperazinyl ring (Figure 7.5).

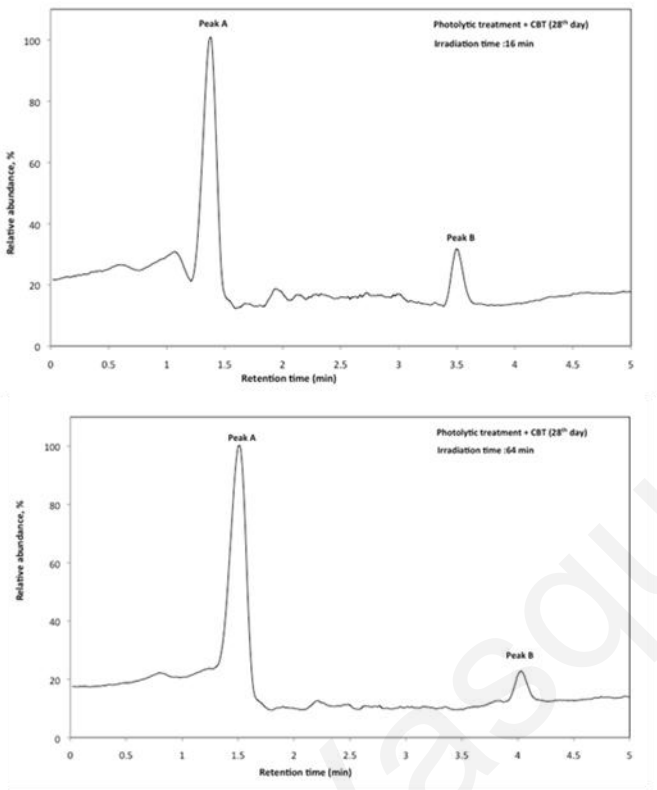


Figure 7.6 Total ion chromatogram of mass spectra for 16 min the photolytic treatment of ofloxacin (A) and 64 min (B) after the application of the Closed Bottle test.

TP1 with 348 m/z (RI=20-60%) may be formed after demethylation of the piperazinyl ring. Subsequent oxidation treatment could lead to the formation of an analogue of ofloxacin (TP2) with m/z=318 (RI=20-70%) which may be formed after decarboxylation.

The formation of TP3(A) (RI=30-50%) with m/z value 304 could be attributed to the opening of the N-piperazine ring. TP3(B) with m/z value 304 (RI=30-50%) may be formed due to decarboxylation of TP1. TP4 product with 391 m/z (RI=15-30%) was observed only after the Closed Bottled test representing a biotransformation product and may result from oxidation of N-piperazinyl ring, giving an aldehyde-derivative.

The total ion chromatogram of mass spectra for the photolytic treatment of ofloxacin for two indicative periods (16 and 64 min) after the application of the Closed Bottle test are presented in Figure 7.6.

The corresponding full-scan mass spectra of peak A and peak B (Figure 7.6 (A)) is presented in Figure 7.7.

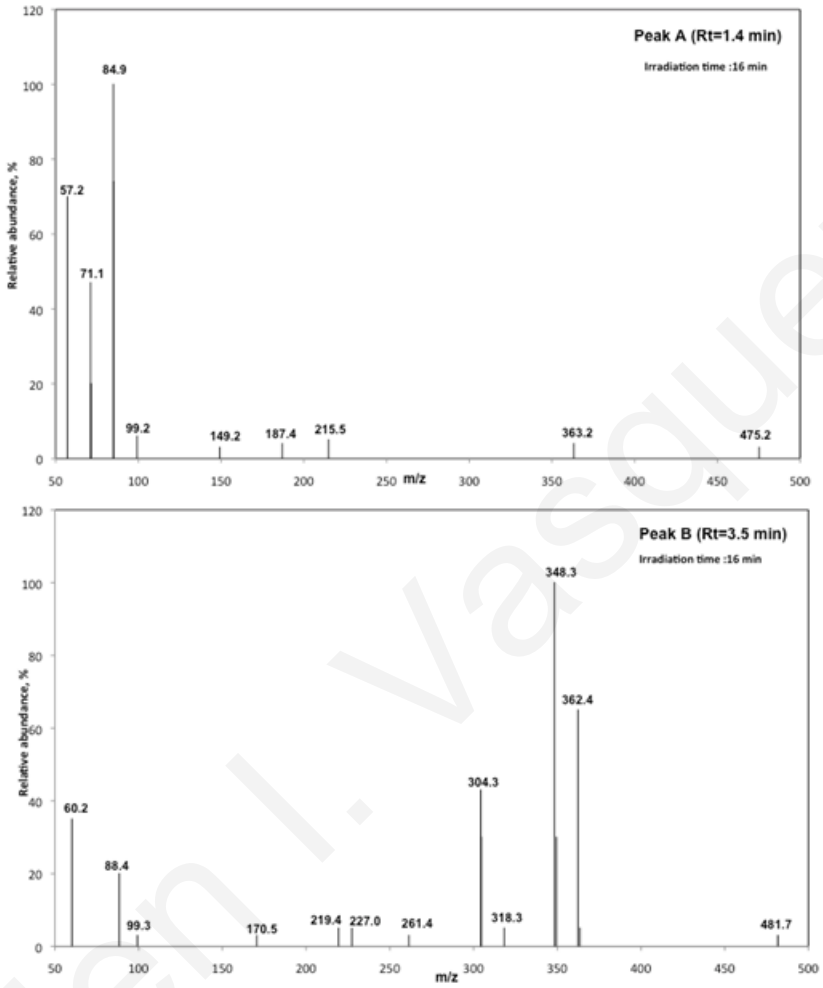


Figure 7.7 Full-scan mass spectra corresponding to peak A (A) and peak B (B) for ofloxacin photolytic treatment of 16 min after the application of the Closed Bottle test

The corresponding full-scan mass spectra of peak A and peak B (Figure 7.6 (B)) is presented in Figure 7.8.

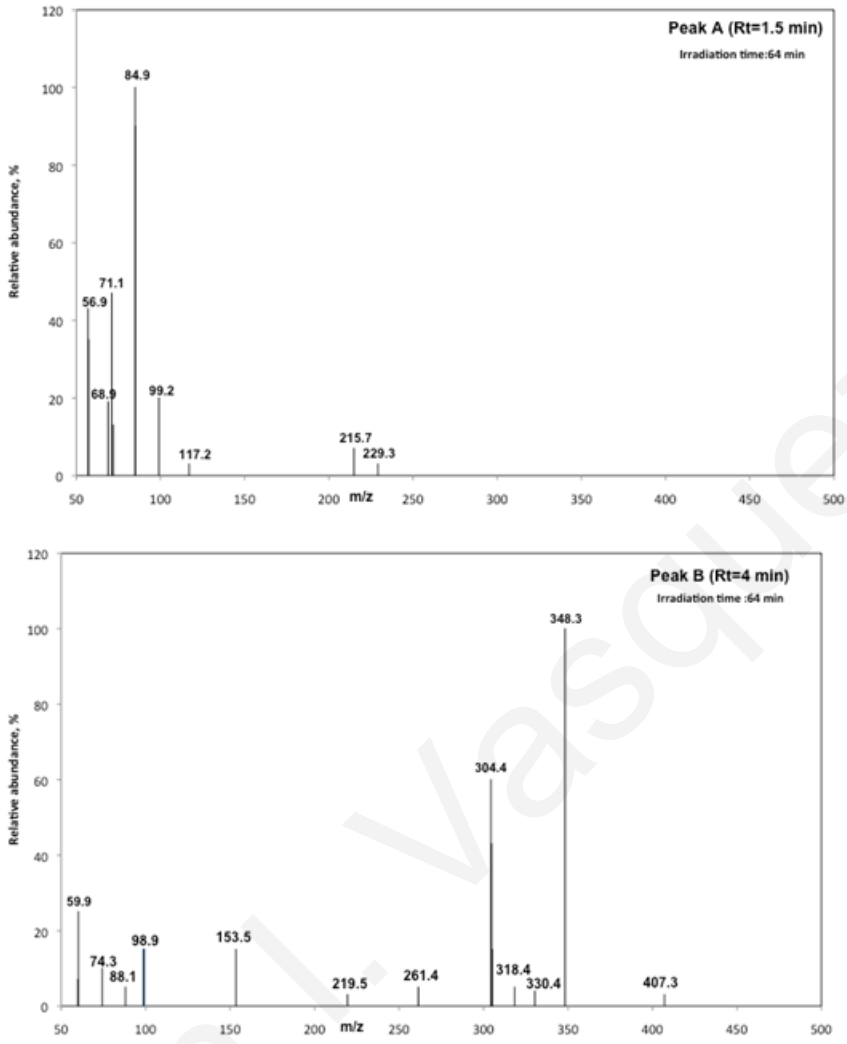


Figure 7.8 Full-scan mass spectra corresponding to peak A (A) and peak B (B) for ofloxacin photolytic treatment of 64 min after the application of the Closed Bottle test

As a summary, the main fragmentation modes of ofloxacin after photo(cata)lytic treatment and the application of the Closed Bottle test are shown in Figure 7.9.

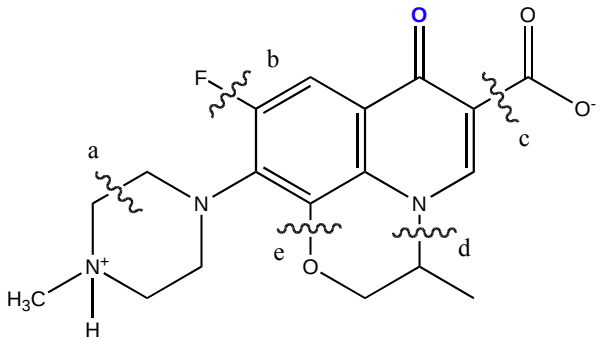


Figure 7.9. Fragmentation modes of ofloxacin

All experiments were performed at pH close to 7, a fact that is quite important, as pH affects the nature of the light-absorbing species in aqueous matrices. Since fluoroquinolones contain two proton-binding sites, pH can affect their photo-transformation [348].

In specific, ofloxacin can be present in three different forms depending on the pH value. As seen in Figure 7.10, irradiation of the zwitterionic species lead to a decarboxylation procedure, followed by protonation [349,350].

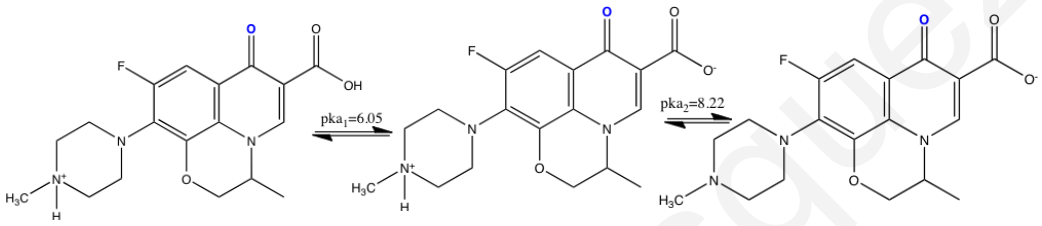


Figure 7.10. Prototropic equilibria of ofloxacin

As it is known, under neutral conditions no alkyloamino side chain cleavage took place. Additionally, cleavage products TP9 and TP10 were detected at photo(cata)lytic treatment and remained until the end of the treatment. TP9 with m/z 169 could be formed from the loss of $C_{11}H_{15}FN_2$ and the TP10 with m/z value 153 after the removal of an oxygen atom. Moreover, products with molecular mass lower than 100 Da such as, small-chain carboxylic acids were observed during all treated solutions.

7.2.3 Assessment of effects of photo(cata)lytically treated active pharmaceutical ingredients' solutions

The assessment of the effects was performed to photo(cata)lytically treated APIs solutions of various irradiation times in order to evaluate the efficiency of these processes, not only by the removal of the parent compounds, but also by investigations on the potential adverse effects to biological systems of the various by-products formed due to the partial oxidation. It should be noted that the assessment was performed to the mixture of the photo-transformation products formed at different irradiation times as a whole, and not to individual photo-transformation products, as these compounds in some cases are not known and when they are, commercially available standards are not available. In any way, the purpose of the thesis was

to assess the treatment processes' efficiency and therefore, evaluating the effects of the treated solution as such, was considered quite important.

7.2.3.1 *Vibrio fischeri*

The chronic growth inhibition for *V. fischeri*, as means of optical density (OD), is presented in Figure 7.11.

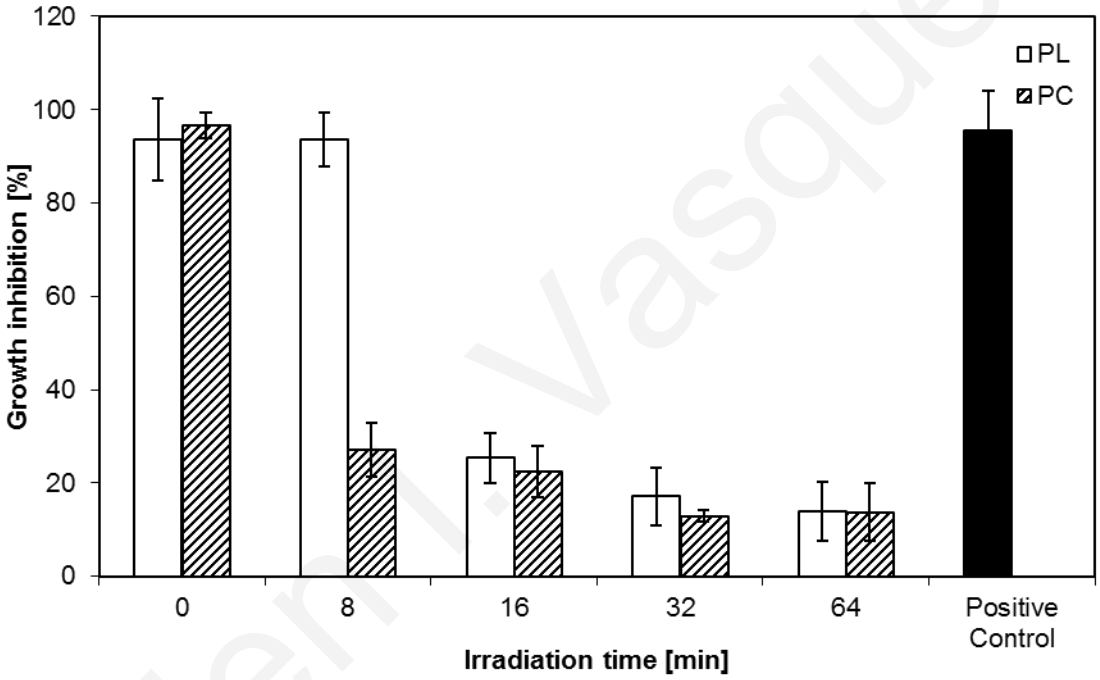


Figure 7.11 Percentage of growth inhibition for *Vibrio fischeri* 24 h exposure time to ofloxacin during photo(cata)lytic treatment processes

The untreated samples were found to be toxic (15 μg/L). During photolytic treatment, irradiation for 8 min did not cause any significant reduction (93±6% inhibition). At 16 min, a significant reduction of the growth inhibition was observed as the value dropped to 25±5%. A further increase of the irradiation time to 32, 64 and 128 min caused a small decrease in the growth inhibition with values ranging at 17±6%.

During photocatalytic treatment, a significant decrease of the growth inhibition effect was observed even after 8 min of irradiation time (27±6% of inhibition). A further increase in the irradiation time to 16 min, did not lead to any significant reduction in the inhibition. At 32 and

64 min a decrease to 13±6% was observed. The positive control caused 95±8% inhibition, whereas the negative control tests, showed no inhibition to the growth of *V. fischeri* (4±3%) (data not shown).

The inhibition results for *V. fischeri* when the bioluminescence induction was the endpoint monitored did not follow the same trend as previously described (Figure 7.12).

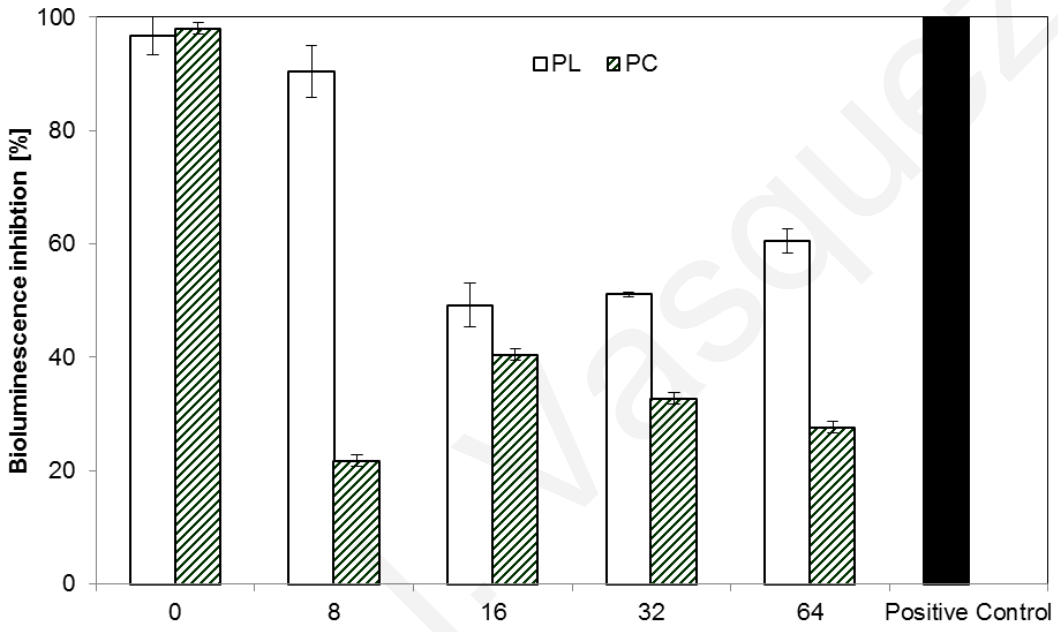


Figure 7.12 Percentage of bioluminescence inhibition for *Vibrio fischeri*, 24 h exposure time to ofloxacin during photo(cata)lytic treatment processes

The untreated samples caused an inhibition of 90±5%. During the photolytic treatment, irradiation for 16, 32 and 64 min caused an inhibition of 51±10%. Irradiation of the samples for 128 min was needed to reduce the toxicity to *V. fischeri* to 19±3%.

The photocatalytic treatment, on the contrary, caused a significant decrease of the bioluminescence inhibition at 8 min with a value of 21±7%. At 16 min of irradiation a significant increase of the bioluminescence inhibition followed by a decrease at 32 and 64 min was observed with values 40±9%, 33±4% and 28±4%, respectively.

The inhibition values for *V. fischeri* and/or its log-transformed values from both endpoints examined were poorly correlated to the concentration of ofloxacin using linear regression,

suggesting that additional factors involved e.g., presence of toxic transformation products had an impact on the inhibition pattern observed.

7.2.3.2 *Pseudomonas putida*

The growth inhibition for *P. putida* to ofloxacin samples after photo(cata)lytic treatment is shown in Figure 7.13.

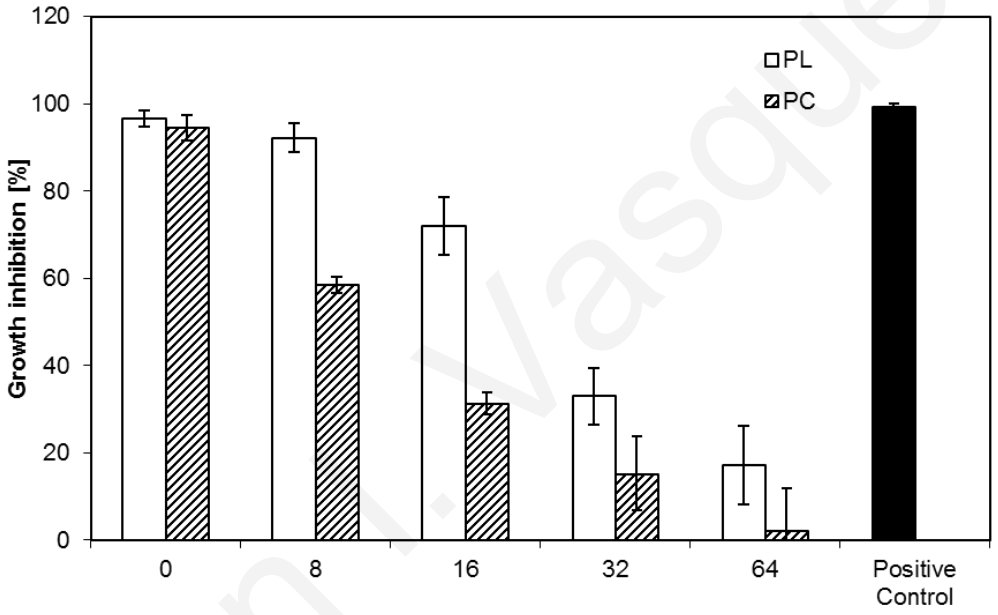


Figure 7.13 Percentage of growth inhibition for *Pseudomonas putida*, 16 h exposure time to ofloxacin during photo(cata)lytic treatment processes

The untreated samples (400 µg/L) were highly toxic to *P. putida* causing a growth inhibition greater than 94±2.9%. During the photolytic treatment, a decrease of the inhibitory effect was observed after 16 min and 32 min of irradiation with values of 72±7% and 33±7%, respectively. When the irradiation time increased to 64 min, the growth inhibition was 17±9%. The same inhibition was observed even when the irradiation time was increased to 128 min (17±8%) (data not shown). The growth inhibition in *P. putida* could be modelled during the photolytic treatment by the equation: $I = 18.78 + 29.6 \times \log C$ ($R^2 = 0.96$).

During the photocatalytic treatment, irradiation for 8 min caused a growth inhibition of 58±2%. After 16 min and 32 min of irradiation, the inhibition decreased to 31±2% and

15±8%. At the end of the experiment (64 min), the growth inhibition was negligible (2±9%). The growth inhibition in *P. putida* during photocatalytic treatment could be explained by the equation: $I = 11.15 + 0.216 \times C$ ($R^2 = 0.97$). The positive control experiments caused 99±1% growth inhibition, whereas the negative control experiments showed no inhibitory effect (1±2%) for *P. putida* (data not shown).

7.2.3.3 *Daphnia magna*

Ofloxacin and atenolol solutions (10 mg/L) were subjected to photocatalytic treatment with the optimum concentrations of 500 and 150 mg/L TiO_2, respectively, and then evaluated for their toxicity, whose evolution, alongside substrate concentration, is shown in Figure 7.14. The methodology used for the photo(cata)lytic treatment of atenolol and propranolol is provided by Hapeshi et al. [278].

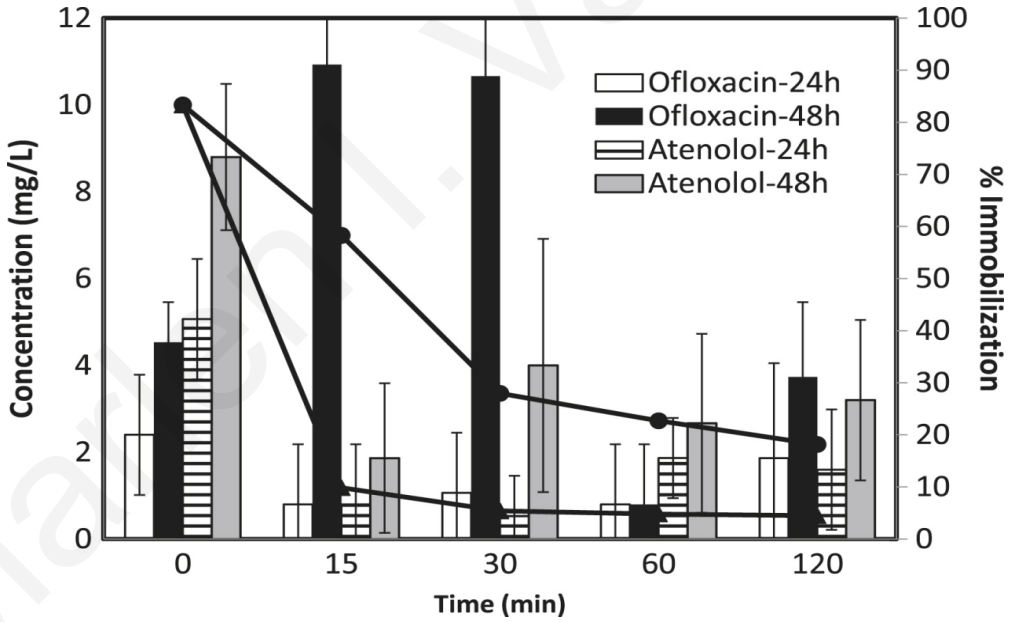


Figure 7.14 Atenolol and ofloxacin photocatalytically treated sample toxicity (bars) and concentration of the substrate (lines)

Short photocatalytic treatment of 15-30 min was capable of reducing substantially the atenolol toxicity and this (i) was associated with the fact that the residual atenolol concentration of about 4 mg/L was relatively non-toxic and (ii) implied that reaction transformation products were not toxic to *D. magna*.

Ofloxacin decomposes rapidly to transformation products, whose 24 h toxicity was low throughout the experiment. Interestingly, its early reaction intermediates induced considerable 48 h toxicity, which was though eliminated upon prolonged treatment. Overall, photocatalytic treatment of ofloxacin and atenolol at the conditions in question can reduce sample toxicity to values with no statistically significant difference from the control ones. Longer irradiation periods did not reduce the toxicity significantly.

7.2.3.4 *Pseudokirchneriella subcapitata*

The effects of ofloxacin solution (20 mg/L) after photo(cata)lytic treatment processes with TiO_2 (1 g/L) demonstrated high toxicity to untreated samples as shown in Figure 7.15.

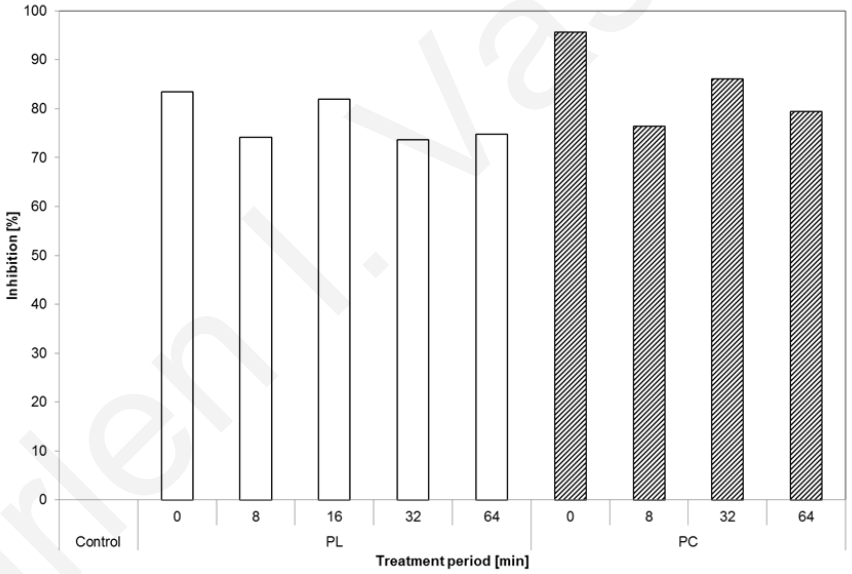


Figure 7.15 Inhibition percentage of ofloxacin solution after photo(cata)lytic treatment processes

Both the photolytic and photocatalytic treatment processes were not able to substantially reduce the toxicity of ofloxacin and its transformation products formed during both processes. The inhibition values were greater than 70% in all treatment periods evaluated, indicating the formation of potent transformation products.

7.2.4 Biological persistence of photo-transformation products of ofloxacin

No biodegradation was practically observed for ofloxacin and its treated samples after photo(cata)lytic treatment processes during the 28 d of exposure time, as shown in Table 7.3.

In specific, the biodegradation percentage of photolytically treated samples ranged from -1 to 8% and -2 to 9% for 14 and 28 d, respectively. Similarly, biodegradation percentage of photocatalytically treated samples ranged from 0 to 6% and 0 to 7% for 14 and 28 d, respectively.

Table 7.3 Biodegradation achieved by the Closed Bottle test

Irradiation time (min)	Biodegradation of test suspension series (%)		Biodegradation of toxicity control series (%)		Transformation of ofloxacin** (%)	
	14 d	28 d	14 d	28 d	0 d	28 d
Photolytic treatment						
0	-1	-2	27	28	0	0
16	2	1	54*	59*	81	82
32	5	5	72*	68*	95	99
64	7	9	72*	68*	>99	>99
128	8	9	42*	45*	>99	>99
Photocatalytic treatment						
0	2	2	28	30	0	0
8	0	0	27	27	78	82
32	6	7	38*	38	99	99
64	6	7	38*	40*	>99	>99

* Calculated values are greater tha predicted ones, based on the percentage of biodegradability of the test solutions and sodium acetate alone. The (-) values are due to consumption of oxygen in the control$_1$

It can be observed that during photolytic treatment, the biodegradation percentage ranged from -10 to +10%, while during photocatalytic treatment from -5 to +10%. These values demonstrate that neither ofloxacin nor the treated samples containing the transformation products for the irradiation periods tested and the conditions applied were readily biodegradable.

It can be assumed that no abiotic transformation took place as the removal of ofloxacin as quantified by UPLC-MS/MS did not change after the 28 d exposure time at 0 min photo(cata)lytically treated samples.

The transformation of the parent compound was monitored by UPLC-MS/MS analysis. After 16 min of photolytic treatment, 74%, and after 8 min of photocatalytic treatment, 58% of ofloxacin was transformed, while mineralization was very low and in specific 2.1% and 6.3%, respectively, based on the NPOC removal (data not shown).

Even though formation of more biodegradable transformation products seems to take place during photo(cata)lytic treatment processes, the difference observed in the values have no statistical significance if the accuracy of the test system is taken into account (±5% variation).

The biodegradation of photo(cata)lytic treated solutions remained at values close to 0. In the toxicity control series, the values were slightly higher than 25% (27% in photolytic and 34% in photocatalytic treatment).

It is worthnoting that the biodegradability of the samples obtained after 16 and 32 min of photolytic treatment, was higher than the levels of the quality control series; hence the presence of sodium acetate probably facilitated the biodegradation of ofloxacin and its treated solutions.

An explanation may be that a type of co-metabolism takes place, during which the biodegradation of ofloxacin and its treated samples depends on the presence of sodium acetate. Another explanation could be that in the presence of sodium acetate, bacteria able to degrade some of the photolytic products grow faster, firstly by feeding on the sodium acetate and after having reached a critical mass, start feeding on the transformation products.

This observation was not made in the samples obtained after the photocatalytic treatment, suggesting that the treated solutions at 8 and 32 min contained less biodegradable products. It should be mentioned that during the negative $control_2$ test, the biodegradability values remained close to zero demonstrating that no biodegradability took place. Furthermore, in the toxicity control series the values approached the expected ones, based on the percentage of biodegradability of negative $control_2$ and sodium acetate alone; hence the possibly TiO_2 particles present after filtration were considered as non-toxic to the inoculum.

A detailed presentation of the biodegradation percentage, as recorded daily, is presented in Figure 7.16-7.19. The diagrams represent the mean values of two independent experiments.

Each experiment was performed in duplicates and the SD was never higher than 5%. The irradiation periods to be examined were definitized taking into account the DOC removal and after performing some preliminary tests. As a general remark, it can be observed that during photolytic treatment the biodegradation percentage ranged from -10 to +10%, while during photocatalytic treatment from -5 to +10%. These values demonstrate that neither ofloxacin nor its transformation products formed during the experiments performed and the conditions applied are readily biodegradable for the irradiation periods tested.

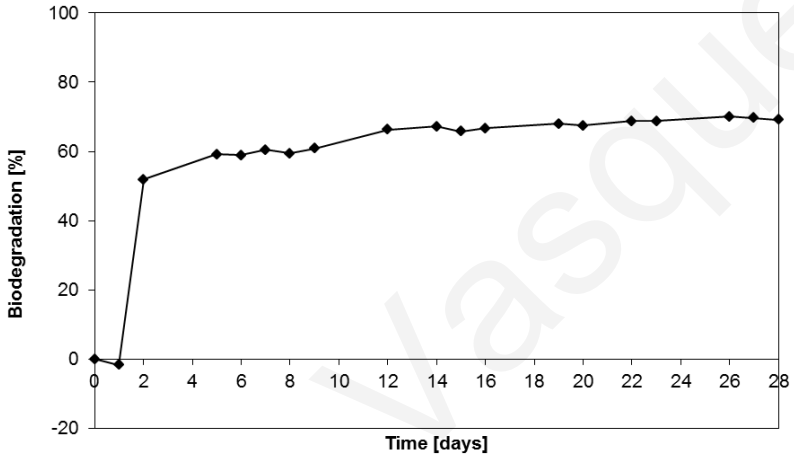


Figure 7.16 Biodegradation percentage in the quality control test

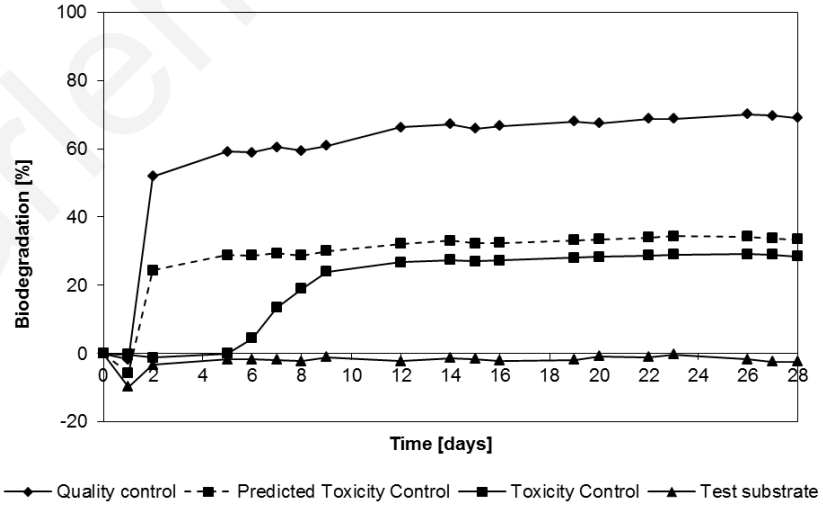


Figure 7.17 Biodegradation rate of ofloxacin after photolytic irradiation of 0 min

During the quality control test biodegradation greater than 60% was observed, meeting the quality criteria, as mentioned in the Chapter 3. An increased biodegradation rate was determined from day 2 (52%) ensuring that the microorganisms were active during the test period. The 60% biodegradation was achieved at day 7, as shown in Figure 7.17.

Ofloxacin is considered as non-readily biodegradable as already mentioned (Figure 7.17). The biodegradation rate was close to zero values during the 28 d exposure time. It was observed that a slower percentage of biodegradability than that predicted, based on the percentage of biodegradability of the substrate and sodium acetate alone, was observed on the toxicity control for the first 5 d of the experiment. This underlines that ofloxacin is slightly toxic to the microorganisms at the concentrations tested and a delay to get adapted to ofloxacin is required. The microorganisms were not killed as the biodegradation rate in the toxicity control test increased after the first 5 d of the experiment to values close to the predicted ones.

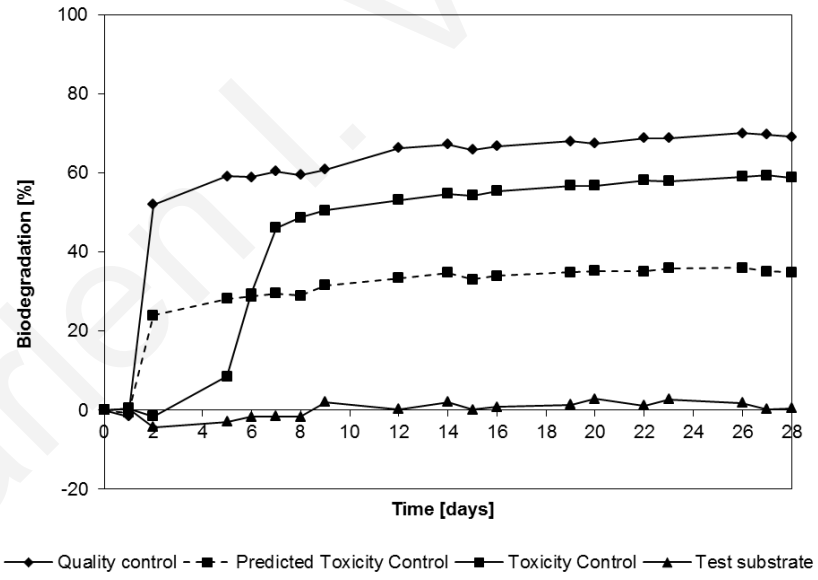


Figure 7.18 Biodegradation rate of ofloxacin and its transformation products after photolytic irradiation of 16 min

Ofloxacin and its transformation products created after a photolytic irradiation of 16 min are considered as non-readily biodegradable (Figure 7.18). However, in the toxicity control test, after an exposure time of 7 d the biodegradation rate increased to 46%. The predicted

value for this exposure was approximately 30%. By the end of the exposure time the biodegradation reached 59%; whereas the predicted value was 35%.

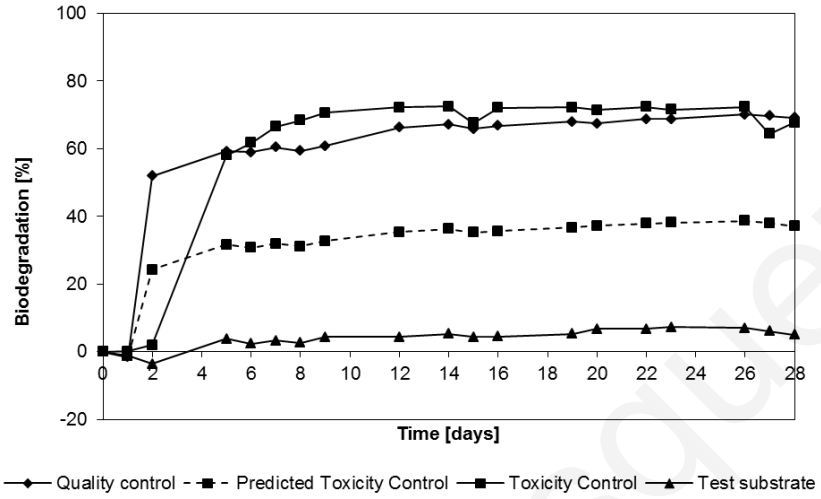


Figure 7.19 Biodegradation rate of the transformation products of ofloxacin after photolytic irradiation of 32 min

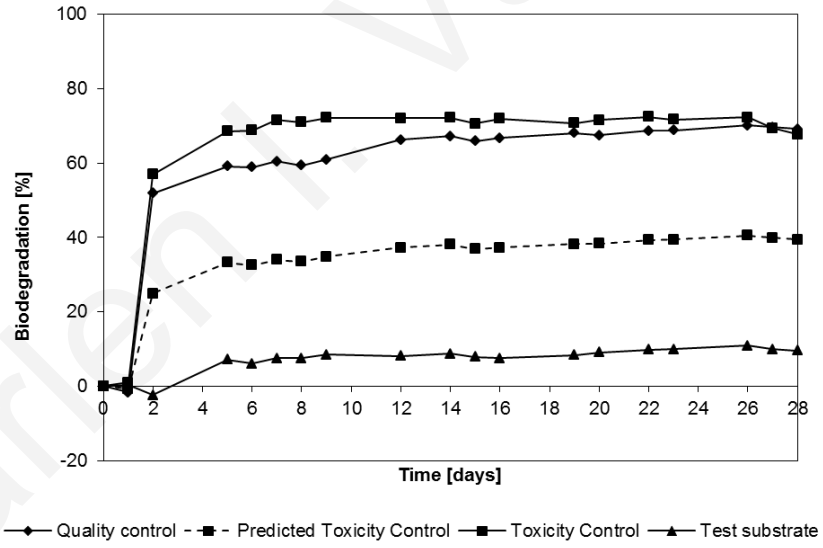


Figure 7.20 Biodegradation rate of the transformation products of ofloxacin after photolytic irradiation of 64 min

The biodegradability rate of the transformation products of ofloxacin after a photolytic irradiation time of 32 min is presented in Figure 7.19. The transformation products present are considered as non-readily biodegradable. It should be mentioned that as ofloxacin removal,

quantified by chromatographic analysis, was 99% (Table 7.3), the biodegradation rate investigated refers mainly to the transformation products and not the parent compound.

The biodegradability rate of transformation products of ofloxacin after 64 min irradiation time is shown in Figure 7.20. The transformation products achieved a biodegradability of 10%. However, they are still considered as non-readily biodegradable. The behaviour of the biodegradability in the toxicity control is comparable to the one observed at 32 min. Even at day 2, the biodegradation was higher than the one of the quality control experiment. The values were maintained high for all the experiment's period.

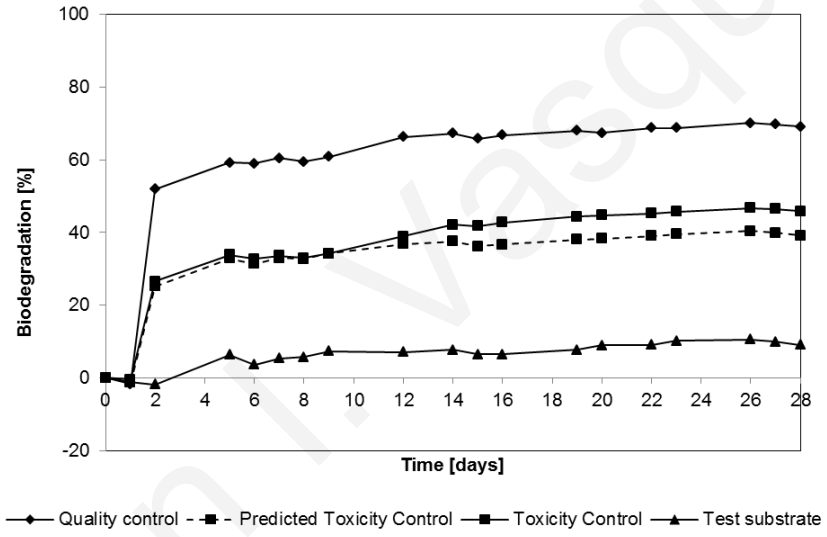


Figure 7.21 Biodegradation rate of the transformation products of ofloxacin after photolytic irradiation of 128 min

The biodegradation rate of the transformation products of ofloxacin were maintained at approximately 10% (Figure 7.21), as for the 64 min photolytic treatment. The biodegradation of the toxicity control was higher but close to the predicted values. Approximately 47% biodegradation was observed at the end of the experiment.

During the negative control$_2$ test, in which Milli-Q water with TiO_2 at 1g/L was irradiated with UV for 64 min and filtered, the biodegradability values remained close to zero and in the toxicity control series of the negative control$_2$ the values approached the predicted ones,

demonstrating that no biodegradability took place and the possible TiO_2 particles present after filtration were not toxic to the inoculum (Figure 7.22).

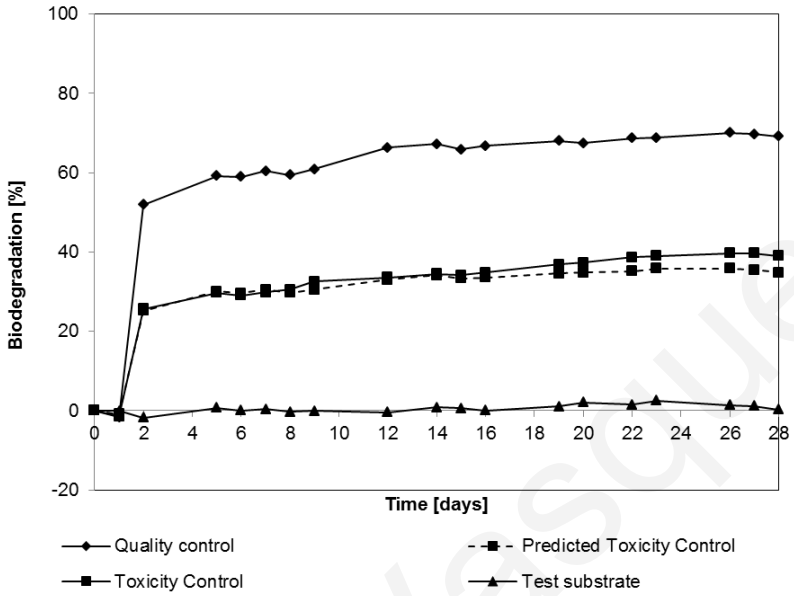


Figure 7.22 Biodegradation rate of negative control$_2$

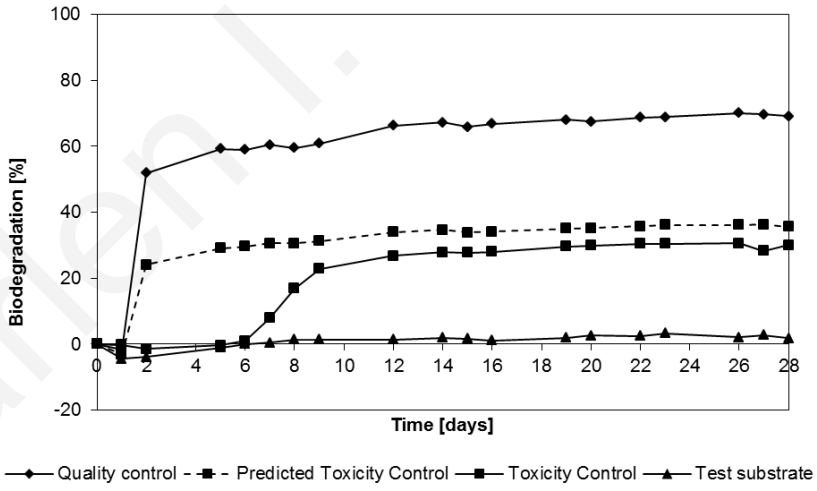


Figure 7.23 Biodegradation rate of ofloxacin after photocatalytic irradiation of 0 min

The biodegradation rate for ofloxacin at the beginning of the photocatalytic treatment (Figure 7.23) is the same as the biodegradation rate for ofloxacin at the beginning of the photolytic treatment (Figure 7.17).

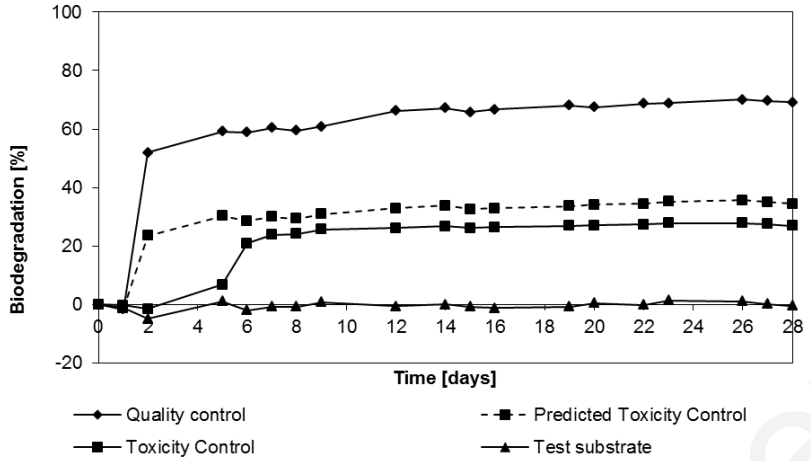


Figure 7.24 Biodegradation rate of ofloxacin and its transformation products after photocatalytic irradiation of 8 min

The biodegradation rate of ofloxacin and its transformation after an irradiation period of 8 min are considered as non-readily biodegradable as the values were maintained close to zero (Figure 7.24). In the toxicity control experiment a delay on the biodegradation was observed for the first 6 d and then the biodegradation approached predicted values, suggesting that no toxic by-products were present.

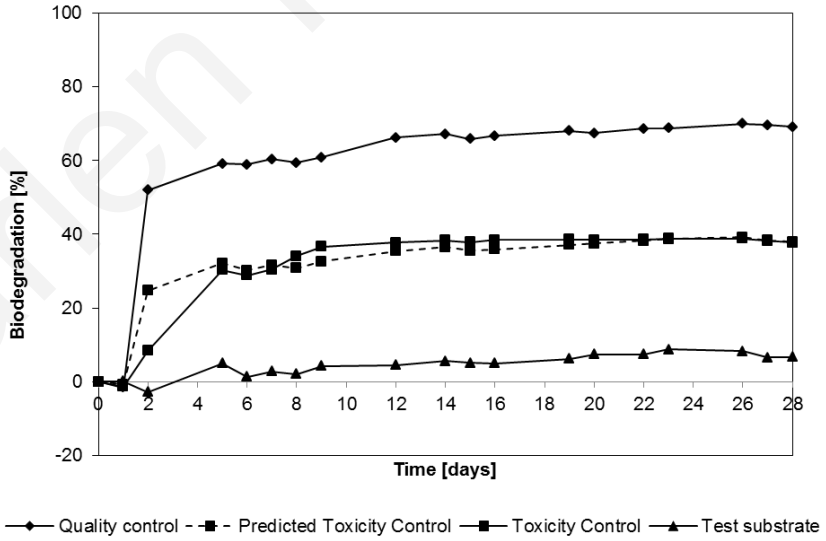


Figure 7.25 Biodegradation rate of ofloxacin and its transformation products after photocatalytic irradiation of 32 min

The biodegradation rate of ofloxacin and its transformation products are again considered as non-readily biodegradable after 32 min photocatalytic treatment. At the end of the exposure time approximately 7% biodegradation was calculated. The toxicity control was higher but close to the predicted toxicity control values (Figure 7.25).

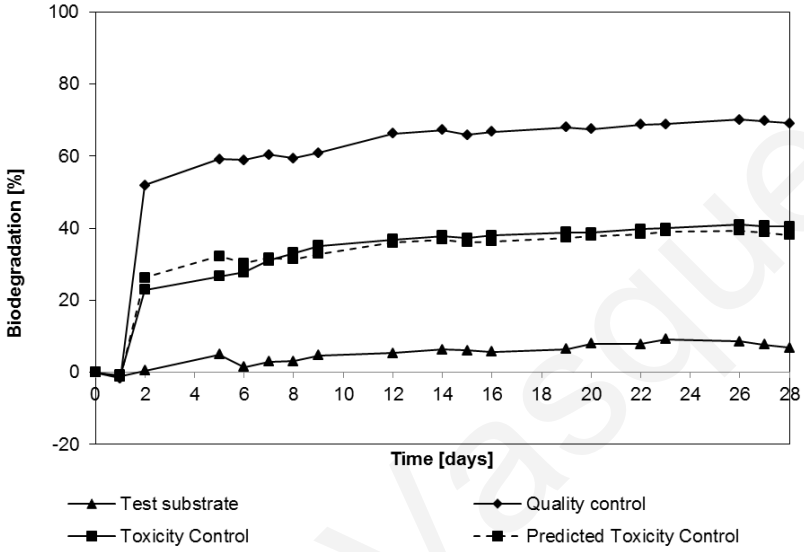


Figure 7.26 Biodegradation rate of ofloxacin and its transformation products after photocatalytic irradiation of 64 min

The biodegradation rate of the transformation products of ofloxacin are also considered as non-readily biodegradable after 64 min photocatalytic treatment. At the end of the exposure time approximately 7% biodegradation was calculated (Figure 7.26).

To summarize, the biodegradation of ofloxacin remained at values close to 0 during both photo(cata)lytic treatment. In the toxicity control series the values were higher than 25%, hence meeting the validity criteria for the test. However, the values in the toxicity control were close to that value, 27% in photolytic and 34% in photocatalytic treatment, indicating a marginal toxicity for the bacteria.

It was observed that a lower percentage of biodegradability and a slower biodegradation rate than the ones predicted, based on the percentage of biodegradability of the substrate and sodium acetate alone, were observed on the toxicity control series at 0 and 16 min of photolytic and 0 and 8 min of photocatalytic treatment for the first 5 d of the experiment. This

underlines that ofloxacin was slightly toxic to the microorganisms at the concentrations tested and therefore its biodegradation was poor.

Furthermore, it is worthnoting that, within the toxicity control series of the photolytic treatment, after the delay noted for the first 5 d of the experiment, the biodegradability increased significantly as the irradiation time of the samples increased suggesting that the microbes were not killed by ofloxacin and its transformation products. The microbes had a delay in adapting to the substrate.

The biodegradability percentages of the substrates even reached the percentages of the quality control series; hence the presence of sodium acetate probably facilitated the biodegradation of ofloxacin and its transformation products formed during photolytic treatment. It can be suggested that a type of co-metabolism took place, in which the biotransformation of ofloxacin and its transformation products are depended on the presence of sodium acetate. This phenomenon was not so obvious in the samples after photocatalytic treatment, suggesting that either less biodegradable transformation products and/or biotransformation products are formed.

The transformation products were found to be non-readily biodegradable, probably due to the presence of compounds containing fluorine which have higher toxicity than defluorinated products.

The slight increase of biodegradation percentage observed after 64 min irradiation in photolytically treated samples may be attributed to the presence of decarboxylated products; whereas in photocatalytically treated samples (after 32 and 64 min irradiation), this increase may be due to the formation of products with a molecular mass lower than 100 Da. The presence of decarboxylated products was not coupled by an increase of the observed biodegradation percentage, even though they are considered to have lower antimicrobial activity [351].

Emphasis should be given on the co-metabolism observed, as studies on co-metabolism of APIs are still limited. Co-metabolism has been proved to be one of the major removal mechanisms in activated sludge treatment plants for ibuprofen and naproxen when milk

powder was used as co-substrate [352]. *Rhodococcus rhodochrous* bacteria were also proved to co-metabolize carbamazepine, sulfamethizole and sulfamethoxazole [243,353].

However, co-metabolism in the presence of sodium acetate is still not fully understood. Alexy et al. [243] (2004) found sodium acetate to enhance the biodegradation of trimethoprim, benzylpenicillin and sulfamethoxazole, while in the study by Lahti and Oikari [28] the biodegradation of diclofenac, naproxen and bisoprolol was not enhanced by sodium acetate, meaning that co-metabolism can be substrate specific.

Another interesting example of co-metabolism was studied for sulfamethoxazole, where it could serve both as a carbon or a nitrogen source, depending on the co-substrate. In combination with acetate, sulfamethoxazole behaved as a source of nitrogen, whereas when ammonium was added, sulfamethoxazole remained intact [56,115].

7.2.5 Assessment of effects of photo-transformation products of ofloxacin using the cytokinesis-block micronucleus cytome assay

In order to assess the effects of ofloxacin transformation products after the photo(cata)lytic treatment, a prerequisite was to assess the effects of ofloxacin as parent compound to the cytostasis and the genotoxicity of the system. The average effect of various concentrations of ofloxacin (0.1-100 μM ~ 0.3614-3614 mg/L) compared to the average effect of a solvent and a positive control to the HepG2 cells is shown in Figure 7.27. The positive control (PC) used was EMS at 400 μM and the values presented are the average of three independent experiments with their standard deviations. The statistical significance at the 0.05 probability level was evaluated using an ANOVA analysis and post hoc comparisons **$p<0.05$ versus solvent control (SC, white bar).

The cytostasis as a percentage and the mean proliferation index (NDI) are shown in Figure 7.27. (A). The cytostasis of the solvent control (SC) was 0.0±0.8% and the NDI 2.00±0.02. These values were not statistically different from those obtained for 0.1 μM ofloxacin (cytostasis: 1.4±1.3% and NDI 1.99±0.01). However, further concentration increase was coupled with an increase of the cytostasis with values from 5.8±2.3 to 9.2±2.2% and a

decrease of the NDI with values from 1.95±0.01 to 1.92±0.01, when cells were exposed from 1 to 30 μM ofloxacin, respectively. Both the cytostasis and NDI observed at 100 μM were statistically ($p<0.05$) different to the responses for 1-30 μM, with values of 17.8±1.0% and 1.83±0.01, respectively. The positive control demonstrated a cytostasis of 36.9±1.6% and a NDI of 1.64±0.01.

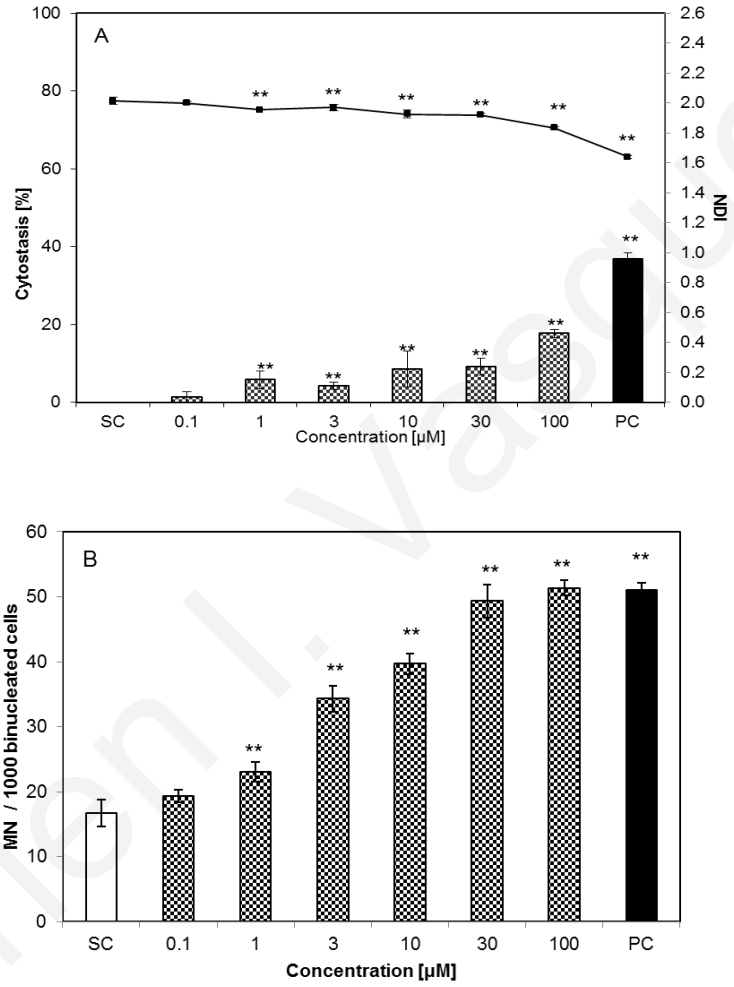


Figure 7.27 (A) Cytostasis (bars), Nuclear Division Index (line) and (B) micronuclei induction to hepatoma cell line (HepG2) from ofloxacin solutions at different concentrations in μM

Regarding the MNi induction, a concentration-dependent increase was observed, as presented in Figure 7.27 B. The average frequency of spontaneous MNi formation (SC) was 16.67±2.08 MNi/1000 BN. A significant ($p<0.05$) difference compared to the SC experiment was observed when HepG2 cells were exposed to 1-100 μM ofloxacin with values ranging between 23.00±2.00-51.33±1.16. The MNi induction of ofloxacin at concentrations higher

than 30 μM ofloxacin was found not to be statistically different to the one of the PC which was 51.00±1.00.

The cytostasis, proliferation index and MNi induction of ofloxacin during photo(cata)lytic treatment can be seen in Figure 7.28. As already mentioned, the initial concentration of ofloxacin was 1.1 μM (equivalent to 397 μg/L) at the beginning of both treatment processes. The cytostasis and NDI were not statistically different from the SC during both treatment processes, as shown in Figure 7.28 (A).

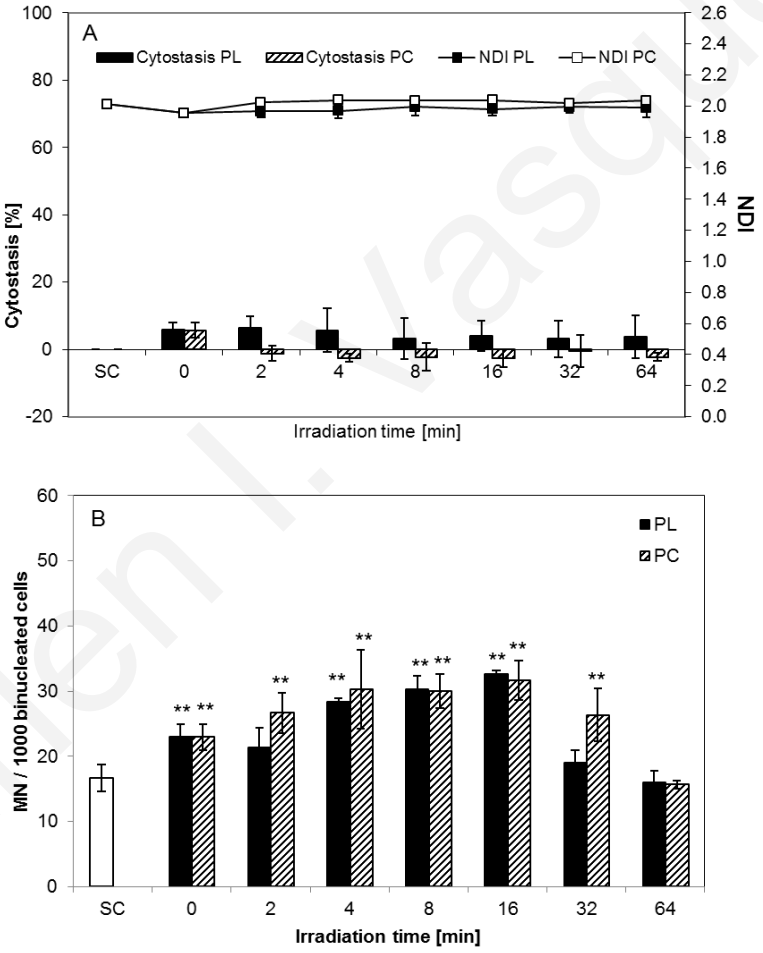


Figure 7.28 (A) Cytostasis (bars), Nuclear Division Index (line) and (B) micronuclei induction to hepatoma cell line from ofloxacin solutions during different photo(cata)lytic treatment periods

The highest level of cytostasis was observed when the HepG2 cells were exposed to untreated ofloxacin samples; whereas during the photolytic treatment it was quite low with

values ranging from 0.0 to 6.3±3.6% and close to 0 during photocatalytic treatment. The NDI remained constant with values ranging from 1.95±0.04 to 1.98±0.04 and from 2.02±0.02 to 2.04±0.01 for the photolytic and photocatalytic treatment processes, respectively.

This was not the case for the MNi induction, in which during the photolytic treatment, after only 4 min of irradiation a significant increase in the number of MNi was observed (28.33±0.58). This increase was even higher when the irradiation time was increased to 8 min (30.33±2.08). However, the greatest MNi induction (32.67±0.58) was observed at 16 min irradiation. The MNi induction during 4-16 min irradiation was statistically greater than the MNi induction at 0-2 min ($p<0.05$). At 32 and 64 min irradiation time the MNi number was reduced significantly and was not statistically different to the SC.

Similarly, during the photocatalytic treatment MNi were overexpressed at 4-16 min. Furthermore, the induction at 32 min irradiation was not statistically different from the induction of untreated samples. A 64 min irradiation was needed to reduce MNi number to values similar to the SC.

As a general remark it can be suggested, that MNi presence was more intense during the photocatalytic treatment than during the photolytic treatment. The negative control experiment showed no increase in the number of MNi observed (16.00±1.00) or in the cytostasis (0.8±1.7%) and NDI (2.01±0.01) calculated (data not shown).

7.3 Discussion

In the present Chapter the effects of photo(cata)lytically treated solutions of ofloxacin were investigated using a battery assay in which acute (*D. magna*) and chronic ecotoxicity tests (*V. fischeri, P. putida, P. subcapitata*) were used. The effects were also evaluated with regard to the cytostatic and genotoxic potential to the hepatoma cell line. The advantages and disadvantages of using bioassays to evaluate oxidation processes have been recently comprehensively reviewed by Rizzo [354].

Compounds containing fluorine were present in all photolytically and photocalytically treated solutions; a fact that could potentially explain the lack of the biodegradability potential

observed. A previous study by Fasani et al. [355] demonstrated that ofloxacin was slowly degraded by photolytic treatment primarily through defluorination; whereas ciprofloxacin, enrofloxacin and norfloxacin were more quickly degraded undergoing dealkylation. However, defluorination of ciprofloxacin was reported after photocatalytic treatment [356], and after photolytic treatment at pH 9 [357].

In the present thesis, after photo(cata)lytic treatment of 8, 16 and 32 min, fluoride liberation was observed, through C-F fragmentation. No hydroquinolones analogous, created after substitution of fluorine by water or hydroxyl through the C-F fragmentation were isolated after its photolytic treatment, as reported Albini and Monti [358].

An increasing number of studies are being performed in which the assessment of effects of transformation products of APIs is taken into account. In the majority of cases the transformation products tend to be less toxic. More polar, more mobile and less toxic transformation products are usually observed [60,259]. However, some exceptions have been recognized that stress the importance of investigating the effects of transformation products when suspected that may cause similar or greater effects compared to the parent compounds. For instance increased phytotoxicity of photo-transformation products of diclofenac were related to increased hydrophobicity [359]. Sulfamethoxazole photo-transformation products were more toxic to *D. magna* [360].

The ecotoxicity to *D. magna* at 48 h exposure time provided hints that during the photocatalytic treatment of ofloxacin resulted in the formation of photo-transformation products that retained the toxicity [278]. Moreover, *P. subcapitata* exposed for 72 h, as investigated in the present thesis, indicated that photo(cata)lytic treatment was unable to reduce the toxicity of ofloxacin and its photo-transformation products.

The growth inhibition for Gram negative bacteria, investigated in this thesis, was reduced proportionally to the concentration of the parent compound present. Interestingly, the bioluminescence inhibition of *V. fischeri* demonstrated a similar pattern with the genotoxicity stimulation in the hepatoma cell line. This indicated that photo(cata)lytic treatment may interrupt the normal gene expression of the *lux* operon, which is directly connected to the cellular ATP-level (cAMP/CRP regulation). It should be emphasized that luminescence

inhibition is not a direct indicator of growth inhibition. It depends on cell density, but also on the physiological state of the cells [361].

This thesis has revealed that during photo(cata)lytic treatment more genotoxic photo-transformation products may be formed [361]. After photo-excitation of ofloxacin, a one-electron transfer (type I reaction) or reaction with molecular oxygen (type II reaction) takes place. Type II reactions usually generate ROS: singlet oxygen (by triplet-triplet energy transfer) or superoxide (by one-electron transfer) [362].

Viola et al. [363] tried to explain the phototoxicity mechanism for levofloxacin through the involvement of free radicals and ROS by concurrently irradiating 3T3 cells and various concentrations of levofloxacin. A decrease of the phototoxic effects (cytotoxicity and lipid peroxidation) by using suitable scavengers and in the absence of oxygen indicated the formation of radical species under UVA radiation. These findings strongly suggest that HO• generation played the main role in the phototoxicity induced by levofloxacin.

However in the present thesis, the irradiation experiments were done prior to the bioassay exposure time. Furthermore, the irradiated samples treatment and storage were expected to reduce the effects of short-living photo-transformation products, free radicals and ROS, that could damage DNA in the immediate vicinity of their site of generation [364]. Therefore, the increase of genotoxicity could be partly explained by more stable compounds such as the photo-transformation products proposed in the present thesis.

Alternatively, a mechanism in which other cellular compounds are damaged *i.e.*, fatty or amino acids increasing thus the endogenous ROS production could be suggested [362,365]. Ofloxacin was found to induce cytostasis at concentrations greater than 150 mg/L by reducing cellular DNA replication of primary human kidney cells [366]; whereas in our study this effect was observed at 36.14 mg/L. The use of different cell types, exposure times and experimental settings may explain this variation.

A similar trend of the genotoxicity of ciprofloxacin to HepG2 cells after photolytic treatment was assessed and reported by Garcia-Käufer et al. [367]. However, ciprofloxacin irradiated for 32 min was found to induce the highest genotoxicity and an irradiation of 128

min was necessary for detoxification. From this comparison, it can be suggested that ciprofloxacin and the photo-transformation products created during photolytic treatment are more persistent, than ofloxacin and the photo-transformation products addressed in the present thesis.

It should also be mentioned that the major reaction mechanisms in the photo-transformation of ofloxacin are similar to those of other fluoroquinolones such as levofloxacin, ciprofloxacin etc. [357,368]. In the present thesis it has been shown that ofloxacin is sensitive to photo-transformation, with oxidation of the substituent on the C-7. The decarboxylation of ofloxacin and the loss of fluorine relate mainly with the gyrase binding potential. Furthermore, according to Sanchez et al. [369], highly reactive carbene species via defluorination may enhance the genotoxicity observed.

In the study by Garcia-Käufer et al. [367], genotoxicity of UV-irradiated ciprofloxacin samples was observed, attributed to additional transformation products with smaller mass or by-products not detectable by electrospray ionisation. These products are suggested to have a telomerase-disturbing activity and could cause oxidative damage to human cells. The constraints of isolating the various PTPs, though, do not permit a more concrete identification of the genotoxic products and this should be addressed in further studies.

The biopersistence of the photo-transformation products of ofloxacin were investigated in this thesis. Low biodegradation potential was observed for all the treatment periods evaluated, regardless the treatment applied [370]. Previous studies dealing with the photo(cata)lytic treatment of fluoroquinolones did not assess the biodegradability of the by-products formed and concentrated mainly on the removal of the parent compound and the identification of transformation products.

In this thesis the identification of the biotransformation products was addressed. The findings in this study are in accordance with previous studies, in which the UV photocatalytic treatment of other fluoroquinolones was investigated and similar transformation mechanisms (e.g., opening of piperazinyl ring and loss of fluorine), were observed [356,371].

Hydroxylation however, was not observed in the present thesis although observed in the abovementioned studies. Dealkylation of ofloxacin was also reported after oxidation by manganese oxide [372]. Some of the transformation products identified in the present study have not been previously reported (TP1, TP3, TP4, TP6 and TP8), indicating that a plethora of transformation products may occur depending on the experimental and analytical set up.

Among fluoroquinolones, ofloxacin can be transformed after UV photolytic treatment; whereas norfloxacin and ciprofloxacin are more persistent. Furthermore, the presence of oxygen or another electron receptor (e.g., bromate ions) is necessary for fluoroquinolones transformation [278,356,361,373].

The effects of the phototransformation products as means of immobilization for *D. magna* [278], growth inhibition for *Pseudomonas putida*, *Vibrio fischeri* [361] and *Escherichia coli* [374] indicated a reduction of toxicity on the photo(cata)lytically treated samples as irradiation time increased. The formation of more biodegradable products though, was not verified in the present study probably to the preservation of the core quinolone structure.

Attention should be given to the suggested co-metabolism, as studies on co-metabolism of APIs are still limited. Co-metabolism was one of the major removal mechanisms in activated sludge treatment plants for ibuprofen and naproxen when milk powder was used as co-substrate [352]. *Rhodococcus rhodochrous* bacteria could also co-metabolize carbamazepine, sulfamethizole and sulfamethoxazole [353].

However, co-metabolism in the presence of sodium acetate is still not fully understood. Alexy et al. [243] found sodium acetate to enhance the biodegradation of trimethoprim, benzylpenicillin and sulfamethoxazole, while in the study by Lahti and Oikari [28] the biodegradation of diclofenac, naproxen and bisoprolol was not enhanced by sodium acetate, indicating that co-metabolism can be substrate specific.

Sodium acetate was not found to improve biodegradability of ciprofloxacin and its photolytically treated samples at pH 9 [375]. Another interesting example of co-metabolism was studied for sulfamethoxazole, where it could serve both as a carbon or a nitrogen source, depending on the co-substrate. In combination with acetate, sulfamethoxazole behaved as a

source of nitrogen, whereas when ammonium was added, sulfamethoxazole remained intact [115].

The chemical structure of each API and the matrix composition seem to play a key role with regard to co-metabolism process. Partial biodegradation and mineralization of antibiotics through co-metabolism have been reported in sewage treatment plants [376]. The chemical structure of each API and the matrix composition seem to play a key role with regard to co-metabolism process. Further studies are required however, for an in-depth investigation for such processes.

The application of Advanced Oxidation Processes has been associated with an increase of the toxicity in the early min of the treatment due to oxidation intermediates which are more toxic than the parent compounds [305,307,377]. In fact a recent review by Rizzo [354] recognized that when wastewater was treated with advanced oxidation processes the toxicity was not reduced and could even increase. The reason for this is the composition of the complex mixture in which a great number of pollutants may be present.

A risk assessment for transformation products should be applied if they (i) are formed with a high yield, (ii) possess characteristics of being more persistent, bioaccumulative or mobile compared to the parent compound; or (iii) are highly toxic, genotoxic, mutagenic or cause any other adverse effect [378]. The need to include transformation products of APIs in the risk assessment has also been mentioned in the EMEA guidelines [13].

7.4 Conclusions

The presence of APIs in the environment is a triggering issue and a great effort is directed towards developing treatment processes capable of removing these substances, as well as other xenobiotics present in the wastewater cycle. The efficiency of these processes though should be evaluated, not only by the removal of the parent compounds, but complemented by investigations on the potential adverse effects to biological systems of the various by-products formed due to the partial oxidation. The assessment of the effects to biological system should

be an integrated one and address, not only acute effects, but also chronic, cytotoxic and genotoxic effects which may be more sensitive ones.

One important finding of the present thesis is that even when using strong oxidatives such as HO•, certain irradiation time is required to eliminate possible toxic/genotoxic effects of the oxidation products of APIs. In relevant technical applications, the residence time during treatment should receive special attention. The biodegradability of these products should be addressed as persistent photo- and biotransformation products may occur. The conditions enhancing biodegradability, for instance the presence of a readily biodegradable carbon source, should be investigated so as to increase the understanding of the conditions that may lead to less potent and persistent substances during the application of such engineered or natural processes.

CHAPTER 8. CONCLUSIONS - FUTURE WORK

The investigation of the real matrices, APIs and organisms and their intercorrelations were the main focus of this thesis, as illustrated in Figure 8.1. A methodology was developed for the assessment of the effects of environmental matrices (*i.e.*, water, wastewater), APIs (e.g., atenolol, diclofenac, ofloxacin, etc.) and treatment processes (*i.e.*, photo(cata)lytic treatment) using a battery assay with organisms from different trophic levels, and evaluating a plethora of endpoints by acute and chronic testing. The main advantage of the methodology developed is its flexibility, in which a selection of the most relevant endpoints can be performed for assessing the effects of the solution/matrix under study. The methodology developed may also be used for assessing the effects of different environmental matrices (e.g., agro-industrial, industrial wastewater), pollutants and treatment processes.

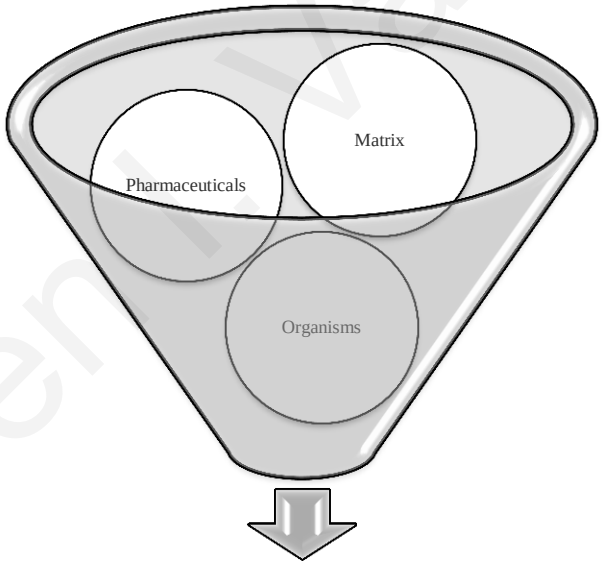


Figure 8.1 Main ideas addressed in the present thesis

Figure 8.2 summarizes the proposed approach and the most significant findings of the present thesis. The first subdivision refers to the matrix composition, whether the environmental matrix used was a complex (e.g., wastewater) or a synthetic one (e.g., aqueous solutions of APIs, photo(cata)lytically treated solutions of APIs). The second subdivision

indicates the type of assessment followed (e.g., physicochemical assessment, assessment of effects, etc.) and the third subdivision annotates the parameters/ endpoints evaluated. The final subdivision provides an answer to whether a significant risk is to be expected for the aspects evaluated according to the results obtained from the present study based on the various conditions applied. "YES" is used in instances where the risk is significant, "NO" when the risk is negligible and "NO?" when further studies are needed to fully evaluate the effects examined.

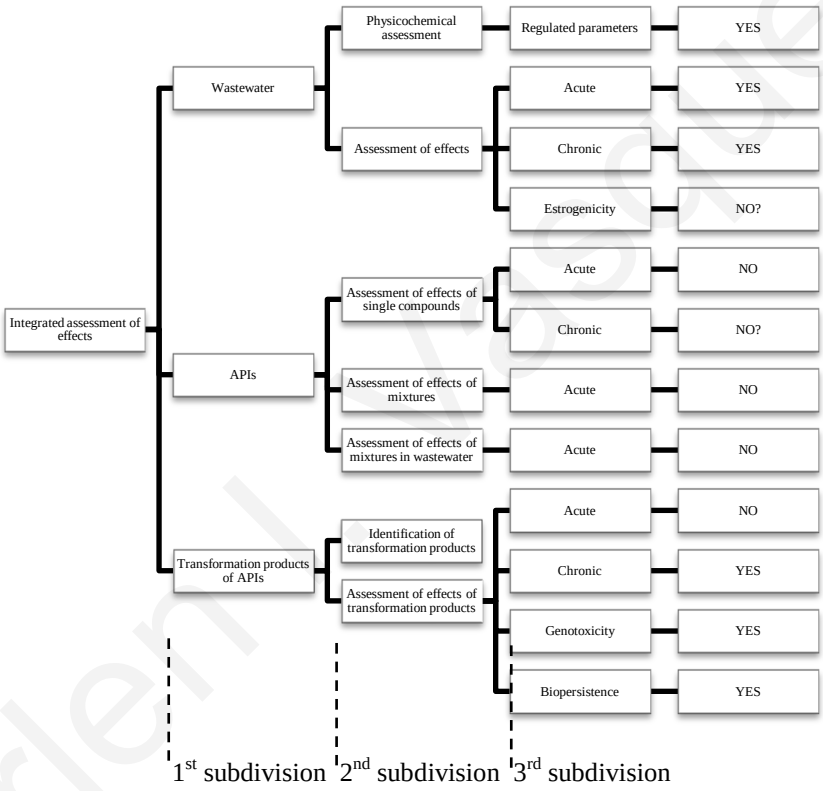


Figure 8.2 Summary of the most significant aspects evaluated

The most significant findings of this thesis followed by suggestions for future investigation are provided below.

- The physicochemical characterization of the wastewater in which all regulated parameters were monitored could not be correlated in a unanimous way, with the ecotoxicity present in the wastewater in a uniform manner. Some correlations could be attributed to the deficiency of nutrients, instead of the presence of toxic substances. This finding emphasizes the fact that in a multi-component mixture, both stimulating

and inhibiting substances, co-exist and potentially interact. Consequently, the ecotoxicity could originate from the combined effects of regulated parameters. Additionally some non-regulated parameters may enhance the ecotoxic effect observed.

- The dynamic composition of treated wastewater is one of the greatest enigmas when it comes to the assessment of its effects. Seasonal variation, specifically higher ecotoxicity during summer period and lower ecotoxicity during winter period, was found in all the samples of all sewage treatment plants evaluated. Spatial variation, specifically between the qualities of the wastewater of the three sewage treatment plants monitored (inlets, the secondary and the tertiary treatment processes), was identified. The increase of the toxicity during the summer season may indicate a greater impact to the environment also due to the fact that treated wastewater may be less diluted in the receiving water bodies. A quantitative approach regarding the dilution occurring in the environment may provide information on the risk associated with releasing toxic wastewater to the environment.

- A cost and time-effective battery assay was developed for an integrated assessment of the effects of wastewater, in which all trophic levels were investigated by applying a multi-species and multi-endpoint approach. The hazard classification applied for the treated wastewater identified a higher hazard for freshwater organisms (*P. subcapitata*, *D. magna*), than for marine (*V. fischeri, A. salina*) and terrestrial (*L. sativum*) organisms. The estrogenicity of the treated wastewater could be detected; however, it was not quantified due to the low concentration of the compounds causing the estrogenicity.

A Toxicity Identification Evaluation scheme is proposed to be implemented in future studies, to complement the findings of this thesis. Briefly described, the process includes three phases; (i) a characterization step, in which the physicochemical nature of the constituents causing the toxicity by handling the samples (e.g., solubility, volatility, etc.) is applied, (ii) an identification step, in which methods for identifying ammonia, non-polar compounds or

metals, is developed and (iii) a confirmation step, in which methods to confirm the suspected toxicants proposed, is performed [379].

A tailored-made assessment of the effects of each sewage treatment plant, in which a selection of the endpoints to be evaluated and the frequency of the samplings are adapted to the specific characteristics of the treatment plant, could provide a better understanding and improvement of the treatment processes. A set of criteria should be established that would take into account the discharge amounts of wastewater, the dilution occurring in the environment, the ecosystems affected by the discharge, the reuse practices, etc.

The assessment of the effects of the APIs revealed the following:

- The APIs evaluated in the present thesis were not able to cause acute effects, with the exception of propranolol for *D. magna*. Erythromycin, ofloxacin, sulfamethoxazole and propranolol were able to provoke chronic toxicity to *P. subcaptitata.* When the concentrations affecting the organisms were compared to the environmental concentrations determined in Cyprus (PEC and MEC), as suggested by the EMEA [13], propranolol was found to possess a risk for *P. subcapitata*. Ofloxacin was also found to possess a risk in chronic tests with *V. fischeri* at the environmental concentrations monitored.

- The effects of APIs to *D. magna* and *P. subcapitata* could be positively correlated at the 0.05 probability level with a R^2 of 0.61, indicating that to a limited extent extrapolations from acute to chronic effects and between different organisms are probable.

- The evaluation of the mixture toxicity of APIs is a complex issue, in which the chemical structure of the components and their mode of action have a crucial role. Synergistic acute effects were observed only in binary mixtures of non-steroidal anti-inflammatory APIs. Furthermore, additive acute effects were observed for β-blockers. All other mixtures evaluated, had an antagonistic behaviour, especially at the lowest

concentrations examined. The antagonistic behaviour was enhanced when the mixtures were present in treated wastewater, indicating less bioavailability of the compounds.

- APIs are present in the environment, not only in the form of the parent compound but also as transformation products. The photo-transformation porducts were addressed in this thesis. Ofloxacin, as a model compound, was photo(cata)lytically treated. The ecotoxicity of treated samples demonstrated a time-dependent response with photocatalytic treatment being more effective than photolytic treatment in reducing the inhibition for both *P. putida* and *V. fischeri*. Sixteen to 32 min of irradiation was needed to reduce the inhibition to non-toxic effects for both species.

- The toxicity of photo(cata)lytically treated samples towards *V. fischeri*, when bioluminescence inhibition was used as an endpoint, increased and subsequently decreased with increasing irradiation time. This suggests that photo(cata)lytic treatment may interrupt the normal gene expression of the *lux operon***,** which is directly connected to the cellular ATP-level (cAMP/CRP regulation).

- During photo(cata)lytic treatment, genotoxic by-products that are even more genotoxic than the parent compound, may be formed. The increase of genotoxicity could be partly explained by more stable compounds such as the photo-transformation products proposed in the present study. Ofloxacin was found to induce cytostasis at 36.14 mg/L, which is the lowest concentration reported so far in the literature for this effect.

- Ofloxacin was found to be sensitive to photo-transformation, with oxidation of the substituent on the C-7. Nine compounds were tentatively identified as its photo-transformation products formed during the photo(cata)lytic treatment. Two major pathways were suggested, in which piperazinyl dealkylation and decarboxylation are described as the major transformation mechanisms.

- The biopersistence of the transformation products of ofloxacin after photo(cata)lytic treatment were evaluated using the Closed Bottle test. None of the treated samples

were readily biodegradable, suggesting high biopersistence in the environment. However, in the presence of sodium acetate, a readily degradable organic compound, the biodegradation potential significantly increased for some photolytically treated samples. The biodegradability of the samples obtained after 16 and 32 min of photolytic treatment was higher than the levels determined with the quality control series; hence, the presence of sodium acetate probably facilitated the biodegradation of ofloxacin and its treated solutions. A possible explanation may be that a type of co-metabolism takes place, during which the biodegradation of ofloxacin and its treated samples depend on the presence of sodium acetate. Another possible explanation is that in the presence of sodium acetate, bacteria that are able to degrade some of the photolytic products grow faster, first by feeding on the sodium acetate, and then, after having reached a critical mass, by feeding on the transformation products. This observation was not seen in the samples obtained after the photocatalytic treatment, suggesting that the treated solutions at 8 and 32 min contained less biodegradable products.

- Photo- and bio-transformation products of ofloxacin, identified by UPLC-MS/MS, indicate three major transformation pathways: demethylation, decarboxylation and opening of piperazinyl ring.

In order to increase our understanding of APIs as multi-component mixtures, the following suggestions for future investigation are proposed:

- The assessment of the effects of transformation products of different mixtures of APIs. The potentially additive, antagonistic and synergetic effects of these compounds when present in mixtures with parent compounds and other naturally occurring compounds and chemical elements need to be investigated. This assessment should be performed both for acute and, more importantly, chronic effects, focusing not only on toxicity but also on genotoxicity, mutagenicity and other relevant potential effects. Fractional factorial designs, in which a decreased number of experiments are used but the interactions between components are addressed, are proposed.

- The in-depth analysis of the cause of genotoxicity at a molecular level, e.g., with the implementation of microarray techniques could reveal which genes are activated along with the possible mechanisms responsible for the effects observed.

- The investigation of mutagenicity as an alternative endpoint of APIs and their transformation products could improve our understanding on whether the observed genotoxicity can be inherited to daughter cells as detectable malfunctions.

- The investigation of other chronic endpoints, such as fecundity, in order to better understand the effects due long-term exposure, that better simulate environmental conditions.

CHAPTER 9. REFERENCES

[1] Madden JC, Enoch SJ, Hewitt M, Cronin MTD. 2009. Pharmaceuticals in the environment: Good practice in predicting acute ecotoxicological effects. *Toxicol Lett* 185:85-101.

[2] Pharmaceutical Services. 2009. *Pharmaceutical Products in Cyprus.* 1. Pharmaceutical Services, Nicosia, Cyprus.

[3] Fatta-Kassinos D, Hapeshi E, Achilleos A. 2008. *Amounts of Pharmaceuticals that are Sold and Consumed in Cyprus, Determination of their Active Compounds, Evaluation of their Mass and the Expected Concentration in Wastewater*. Deliverable Report No 2. PHAREM ΑΕΙΦΟ/0506/16, Nicosia, Cyprus.

[4] Nikolaou A, Meriç S, Fatta-Kassinos D. 2007. Occurrence patterns of pharmaceuticals in water and wastewater environments. *Anal Bioanal Chem* 387:1225-1234.

[5] Tabak HH, Bunch RL. 1970. Steroid hormones as water pollutants I: Metabolism of natural and synthetic ovulation-inhibiting hormones by micro-organisms of activated sludge and primary settled sludge. *Dev Ind Microbiol* 11:367-376.

[6] Norpoth K, Nehrkorn A, Kirchner M, Holsen H, Teipel H. 1973. Untersuchungen zur frage der löslichkeit und stabilität ovulationshemmender steroide in wasser, abwässern und belebtschlamm. *Zbl Bakt Hyg I Abt Orig B* 156:500-511.

[7] Fielding M, Gibson TM, James HA, McLoughlin K, Steel CP. 1981. *Organic Micropollutants in Drinking Water.* Technical report TR159. Water Research Center, Marlow, UK.

[8] Kümmerer K. 2009. The presence of pharmaceuticals in the environment due to human use – present knowledge and future challenges. *J Environ Manage* 90:2354-2366.

[9] Richman C, Castensson S. 2008. Impact of waste pharmaceuticals: an environmental hazard or "greenwash"? *Pharm J* 280:335-336.

[10] Fatta-Kassinos D, Hapeshi E, Achilleos A, Meriç S, Gros M, Petrovic M, Barceló D. 2011. Existence of pharmaceutical compounds in tertiary treated urban wastewater that is utilized for reuse applications. *Water Resour Manage* 25:1183-1193.

[11] Makris C, Snyder A. 2010. Screening of pharmaceuticals and endocrine disrupting compounds in water supplies of Cyprus. *Water Sci Technol* 62:2720-2728.

[12] Council Regulation (EEC) No 2309/93 for the authorization and supervision of medicinal products for human and veterinary use and establishing a European Agency for the Evaluation of Medicinal Products. 1993. *Official Journal* L214, 24/8/1993:1-21.

[13] Committee for Medicinal Products for Human Use. 2006. *European Medicines Agency Pre-Authorisation Evaluation of Medicines for Human use. Guideline on the Environmentalriskassessment of Medicinalproducts for Human use*. EMEA/CHMP/SWP/4447/00. EMEA, London.

[14] Richardson SD, Ternes TA. 2011. Water analysis: emerging contaminants and current issues. *Anal Chem* 83:4614-4648.

[15] Cunningham VL. 2004. Special characteristics of pharmaceuticals related to environmental fate. In Kümmerer K, ed, *Pharmaceuticals in the Environment*, 2nded. Springer-Verlag, Berlin Heidelberg, pp 21-33.

[16] Martin D, Shen J, Griener J, Raasch R, Patterson J, Cascio W. 1996. Effects of ofloxacin on the pharmacokinetics and pharmacodynamics of procainamide. *J Clin Pharmacol* 36:85-91.

[17] Goyne KW, Chorover J, Kubicki JD, Zimmerman AR, Brantley SL. 2005. Sorption of the antibiotic ofloxacin to mesoporous and nonporous alumina and silica. *J Colloid Interface Sci* 283:160-170.

[18] Klaassen C, Watkins J. 2003. *Essentials of Toxicology*. MacGraw-Hill, United States of America.

[19] Kah M, Brown CD. 2008. Log D: Lipophilicity for ionisable compounds. *Chemosphere* 72:1401-1408.

[20] Escher BI, Schwarzenbach R, Westall JC. 2000. Evaluation of liposome-water partitioning of organic acids and bases. 1. Development of a sorption model. *Environ Sci Technol* 34:3954-3961.

[21] Ternes TA, Herrmann N, Bonerz M, Knacker T, Siegrist H, Joss A. 2004. A rapid method to measure the solid–water distribution coefficient (K_d) for pharmaceuticals and musk fragrances in sewage sludge. *Water Res* 38:4075-4084.

[22] Walker CH, Hopkin SP, Sibly RM, Peakall DB. 2001. *Principles of Ecotoxicology*. Taylor & Francis Press, London.

[23] Lindman U. 2005. The nature and chemistry of toxicants. In Thompson KC, Wadhia K, Loibner AP, eds, *Environmental Toxicity Testing*. Blackwell Publishing CRC Press, Oxford, UK.

[24] Bort R, Macé K, Boobis A, Gómez-Lechón M, Pfeifer A, Castell J. 1999. Hepatic metabolism of diclofenac: role of human CYP in the minor oxidative pathways. *Biochem Pharmacol* 58:787-796.

[25] Reeves PR, McAinsh J, McIntosh DAD, Winrow MJ. 1978. Metabolism of atenolol in man. *Xenobiotica* 8:313-320.

[26] Ternes TA, Kreckel P, Mueller J. 1999. Behaviour and occurrence of estrogens in municipal sewage treatment plants — II. Aerobic batch experiments with activated sludge. *Sci Total Environ* 225:91-99.

[27] Urase T, Kikuta T. 2005. Separate estimation of adsorption and degradation of pharmaceutical substances and estrogens in the activated sludge process. *Water Res* 39:1289-1300.

[28] Lahti M, Oikari A. 2011. Microbial transformation of pharmaceuticals naproxen, bisoprolol, and diclofenac in aerobic and anaerobic environments. *Arch Environ Contam Toxicol* 61:202-210.

[29] Onesios KM, Yu JT, Bouwer EJ. 2009. Biodegradation and removal of pharmaceuticals and personal care products in treatment systems: A review. *Biodegradation* 20:441-466.

[30] Kümmerer K, Ericson JF, Hannah R, Johnson A, Sedlak DL, Weston JJ. 2005. Environmental fate and transport of human pharmaceuticals. In Williams RT, ed, *Human Pharmaceuticals: Assessing the Impacts on Aquatic Ecosystems*, 1sted. SETAC Press, Pensacola, Florida, USA, pp 111-148.

[31] Kinney CA, Furlong ET, Kolpin DW, Burkhardt MR, Zaugg SD, Werner SL, Bossio JP, Benotti MJ. 2008. Bioaccumulation of pharmaceuticals and other anthropogenic waste indicators in earthworms from agricultural soil amended with biosolid or swine manure. *Environ Sci Technol* 42:1863-1870.

[32] Tonnelier A, Coecke S, Zaldívar JM. 2012. Screening of chemicals for human bioaccumulative potential with a physiologically based toxicokinetic model. *Arch Toxicol* 86:393-403.

[33] Sherer JT. 2006. Pharmaceuticals in the environment. *Am J Health Syst Pharm* 63:174-178.

[34] Vernouillet G, Eullaffroy P, Lajeunesse A, Blaise C, Gagné F, Juneau P. 2010. Toxic effects and bioaccumulation of carbamazepine evaluated by biomarkers measured in organisms of different trophic levels. *Chemosphere* 80:1062-1068.

[35] Cleuvers M. 2004. Mixture toxicity of the anti-inflammatory drugs diclofenac, ibuprofen, naproxen, and acetylsalicylic acid. *Ecotoxicol Environ Saf* 59:309-315.

[36] Backhaus T, Sumpter J, Blanck H. 2008. On the ecotoxicology of pharmaceutical mixtures. In Kümmerer K, ed, *Pharmaceuticals in the Environment*, 3rded. Springer Berlin Heidelberg, pp 257-276.

[37] Owen SF, Giltrow E, Huggett DB, Hutchinson TH, Saye J, Winter MJ, Sumpter JP. 2007. Comparative physiology, pharmacology and toxicology of β-blockers: Mammals versus fish. *Aquat Toxicol* 82:145-162.

[38] Knox C, Law V, Jewison T, Liu P, Ly S, Frolkis A, Pon A, Banco K, Mak C, Neveu V, Djoumbou Y, Eisner R, Guo AC, Wishart DS. 2011. DrugBank 3.0: a comprehensive resource for 'Omics' research on drugs. *Nucleic Acids Res* 39:1035–-1041.

[39] Wishart DS, Knox C, Guo AC, Cheng D, Shrivastava S, Tzur D, Gautam B, Hassanali M. 2008. DrugBank: a knowledgebase for drugs, drug actions and drug targets. *Nucleic Acids Res* 36:901-906.

[40] Wishart DS, Knox C, Guo AC, Shrivastava S, Hassanali M, Stothard P, Chang Z, Woolsey J. 2006. DrugBank: a comprehensive resource for in silico drug discovery and exploration. *Nucleic Acids Res* 34:668-672.

[41] Stevens-Garmon J, Drewes JE, Khan SJ, McDonald JA, Dickenson ERV. 2011. Sorption of emerging trace organic compounds onto wastewater sludge solids. *Water Res* 45:3417-3426.

[42] ChemSpider. 2012. Available at: www.chemspider.com

[43] Escher BI, Bramaz N, Richter M, Lienert J. 2006. Comparative ecotoxicological hazard assessment of beta-blockers and their human metabolites using a mode-of-action-based test battery and a QSAR approach. *Environ Sci Technol* 40:7402-7408.

[44] Maurer M, Escher BI, Richle P, Schaffner C, Alder AC. 2007. Elimination of β-blockers in sewage treatment plants. *Water Res* 41:1614-1622.

[45] Ramil M, El Aref T, Fink G, Scheurer M, Ternes TA. 2010. Fate of beta blockers in aquatic-sediment systems: Sorption and biotransformation. *Environ Sci Technol* 44:962-970.

[46] Belpaire FM, Wijnant P, Temmerman A, Rasmussen BB, Brøsen K. 1998. The oxidative metabolism of metoprolol in human liver microsomes: Inhibition by the selective serotonin reuptake inhibitors. *Eur J Clin Pharmacol* 54:261-264.

[47] Borg KO, Carlsson E, Hoffmann KJ. 1975. Metabolism of metoprolol (3H) in man, the dog and the rat. *Acta Pharmacol Toxicol* 36:125-135.

[48] Hirvonen J, Guy RH. 1997. lontophoretic delivery across the skin: electroosmosis and its modulation by drug substances. *Pharm Res* 14:1258-1263.

[49] Avdeef A. 2003. *Absorption and Drug Development: Solubility, Permeability, and Charge State.* John Wiley & Sons Inc, Hoboken, New Jersey, USA.

[50] Kolpin DW, Furlong ET, Meyer MT, Thurman EM, Zaugg SD, Barber LB, Buxton HT. 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: A national reconnaissance. *Environ Sci Technol* 36:1202-1211.

[51] Masubuchi Y, Hosokawa S, Horie T, Suzuki T, Ohmori S, Kitada M, Narimatsu S. 1994. Cytochrome P450 isozymes involved in propranolol metabolism in human liver microsomes: The role of CYP2D6 as ring-hydroxylase and CYP1A2 as N- desisopropylase. *Drug Metab Dispos* 22:909-915.

[52] Walle T, Walle UK, Olanoff LS. 1985. Quantitative account of propranolol metabolism in urine of normal man. *Drug Metab Dispos* 13:204-209.

[53] Uwai K, Tani M, Ohtake Y, Abe S, Maruko A, Chiba T, Hamaya Y, Ohkubo Y, Takeshita M. 2005. Photodegradation products of propranolol: The structures and pharmacological studies. *Life Sci* 78:357-365.

[54] Liu X, Tu M, Kelly RS, Chen C, Smith BJ. 2004. Development of a computational approach to predict blood-brain barrier permeability. *Drug Metab Dispos* 32:132-139.

[55] Lin AYC, Lin CA, Tung HH, Chary NS. 2010. Potential for biodegradation and sorption of acetaminophen, caffeine, propranolol and acebutolol in lab-scale aqueous environments. *J Hazard Mater* 183:242-250.

[56] Drillia P, Stamatelatou K, Lyberatos G. 2005. Fate and mobility of pharmaceuticals in solid matrices. *Chemosphere* 60:1034-1044.

[57] Lin AYC, Reinhard M. 2005. Photodegradation of common environmental pharmaceuticals and estrogens in river water. *Environ Toxicol Chem* 24:1303-1309.

[58] Yu-Chen A, Reinhard M. 2005. Erratum: Photodegradation of common environmental pharmaceuticals and estrogens in river water (Environmental Toxicology and Chemistry (2005) 24 (1303-1309)). *Environ Toxicol Chem* 24:3000.

[59] Andreozzi R, Raffaele M, Nicklas P. 2003. Pharmaceuticals in STP effluents and their solar photodegradation in aquatic environment. *Chemosphere* 50:1319-1330.

[60] Liu QT, Williams TD, Cumming RI, Holm G, Hetheridge MJ, Murray-Smith R. 2009. Comparative aquatic toxicity of propranolol and its photodegraded mixtures: Algae and rotifer screening. *Environ Toxicol Chem* 28:2622-2631.

[61] Rowley AF, Vogan CL, Taylor GW, Clare AS. 2005. Prostaglandins in non-insectan invertebrates: Recent insights and unsolved problems. *J Exp Biol* 208:3-14.

[62] Gambaro G, Perazella MA. 2003. Adverse renal effects of anti-inflammatory agents: evaluation of selective and nonselective cyclooxygenase inhibitors. *J Intern Med* 253:643-652.

[63] Sawchuk RJ, Maloney JA, Cartier LL, Rackley RJ, Chan KKH, Lau HSL. 1995. Analysis of diclofenac and four of its metabolites in human urine by HPLC. *Pharm Res* 12:756-762.

[64] Seitz S, Boelsterli UA. 1998. Diclofenac acyl glucuronide, a major biliary metabolite, is directly involved in small intestinal injury in rats. *Gastroenterology* 115:1476-1482.

[65] Stülten D, Lamshöft M, Zühlke S, Spiteller M. 2008. Isolation and characterization of a new human urinary metabolite of diclofenac applying LC–NMR–MS and high-resolution mass analyses. *J Pharm Biomed Anal* 47:371-376.

[66] Zhang Y, Geißen S, Gal C. 2008. Carbamazepine and diclofenac: Removal in wastewater treatment plants and occurrence in water bodies. *Chemosphere* 73:1151-1161.

[67] Kallio J, Lahti M, Oikari A, Kronberg L. 2010. Metabolites of the aquatic pollutant diclofenac in fish bile. *Environ Sci Technol* 44:7213-7219.

[68] Carballa M, Fink G, Omil F, Lema JM, Ternes T. 2008. Determination of the solid–water distribution coefficient (K_d) for pharmaceuticals, estrogens and musk fragrances in digested sludge. *Water Res* 42:287-295.

[69] Al-Rajab AJ, Sabourin L, Lapen DR, Topp E. 2010. The non-steroidal anti-inflammatory drug diclofenac is readily biodegradable in agricultural soils. *Sci Total Environ* 409:78-82.

[70] Gröning J, Held C, Garten C, Claußnitzer U, Kaschabek SR, Schlömann M. 2007. Transformation of diclofenac by the indigenous microflora of river sediments and identification of a major intermediate. *Chemosphere* 69:509-516.

[71] González S, Müller J, Petrovic M, Barceló D, Knepper TP. 2006. Biodegradation studies of selected priority acidic pesticides and diclofenac in different bioreactors. *Environ Pollut* 144:926-932.

[72] Packer JL, Werner JJ, Latch DE, McNeill K, Arnold WA. 2003. Photochemical fate of pharmaceuticals in the environment: Naproxen, diclofenac, clofibric acid, and ibuprofen. *Aquat Sci* 65:342-351.

[73] Schmitt-Jansen M, Bartels P, Adler N, Altenburger R. 2007. Phytotoxicity assessment of diclofenac and its phototransformation products. *Anal Bioanal Chem* 387:1389-1396.

[74] Schmidt W, O'Rourke K, Hernan R, Quinn B. 2011. Effects of the pharmaceuticals gemfibrozil and diclofenac on the marine mussel (*Mytilus spp*.) and their comparison with standardized toxicity tests. *Mar Pollut Bull* 62:1389-1395.

[75] Adams SS, Bresloff P, Mason CG. 1976. Pharmacological differences between the optical isomers of ibuprofen: evidence for metabolic inversion of the (-) isomer. *J Pharm Pharmacol* 28:256-257.

[76] Hutt AJ, Caldwell J. 1983. The metabolic chiral inversion of 2-arylpropionic acids. A novel route with pharmacological consequences. *J Pharm Pharmacol* 35:693-704.

[77] Lockwood GF, Albert KS, Gillespie WR. 1983. Pharmacokinetics of ibuprofen in man. I. Free and total area/dose relationships. *Clin Pharmacol Ther* 34:97-103.

[78] Paxéus N. 2004. Removal of selected non-steroidal anti-inflammatory drugs (NSAIDs), gemfibrozil, carbamazepine, β-blockers, trimethoprim and triclosan in conventional wastewater treatment plants in five EU countries and their discharge to the aquatic environment. *Water Sci Technol* 50:253-260.

[79] Brown JN, Paxéus N, Förlin L, Larsson DGJ. 2007. Variations in bioconcentration of human pharmaceuticals from sewage effluents into fish blood plasma. *Environ Toxicol Pharmacol* 24:267-274.

[80] Bhandari K, Venables B. 2011. Ibuprofen bioconcentration and prostaglandin E2 levels in the bluntnose minnow *Pimephales notatus*. *Comp Biochem Physiol C Toxicol Pharmacol* 153:251-257.

[81] Nallani GC, Paulos PM, Constantine LA, Venables BJ, Huggett DB. 2011. Bioconcentration of ibuprofen in fathead minnow (*Pimephales promelas*) and channel catfish (*Ictalurus punctatus*). *Chemosphere* 84:1371-1377.

[82] Chambers H. 2001. Antimicrobial agents - general considerations. In Hardman J, Limbird L, eds, *The Pharmacological Basis of Therapeutics*, 10thed. Goodman & Gilman's, McGraw-Hill, New York, USA, pp 1143-1170.

[83] Segura PA, François M, Gagnon C, Sauvé S. 2009. Review of the occurrence of anti-infectives in contaminated wastewaters and natural and drinking waters. *Environ Health Perspect* 117:675-684.

[84] Monteiro SC, Boxall ABA. 2010. Occurrence and fate of human pharmaceuticals in the environment. In Whitacre DM, ed, *Reviews of Environmental Contamination and Toxicology*, Vol202. Springer New York, New York, pp 53-154.

[85] An A-Z of Medicinal Drugs. 2010. Available at: http://www.oxfordreference.com/views/ENTRY.html?subview=Main&entry=t61.e2291

[86] Alighardashi A, Pandolfi D, Potier O, Pons MN. 2009. Acute sensitivity of activated sludge bacteria to erythromycin. *J Hazard Mater* 172:685-692.

[87] Goldman RC, Fesik SW, Doran CC. 1990. Role of protonated and neutral forms of macrolides in binding to ribosomes from gram-positive and gram-negative bacteria. *Antimicrob Agents Chemother* 34:426-431.

[88] Fisk PR, Wildey RJ, Livingstone DJ, Boxall AB. Development and assessment of risk profiles for pharmaceuticals. 2012.

[89] Reungoat J, Escher BI, Macova M, Keller J. 2011. Biofiltration of wastewater treatment plant effluent: Effective removal of pharmaceuticals and personal care products and reduction of toxicity. *Water Res* 45:2751-2762.

[90] Hirsch R, Ternes T, Haberer K, Kratz K. 1999. Occurrence of antibiotics in the aquatic environment. *Sci Total Environ* 225:109-118.

[91] Halling-Sørensen B, Nors Nielsen S, Lanzky PF, Ingerslev F, Holten Lützhøft HC, Jørgensen SE. 1998. Occurrence, fate and effects of pharmaceutical substances in the environment- A review. *Chemosphere* 36:357-393.

[92] Xu W, Zhang G, Li X, Zou S, Li P, Hu Z, Li J. 2007. Occurrence and elimination of antibiotics at four sewage treatment plants in the Pearl River Delta (PRD), South China. *Water Res* 41:4526-4534.

[93] Minh TB, Leung HW, Loi IH, Chan WH, So MK, Mao JQ, Choi D, Lam JCW, Zheng G, Martin M, Lee JHW, Lam PKS, Richardson BJ. 2009. Antibiotics in the Hong Kong metropolitan area: Ubiquitous distribution and fate in Victoria Harbour. *Mar Pollut Bull* 58:1052-1062.

[94] Tamtam F, Mercier F, Le Bot B, Eurin J, Tuc Dinh Q, Clément M, Chevreuil M. 2008. Occurrence and fate of antibiotics in the Seine River in various hydrological conditions. *Sci Total Environ* 393:84-95.

[95] Fujimoto T, Mitsuhashi S. 1990. *In vitro* antibacterial activity of DR-3355, the S-(-)-isomer of ofloxacin. *Chemotherapy* 36:268-276.

[96] Ferrari B, Paxéus N, Giudice RL, Pollio A, Garric J. 2003. Ecotoxicological impact of pharmaceuticals found in treated wastewaters: Study of carbamazepine, clofibric acid, and diclofenac. *Ecotoxicol Environ Saf* 55:359-370.

[97] Santos LHMLM, Araújo AN, Fachini A, Pena A, Delerue-Matos C, Montenegro MCBSM. 2010. Ecotoxicological aspects related to the presence of pharmaceuticals in the aquatic environment. *J Hazard Mater* 175:45-95.

[98] Jung CM, Heinze TM, Strakosha R, Elkins CA, Sutherland JB. 2009. Acetylation of fluoroquinolone antimicrobial agents by an *Escherichia coli* strain isolated from a municipal wastewater treatment plant. *J Appl Microbiol* 106:564-571.

[99] Boreen AL, Arnold WA, McNeill K. 2003. Photodegradation of pharmaceuticals in the aquatic environment: A review. *Aquatic Sci* 65:320-341.

[100] Lam MW, Mabury SA. 2005. Photodegradation of the pharmaceuticals atorvastatin, carbamazepine, levofloxacin, and sulfamethoxazole in natural waters. *Aquat Sci* 67:177-188.

[101] Beausse J. 2004. Selected drugs in solid matrices: A review of environmental determination, occurrence and properties of principal substances. *TrAC Trends Anal Chem* 23:753-761.

[102] Langdon KA, Warne MSTJ, Kookanaz RS. 2010. Aquatic hazard assessment for pharmaceuticals, personal care products, and endocrine-disrupting compounds from biosolids-amended land. *Integr Environ Assess Manage* 6:663-676.

[103] Antibiotics and anti-infective drugs. 2001. Available at: http://www.oxfordreference.com/views/ENTRY.html?subview=Main&entry=t185.e29

[104] Petri WA. 2001. Antimicrobial agents: sulfonamides, trimethoprim- sulfamethoxazole, quinolones, and agents for urinary tract infections. In Hardman JG, Limbird LE, eds, *The Pharmacological Basis of Therapeutics*, 10thed. Goodman & Gilman's, McGraw- Hill, New York, USA, pp 1171-1188.

[105] Lam MW, Young CJ, Brain RA, Johnson DJ, Hanson MA, Wilson CJ, Richards SM, Solomon KR, Mabury SA. 2004. Aquatic persistence of eight pharmaceuticals in a microcosm study. *Environ Toxicol Chem* 23:1431-1440.

[106] Brown GM. 1962. The biosynthesis of folic acid. *J Biol Chem* 237:536-540.

[107] Isidori M, Lavorgna M, Nardelli A, Pascarella L, Parrella A. 2005. Toxic and genotoxic evaluation of six antibiotics on non-target organisms. *Sci Total Environ* 346:87-98.

[108] Anderson PO, Knoben JE, Troutman WG. 2002. *Handbook of Clinical Drug Data.* McGraw-Hill, USA.

[109] Göbel A, McArdell CS, Suter MJF, Giger W. 2004. Trace determination of macrolide and sulfonamide antimicrobials, a human sulfonamide metabolite, and trimethoprim in wastewater using liquid chromatography coupled to electrospray tandem mass spectrometry. *Anal Chem* 76:4756-4764.

[110] van der Ven AJ, Vree TB, van Ewijk-Beneken Kolmer EW, Koopmans PP, van der Meer JW. 1995. Urinary recovery and kinetics of sulfamethoxazole and its metabolites in HIV-seropositive patients and healthy volunteers after a single oral dose of sulfamethoxazole. *Br J Clin Pharmacol* 39:621-625.

[111] Yang S, Cha J, Carlson K. 2005. Simultaneous extraction and analysis of 11 tetracycline and sulfonamide antibiotics in influent and effluent domestic wastewater by solid-phase extraction and liquid chromatography-electrospray ionization tandem mass spectrometry. *J Chromatogr A* 1097:40-53.

[112] Avisar D, Lester Y, Ronen D. 2009. Sulfamethoxazole contamination of a deep phreatic aquifer. *Sci Total Environ* 407:4278-4282.

[113] Boreen AL, Arnold WA, McNeill K. 2004. Photochemical fate of sulfa drugs in the aquatic environment: Sulfa drugs containing five-membered heterocyclic groups. *Environ Sci Technol* 38:3933-3940.

[114] Solomon KR, Mabury SA. 2004. Aquatic persistence of eight pharmaceuticals in a microcosm study. *Environ Toxicol Chem* 23:1431-1440.

[115] Drillia P, Dokianakis SN, Fountoulakis MS, Kornaros M, Stamatelatou K, Lyberatos G. 2005. On the occasional biodegradation of pharmaceuticals in the activated sludge process: The example of the antibiotic sulfamethoxazole. *J Hazard Mater* 122:259-265.

[116] Radke M, Lauwigi C, Heinkele G, Mürdter TE, Letzel M. 2009. Fate of the antibiotic sulfamethoxazole and its two major human metabolites in a water sediment test. *Environ Sci Technol* 43:3135-3141.

[117] Doudoroff P, Katz M. 1953. Critical review of literature on the toxicity of industrial wastes and their components to fish. II. The metals as salts. *Sewage Ind Waste* 25:802-839.

[118] Doudoroff P, Katz M. 1950. Critical review of literature on the toxicity of industrial wastes and their components to fish. I. Alkalies, acids, and inorganic gases. *Sewage Ind Waste* 22:1432-1458.

[119] Wharfe J. 2009. Historical perspective and overview. In Thompson KC, Wadhia K, Loibner AP, eds, *Environmental Toxicity Testing*. Blackwell Publishing Ltd., pp 1-32.

[120] Johnson I, Whitehouse P, Crane M. 2009. Effective monitoring of the environment for toxicity. In Thompson KC, Wadhia K, Loibner AP, eds, *Environmental Toxicity Testing*. Blackwell Publishing Ltd., pp 33-60.

[121] Calow P. 1989. The choice and implementation of environmental bioassays. *Hydrobiologia* 188-189:61-64.

[122] van den Brink PJ, Sibley PK, Ratte HT, Baird DJ, Nabholz JV, Sanderson H. 2008. Extrapolation of effects measures across levels of biological organization in ecological risk assessments. In Solomon KR, Sibley PK, Sanderson H, Richards SM, Dyer SD, Brock TCM, van den Brink PJ, Posthuma L, de Zwart D, eds, *Extrapolation Practice for Ecological Effect Characterization of Chemicals (EXPECT)*. SETAC Press, pp 105-134.

[123] Tchobanoglous G, Burton FL, Stensel HD. 2003. *Wastewater Engineering, Treatment, and Reuse.* McGraw-Hill, USA.

[124] USEPA. 2002. *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms.* U.S. Environmental Protection Agency, Office of Water, Washington, DC.

[125] APHA, AWWA, WEF. 1998. *Standard Methods for Examination of Water and Wastewater*. American Public Health Association, American Water Works Association, Water Environment Federation, US.

[126] Markert BA, Breure AM, Zechmeister HG. 2003. *Bioindicators and Biomonitors: Principles, Concepts, and Applications.* Elsevier, Amsterdam, Netherlands.

[127] Farré M, Klöter G, Petrovic M, Alonso MC, de Alda MJL, Barceló D. 2002. Identification of toxic compounds in wastewater treatment plants during a field experiment. *Anal Chim Acta* 456:19-30.

[128] Jones OAH, Voulvoulis N, Lester JN. 2007. The occurrence and removal of selected pharmaceutical compounds in a sewage treatment works utilising activated sludge treatment. *Environ Pollut* 145:738-744.

[129] Stülten D, Zühlke S, Lamshöft M, Spiteller M. 2008. Occurrence of diclofenac and selected metabolites in sewage effluents. *Sci Total Environ* 405:310-316.

[130] Kümmerer K. 2004. *Pharmaceuticals in the Environment: Sources, Fate, Effects and Risks.* Springer-Verlag, Berlin, Germany.

[131] Hernando MD, Petrovic M, Fernández-Alba AR, Barceló D. 2004. Analysis by liquid chromatography-electrospray ionization tandem mass spectrometry and acute toxicity evaluation for β-blockers and lipid-regulating agents in wastewater samples. *J Chromatogr A* 1046:133-140.

[132] Han GH, Hur HG, Kim SD. 2006. Ecotoxicological risk of pharmaceuticals from wastewater treatment plants in Korea: Occurrence and toxicity to *Daphnia magna*. *Environ Toxicol Chem* 25:265-271.

[133] Yu JT, Bouwer EJ, Coelhan M. 2006. Occurrence and biodegradability studies of selected pharmaceuticals and personal care products in sewage effluent. *Agric Water Manage* 86:72-80.

[134] Vieno N, Tuhkanen T, Kronberg L. 2007. Elimination of pharmaceuticals in sewage treatment plants in Finland. *Water Res* 41:1001-1012.

[135] Coetsier CM, Spinelli S, Lin L, Roig B, Touraud E. 2009. Discharge of pharmaceutical products (PPs) through a conventional biological sewage treatment plant: MECs vs PECs? *Environ Int* 35:787-792.

[136] Muñoz I, Gómez-Ramos MJ, Agüera A, Fernández-Alba AR, García-Reyes JF, Molina-Díaz A. 2009. Chemical evaluation of contaminants in wastewater effluents and the environmental risk of reusing effluents in agriculture. *TrAC-Trend Anal Chem* 28:676-694.

[137] Zhou JL, Zhang ZL, Banks E, Grover D, Jiang JQ. 2009. Pharmaceutical residues in wastewater treatment works effluents and their impact on receiving river water. *J Hazard Mater* 166:655-661.

[138] USEPA. 1991. *Technical Support Document for Water Quality-Based Toxics Control.* EPA/505/2–90001. USEPA, Washington, DC, USA.

[139] Farré M, Martínez E, Hernando MD, Fernández-Alba A, Fritz J, Unruh E, Mihail O, Sakkas V, Morbey A, Albanis T, Brito F, Hansen PD, Barceló D. 2006. European ring exercise on water toxicity using different bioluminescence inhibition tests based on *Vibrio fischeri*, in support to the implementation of the water framework directive. *Talanta* 69:323-333.

[140] Hernando MD, Malato O, Farré M, Fernandez-Alba AR, Barceló D. 2006. Application of ring study: Water toxicity determinations by bioluminescence assay with *Vibrio fischeri*. *Talanta* 69:370-376.

[141] Grothe DR, Dickson KL, Reed-Judkins DK. 1996. *Whole Effluent Toxicity Testing: An Evaluation of Methods and Prediction of Receiving System Impacts. Proceedings from a SETAC-Sponsored Pellston Workshop.* Society of Environmental Toxicology and Chemistry, Pensacola, FL, USA.

[142] Mount DI. 1998. Midcourse corrections in WET testing program. *Soc Environ Toxicol Chem News* 18:19-20.

[143] Sarakinos HC, Bermingham N, White PA, Rasmussen JB. 2000. Correspondence between whole effluent toxicity and the presence of priority substances in complex industrial effluents. *Environ Toxicol Chem* 19:63-71.

[144] Costan G, Bermingham N, Blaise C, Ferard JF. 1993. Potential ecotoxic effects probe (PEEP): A novel index to assess and compare the toxic potential of industrial effluents. *Environ Toxicol Water Qual* 8:115-140.

[145] Yi X, Kim E, Jo H, Schlenk D, Jung J. 2009. A toxicity monitoring study on identification and reduction of toxicants from a wastewater treatment plant. *Ecotoxicol Environ Saf* 72:1919-1924.

[146] DLgs 152/2006. 2006. Decreto Legislativo 3 April 2006, No. 152. *Gazzeta Ufficiale* 88:14-04.

[147] Law 106 (I)/2002. 2002. Water and Soil Pollution Control Law in Cyprus, 12th July of 2002 (in Greek). *Cyprus Government Gazette* 3621:2121-2151.

[148] Cleuvers M. 2005. Initial risk assessment for three β-blockers found in the aquatic environment. *Chemosphere* 59:199-205.

[149] Küster A, Alder AC, Escher BI, Duis K, Fenner K, Garric J, Hutchinson TH, Lapen DR, Péry A, Römbke J, Snape J, Ternes T, Topp E, Wehrhan A, Knackerk T. 2010. Environmental risk assessment of human pharmaceuticals in the European union: A case study with the β-blocker atenolol. *Integr Environ assess manage* 6:514-523.

[150] Pascoe D, Karntanut W, Müller CT. 2003. Do pharmaceuticals affect freshwater invertebrates? A study with the cnidarian *Hydra vulgaris*. *Chemosphere* 51:521-528.

[151] Parolini M, Quinn B, Binelli A, Provini A. 2011. Cytotoxicity assessment of four pharmaceutical compounds on the zebra mussel (*Dreissena polymorpha*) haemocytes, gill and digestive gland primary cell cultures. *Chemosphere* 84:91-100.

[152] Huggett DB, Brooks BW, Peterson B, Foran CM, Schlenk D. 2002. Toxicity of select beta adrenergic receptor-blocking pharmaceuticals (β-blockers) on aquatic organisms. *Arch Environ Contam Toxicol* 43:229-235.

[153] Winter MJ, Lillicrap AD, Caunter JE, Schaffner C, Alder AC, Ramil M, Ternes TA, Giltrow E, Sumpter JP, Hutchinson TH. 2008. Defining the chronic impacts of atenolol on embryo-larval development and reproduction in the fathead minnow (*Pimephales promelas*). *Aquat Toxicol* 86:361-369.

[154] DellaGreca M, Iesce MR, Pistillo P, Previtera L, Temussi F. 2009. Unusual products of the aqueous chlorination of atenolol. *Chemosphere* 74:730-734.

[155] Calleja MC, Persoone G, Geladi P. 1994. Human acute toxicity prediction of the first 50 MEIC chemicals by a battery of ecotoxicological tests and physicochemical properties. *Food Chem Toxicol* 32:173-187.

[156] Wharfe ES, Winder CL, Jarvis RM, Goodacre R. 2010. Monitoring the effects of chiral pharmaceuticals on aquatic microorganisms by metabolic fingerprinting. *Appl Environ Microbiol* 76:2075-2085.

[157] Brambilla G, Mattioli F, Robbiano L, Martelli A. 2010. Genotoxicity and carcinogenicity testing of pharmaceuticals: Correlations between induction of DNA lesions and carcinogenic activity. *Mutat Res Rev Mutat Res* 705:20-39.

[158] Télez M, Ortiz-Lastra E, Gonzalez AJ, Flores P, Huerta I, Ramírez JM, Barasoain M, Criado B, Arrieta I. 2010. Assessment of the genotoxicity of atenolol in human peripheral blood lymphocytes: correlation between chromosomal fragility and content of micronuclei. *Mutat Res* 695:46-54.

[159] Triebskorn R, Casper H, Scheil V, Schwaiger J. 2007. Ultrastructural effects of pharmaceuticals (carbamazepine, clofibric acid, metoprolol, diclofenac) in rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*). *Anal Bioanal Chem* 387:1405-1416.

[160] Dzialowski EM, Turner PK, Brooks BW. 2006. Physiological and reproductive effects of beta adrenergic receptor antagonists in *Daphnia magna*. *Arch Environ Contam Toxicol* 50:503-510.

[161] Villegas-Navarro A, Rosas-L E, Reyes JL. 2003. The heart of *Daphnia magna*: Effects of four cardioactive drugs. *Comp Biochem Physiol C Toxicol Pharmacol* 136:127-134.

[162] Carlsson C, Johansson AK, Alvan G, Bergman K, Kühler T. 2006. Are pharmaceuticals potent environmental pollutants? Part I: Environmental risk assessments of selected active pharmaceutical ingredients. *Sci Total Environ* 364:67-87.

[163] Fent K, Escher C, Caminada D. 2006. Estrogenic activity of pharmaceuticals and pharmaceutical mixtures in a yeast reporter gene system. *Reprod Toxicol* 22:175-185.

[164] Okine LKN, Ioannides C, Parke DV. 1983. Studies on the possible mutagenicity of β-adrenergic blocker drugs. *Toxicol Lett* 16:167-174.

[165] Sheila A. D. 1990. The membrane stabilizing and β1-adrenoceptor blocking activity of (+)- and (−)-propranolol on the rat left atria. *Gen Pharmacol-Vasc S* 21:677-680.

[166] Ferrari B, Mons R, Vollat B, Fraysse B, Paxéus N, Lo Giudice R, Pollio A, Garric J. 2004. Environmental risk assessment of six human pharmaceuticals: Are the current environmental risk assessment procedures sufficient for the protection of the aquatic environment? *Environ Toxicol Chem* 23:1344-1354.

[167] Escher BI, Bramaz N, Eggen RIL, Richter M. 2005. *In vitro* assessment of modes of toxic action of pharmaceutical in aquatic life. *Environ Sci Technol* 39:3090-3100.

[168] Calleja MC, Persoone G, Geladi P. 1994. Comparative acute toxicity of the first 50 multicentre evaluation of *in vitro* cytotoxicity chemicals to aquatic non-vertebrates. *Arch Environ Contam Toxicol* 26:69-78.

[169] Ericson H, Thorsén G, Kumblad L. 2010. Physiological effects of diclofenac, ibuprofen and propranolol on Baltic Sea blue mussels. *Aquat Toxicol* 99:223-231.

[170] Christen V, Hickmann S, Rechenberg B, Fent K. 2010. Highly active human pharmaceuticals in aquatic systems: A concept for their identification based on their mode of action. *Aquat Toxicol* 96:167-181.

[171] Solé M, Shaw JP, Frickers PE, Readman JW, Hutchinson TH. 2010. Effects on feeding rate and biomarker responses of marine mussels experimentally exposed to propranolol and acetaminophen. *Anal Bioanal Chem* 396:649-656.

[172] Stanley JK, Ramirez AJ, Mottaleb M, Chambliss CK, Brooks BW. 2006. Enantiospecific toxicity of the β-blocker propranolol to *Daphnia magna* and *Pimephales promelas*. *Environ Toxicol Chem* 25:1780-1786.

[173] Lilius H, Hästbacka T, Isomaa B. 1995. Short Communication: A comparison of the toxicity of 30 reference chemicals to *Daphnia magna* and *Daphnia pulex*. *Environ Toxicol Chem* 14:2085-2088.

[174] Romero V, De La Cruz N, Dantas RF, Marco P, Giménez J, Esplugas S. 2011. Photocatalytic treatment of metoprolol and propranolol. *Catal Today* 161:115-120.

[175] Bartram AE, Winter MJ, Huggett DB, McCormack P, Constantine LA, Hetheridge MJ, Hutchinson TH, Kinter LB, Ericson JF, Sumpter JP, Owen SF. 2011. *In vivo* and *in vitro* liver and gill EROD activity in rainbow trout (*Oncorhynchus mykiss*) exposed to the beta-blocker propranolol. *Environ Toxicol* doi: 10.1002/tox.20684.

[176] Laville N, Aït-Ässa S, Gomez E, Casellas C, Porcher JM. 2004. Effects of human pharmaceuticals on cytotoxicity, EROD activity and ROS production in fish hepatocytes. *Toxicology* 196:41-55.

[177] Kim JW, Ishibashi H, Yamauchi R, Ichikawa N, Takao Y, Hirano M, Koga M, Arizono K. 2009. Acute toxicity of pharmaceutical and personal care products on freshwater crustacean (*Thamnocephalus platyurus*) and fish (*Oryzias latipes*). *J Toxicol Sci* 34:227-232.

[178] Owen SF, Huggett DB, Hutchinson TH, Hetheridge MJ, Kinter LB, Ericson JF, Sumpter JP. 2009. Uptake of propranolol, a cardiovascular pharmaceutical, from water into fish plasma and its effects on growth and organ biometry. *Aquat Toxicol* 93:217-224.

[179] Burleson ML, Milsom WK. 1990. Propranolol inhibits O_2-sensitive chemoreceptor activity in trout gills. *Am J Physiol-Reg I* 258:R1089-R1091.

[180] Giltrow E, Eccles PD, Winter MJ, McCormack PJ, Rand-Weaver M, Hutchinson TH, Sumpter JP. 2009. Chronic effects assessment and plasma concentrations of the β-blocker propranolol in fathead minnows (*Pimephales promelas*). *Aquat Toxicol* 95:195-202.

[181] DeLorenzo ME, Fleming J. 2008. Individual and mixture effects of selected pharmaceuticals and personal care products on the marine phytoplankton species *Dunaliella tertiolecta*. *Arch Environ Contam Toxicol* 54:203-210.

[182] Ferrari B, Paxéus N, Giudice RL, Pollio A, Garric J. 2003. Erratum: Ecotoxicological impact of pharmaceuticals found in treated wastewaters: Study of carbamazepine, clofibric acid, and diclofenac (Ecotoxicology and Environmental Safety (2003) 55 (359-370)). *Ecotoxicol Environ Saf* 56:450.

[183] Oviedo-Gómez DGC, Galar-Martínez M, García-Medina S, Razo-Estrada C, Gómez-Oliván LM. 2010. Diclofenac-enriched artificial sediment induces oxidative stress in *Hyalella azteca*. *Environ Toxicol Pharmacol* 29:39-43.

[184] Schwaiger J, Ferling H, Mallow U, Wintermayr H, Negele RD. 2004. Toxic effects of the non-steroidal anti-inflammatory drug diclofenac. Part I: Histopathological alterations and bioaccumulation in rainbow trout. *Aquat Toxicol* 68:141-150.

[185] Parolini M, Binelli A, Provini A. 2011. Assessment of the potential cyto-genotoxicity of the nonsteroidal anti-inflammatory drug (NSAID) diclofenac on the zebra mussel (*Dreissena polymorpha*). *Water Air Soil Pollut* 217:589-601.

[186] Quinn B, Schmidt W, O'Rourke K, Hernan R. 2011. Effects of the pharmaceuticals gemfibrozil and diclofenac on biomarker expression in the zebra mussel (*Dreissena polymorpha*) and their comparison with standardised toxicity tests. *Chemosphere* 84:657-663.

[187] Mehinto AC, Hill EM, Tyler CR. 2010. Uptake and biological effects of environmentally relevant concentrations of the nonsteroidal anti-inflammatory pharmaceutical diclofenac in rainbow trout (*Oncorhynchus mykiss*). *Environ Sci Technol* 44:2176-2182.

[188] Cuklev F, Kristiansson E, Fick J, Asker N, Förlin L, Larsson DGJ. 2011. Diclofenac in fish: Blood plasma levels similar to human therapeutic levels affect global hepatic gene expression. *Environ Toxicol Chem* 30:2126-2134.

[189] Hoeger B, Köllner B, Dietrich DR, Hitzfeld B. 2005. Water-borne diclofenac affects kidney and gill integrity and selected immune parameters in brown trout (*Salmo trutta f. fario*). *Aquat Toxicol* 75:53-64.

[190] Hong HN, Kim HN, Park KS, Lee S, Gu MB. 2007. Analysis of the effects diclofenac has on Japanese medaka (*Oryzias latipes*) using real-time PCR. *Chemosphere* 67:2115-2121.

[191] Nassef M, Kim SG, Seki M, Kang IJ, Hano T, Shimasaki Y, Oshima Y. 2010. *In ovo* nanoinjection of triclosan, diclofenac and carbamazepine affects embryonic development of medaka fish (*Oryzias latipes*). *Chemosphere* 79:966-973.

[192] Nassef M, Matsumoto S, Seki M, Khalil F, Kang IJ, Shimasaki Y, Oshima Y, Honjo T. 2010. Acute effects of triclosan, diclofenac and carbamazepine on feeding performance of Japanese medaka fish (*Oryzias latipes*). *Chemosphere* 80:1095-1100.

[193] Lee J, Ji K, Lim Kho Y, Kim P, Choi K. 2011. Chronic exposure to diclofenac on two freshwater cladocerans and Japanese medaka. *Ecotoxicol Environ Saf* 74:1216-1225.

[194] Hallare AV, Köhler H-, Triebskorn R. 2004. Developmental toxicity and stress protein responses in zebrafish embryos after exposure to diclofenac and its solvent, DMSO. *Chemosphere* 56:659-666.

[195] van den Brandhof E, Montforts M. 2010. Fish embryo toxicity of carbamazepine, diclofenac and metoprolol. *Ecotoxicol Environ Saf* 73:1862-1866.

[196] Lawrence JR, Swerhone GDW, Topp E, Korber DR, Neu TR, Wassenaar LI. 2007. Structural and functional responses of river biofilm communities to the nonsteroidal anti-inflammatory diclofenac. *Environ Toxicol Chem* 26:573-582.

[197] Paje MP, Kuhlicke UK, Winkler MW, Neu TN. 2002. Inhibition of lotic biofilms by diclofenac. *Appl Microbiol Biotechnol* 59:488-492.

[198] Richards N, Cook G, Simpson V, Hall S, Harrison N, Scott K. 2011. Qualitative detection of the NSAIDs diclofenac and ibuprofen in the hair of Eurasian otters (*Lutra lutra*) occupying UK waterways with GC-MS. *Eur J Wildlife Res* 57:1107-1114.

[199] Oaks JL, Gilbert M, Virani MZ, Watson RT, Meteyer CU, Rideout BA, Shivaprasad HL, Ahmed S, Iqbal Chaudhry MJ, Arshad M, Mahmood S, Ali A, Ahmed Khan A. 2004. Diclofenac residues as the cause of vulture population decline in Pakistan. *Nature* 427:630-633.

[200] Shultz S, Baral HS, Charman S, Cunningham AA, Das D, Ghalsasi GR, Goudar MS, Green RE, Jones A, Nighot P, Pain DJ, Prakash V. 2004. Diclofenac poisoning is widespread in declining vulture populations across the Indian subcontinent. *P Roy Soc Lond B Bio* 271:S458-S460.

[201] Green RE, Newton I, Shultz S, Cunningham AA, Gilbert M, Pain DJ, Prakash V. 2004. Diclofenac poisoning as a cause of vulture population declines across the Indian subcontinent. *J Appl Ecol* 41:793-800.

[202] Naidoo V, Wolter K, Cuthbert R, Duncan N. 2009. Veterinary diclofenac threatens Africa's endangered vulture species. *Regul Toxicol Pharm* 53:205-208.

[203] Hussain I, Khan MZ, Khan A, Javed I, Saleemi MK. 2008. Toxicological effects of diclofenac in four avian species. *Avian Pathol* 37:315-321.

[204] Rattner BA, Whitehead MA, Gasper G, Meteyer CU, Link WA, Taggart MA, Meharg AA, Pattee OH, Pain DJ. 2008. Apparent tolerance of turkey vultures (*Cathartes aura*) to the non-steroidal anti-inflammatory drug diclofenac. *Environ Toxicol Chem* 27:2341-2345.

[205] Haap T, Triebskorn R, Köhler H-. 2008. Acute effects of diclofenac and DMSO to *Daphnia magna*: Immobilisation and hsp70-induction. *Chemosphere* 73:353-359.

[206] Zwiener C, Frimmel FH. 2003. Short-term tests with a pilot sewage plant and biofilm reactors for the biological degradation of the pharmaceutical compounds clofibric acid, ibuprofen, and diclofenac. *Sci Total Environ* 309:201-211.

[207] Farré M, Ferrer I, Ginebreda A, Figueras M, Olivella L, Tirapu L, Vilanova M, Barceló D. 2001. Determination of drugs in surface water and wastewater samples by liquid chromatography-mass spectrometry: Methods and preliminary results including toxicity studies with *Vibrio fischeri*. *J Chromatogr A* 938:187-197.

[208] Brambilla G, Martelli A. 2009. Update on genotoxicity and carcinogenicity testing of 472 marketed pharmaceuticals. *Mutat Res -Rev Mutat* 681:209-229.

[209] Lawrence JR, Swerhone GDW, Wassenaar LI, Neu TR. 2005. Effects of selected pharmaceuticals on riverine biofilm communities. *Can J Microbiol* 51:655-669.

[210] Brain RA, Johnson DJ, Richards SM, Sanderson H, Sibley PK, Solomon KR. 2004. Effects of 25 pharmaceutical compounds to *Lemna gibba* using a seven-day static-renewal test. *Environ Toxicol Chem* 23:371-382.

[211] Cleuvers M. 2003. Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. *Toxicol Lett* 142:185-194.

[212] Pomati F, Netting AG, Calamari D, Neilan BA. 2004. Effects of erythromycin, tetracycline and ibuprofen on the growth of *Synechocystis* sp. and *Lemna minor*. *Aquat Toxicol* 67:387-396.

[213] De Lange HJ, Noordoven W, Murk AJ, Lürling M, Peeters ETHM. 2006. Behavioural responses of *Gammarus pulex* (Crustacea, Amphipoda) to low concentrations of pharmaceuticals. *Aquat Toxicol* 78:209-216.

[214] Quinn B, Gagné F, Blaise C. 2008. An investigation into the acute and chronic toxicity of eleven pharmaceuticals (and their solvents) found in wastewater effluent on the cnidarian, *Hydra attenuata*. *Sci Total Environ* 389:306-314.

[215] Han S, Choi K, Kim J, Ji K, Kim S, Ahn B, Yun J, Choi K, Khim JS, Zhang X, Giesy JP. 2010. Endocrine disruption and consequences of chronic exposure to ibuprofen in Japanese medaka (*Oryzias latipes*) and freshwater cladocerans *Daphnia magna* and *Moina macrocopa*. *Aquat Toxicol* 98:256-264.

[216] Pounds N, Maclean S, Webley M, Pascoe D, Hutchinson T. 2008. Acute and chronic effects of ibuprofen in the mollusc *Planorbis carinatus* (Gastropoda: Planorbidae). *Ecotoxicol Environ Saf* 70:47-52.

[217] Parolini M, Binelli A, Provini A. 2011. Chronic effects induced by ibuprofen on the freshwater bivalve *Dreissena polymorpha*. *Ecotoxicol Environ Saf* 74:1586-1594.

[218] Hayashi Y, Heckmann LH, Callaghan A, Sibly RM. 2008. Reproduction recovery of the crustacean *Daphnia magna* after chronic exposure to ibuprofen. *Ecotoxicology* 17:246-251.

[219] Gagné F, Blaise C, André C. 2006. Occurrence of pharmaceutical products in a municipal effluent and toxicity to rainbow trout (*Oncorhynchus mykiss*) hepatocytes. *Ecotoxicol Environ Saf* 64:329-336.

[220] Flippin JL, Huggett D, Foran CM. 2007. Changes in the timing of reproduction following chronic exposure to ibuprofen in Japanese medaka, *Oryzias latipes*. *Aquat Toxicol* 81:73-78.

[221] Gravel A, Vijayan MM. 2007. Non-steroidal anti-inflammatory drugs disrupt the heat shock response in rainbow trout. *Aquat Toxicol* 81:197-206.

[222] Gravel A, Wilson JM, Pedro DFN, Vijayan MM. 2009. Non-steroidal anti-inflammatory drugs disturb the osmoregulatory, metabolic and cortisol responses associated with seawater exposure in rainbow trout. *Comp Biochem Physiol C Toxicol Pharmacol* 149:481-490.

[223] David A, Pancharatna K. 2009. Developmental anomalies induced by a non-selective COX inhibitor (ibuprofen) in zebrafish (*Danio rerio*). *Environ Toxicol Pharmacol* 27:390-395.

[224] Ragugnetti M, Adams ML, Guimarães ATB, Sponchiado G, De Vasconcelos EC, De Oliveira CMR. 2011. Ibuprofen genotoxicity in aquatic environment: An experimental model using *Oreochromis niloticus*. *Water Air Soil Pollut* 218:361-364.

[225] Richards SM, Cole SE. 2006. A toxicity and hazard assessment of fourteen pharmaceuticals to *Xenopus laevis* larvae. *Ecotoxicology* 15:647-656.

[226] Harada A, Komori K, Nakada N, Kitamura K, Suzuki Y. 2008. Biological effects of PPCPs on aquatic lives and evaluation of river waters affected by different wastewater treatment levels. *Water Sci Technol* 58:1541-1546.

[227] Heckmann LH, Callaghan A, Hooper HL, Connon R, Hutchinson TH, Maund SJ, Sibly RM. 2007. Chronic toxicity of ibuprofen to *Daphnia magna*: Effects on life history traits and population dynamics. *Toxicol Lett* 172:137-145.

[228] Yu TH, Lin AYC, Panchangam SC, Hong PKA, Yang PY, Lin CF. 2011. Biodegradation and bio-sorption of antibiotics and non-steroidal anti-inflammatory drugs using immobilized cell process. *Chemosphere* 84:1216-1222.

[229] Elvers KT, Wright SJL. 1995. Antibacterial activity of the anti-inflammatory compound ibuprofen. *Lett Appl Microbiol* 20:82-84.

[230] Marco-Urrea E, Pérez-Trujillo M, Vicent T, Caminal G. 2009. Ability of white-rot fungi to remove selected pharmaceuticals and identification of degradation products of ibuprofen by *Trametes versicolor*. *Chemosphere* 74:765-772.

[231] Philipose B, Singh R, Khan KA, Giri AK. 1997. Comparative mutagenic and genotoxic effects of three propionic acid derivatives ibuprofen, ketoprofen and naproxen. *Mutat Res Genet Toxicol Environ Mutagen* 393:123-131.

[232] Liu B, Nie X, Liu W, Snoeijs P, Guan C, Tsui MTK. 2011. Toxic effects of erythromycin, ciprofloxacin and sulfamethoxazole on photosynthetic apparatus in *Selenastrum capricornutum*. *Ecotoxicol Environ Saf* 74:1027-1035.

[233] Flaherty CM, Dodson SI. 2005. Effects of pharmaceuticals on *Daphnia* survival, growth, and reproduction. *Chemosphere* 61:200-207.

[234] Kim Y, Cerniglia CE. 2005. Influence of erythromycin A on the microbial populations in aquaculture sediment microcosms. *Aquatic Toxicology* 73:230-241.

[235] Rodgers FG, Tzianabos AO, Elliott TSJ. 1990. The effect of antibiotics that inhibit cell-wall, protein, and DNA synthesis on the growth and morphology of *Legionella pneumophila*. *J Med Microbiol* 31:37-44.

[236] Sofer D, Gilboa-Garber N, Belz A, Garber NC. 1999. "Subinhibitory" erythromycin represses production of *Pseudomonas aeruginosa* lectins, autoinducer and virulence factors. *Chemotherapy* 45:335-341.

[237] Tateda K, Hirakata Y, Furuya N, Ohno A, Yamaguchi K. 1993. Effects of sub-MICs of erythromycin and other macrolide antibiotics on serum sensitivity of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 37:675-680.

[238] Louvet JN, Giammarino C, Potier O, Pons MN. 2010. Adverse effects of erythromycin on the structure and chemistry of activated sludge. *Environ Pollut* 158:688-693.

[239] Eguchi K, Nagase H, Ozawa M, Endoh YS, Goto K, Hirata K, Miyamoto K, Yoshimura H. 2004. Evaluation of antimicrobial agents for veterinary use in the ecotoxicity test using microalgae. *Chemosphere* 57:1733-1738.

[240] Ando T, Nagase H, Eguchi K, Hirooka T, Nakamura T, Miyamoto K, Hirata K. 2007. A novel method using cyanobacteria for ecotoxicity test of veterinary antimicrobial agents. *Environ Toxicol Chem* 26:601-606.

[241] Christensen AM, Ingerslev F, Baun A. 2006. Ecotoxicity of mixtures of antibiotics used in aquacultures. *Environ Toxicol Chem* 25:2208-2215.

[242] Dojmi Di Delupis G, Macri A, Civitareale C, Migliore L. 1992. Antibiotics of zootechnical use: Effects of acute high and low dose contamination on *Daphnia magna straus*. *Aquat Toxicol* 22:53-60.

[243] Alexy R, Kümpel T, Kümmerer K. 2004. Assessment of degradation of 18 antibiotics in the Closed Bottle Test. *Chemosphere* 57:505-512.

[244] Migliore L, Civitareale C, Brambilla G, Delupis GDD. 1997. Toxicity of several important agricultural antibiotics to *Artemia*. *Water Res* 31:1801-1806.

[245] Kümmerer K, Alexy R, Hüttig J, Schöll A. 2004. Standardized tests fail to assess the effects of antibiotics on environmental bacteria. *Water Res* 38:2111-2116.

[246] Amin MM, Zilles JL, Greiner J, Charbonneau S, Raskin L, Morgenroth E. 2006. Influence of the antibiotic erythromycin on anaerobic treatment of a pharmaceutical wastewater. *Environ Sci Technol* 40:3971-3977.

[247] National Toxicology Program. 1988. *National Toxicology Program Toxicology and Carcinogenesis Studies of Erythromycin Stearate (CAS no. 643-22-1) in F344/N Rats and B6C3F1 Mice (Feed Studies).* 338. National Toxicology Program.

[248] Drlica K, Zhao X. 1997. DNA gyrase, topoisomerase IV, and the 4-quinolones. *Microbiol Mol Biol Rev* 61:377-392.

[249] Pan X, Zhang D, Chen X, Mu G, Li L, Bao A. 2009. Effects of levofloxacin hydrochlordie on photosystem II activity and heterogeneity of *Synechocystis sp*. *Chemosphere* 77:413-418.

[250] Smith AJ, Balaam JL, Ward A. 2007. The development of a rapid screening technique to measure antibiotic activity in effluents and surface water samples. *Mar Pollut Bull* 54:1940-1946.

[251] Kümmerer K, Al-Ahmad A, Mersch-Sundermann V. 2000. Biodegradability of some antibiotics, elimination of the genotoxicity and affection of wastewater bacteria in a simple test. *Chemosphere* 40:701-710.

[252] Chételat A, Albertini S, Gocke E. 1996. The photomutagenicity of fluoroquinolones in tests for gene mutation, chromosomal aberration, gene conversion and DNA breakage (Comet assay). *Mutagenesis* 11:497-504.

[253] Hussy P, Maass G, Tümmler B, Grosse F, Schomburg U. 1986. Effect of 4-quinolones and novobiocin on calf thymus DNA polymerase alpha primase complex, topoisomerases I and II, and growth of mammalian lymphoblasts. *Antimicrob Agents Chemother* 29:1073-1078.

[254] Li Q, Peng S, Sheng Z, Wang Y. 2010. Ofloxacin induces oxidative damage to joint chondrocytes of juvenile rabbits: Excessive production of reactive oxygen species, lipid peroxidation and DNA damage. *Eur J Pharmacol* 626:146-153.

[255] Robinson AA, Belden JB, Lydy MJ. 2005. Toxicity of fluoroquinolone antibiotics to aquatic organisms. *Environ Toxicol Chem* 24:423-430.

[256] Backhaus T, Grimme LH. 1999. The toxicity of antibiotic agents to the luminescent bacterium *Vibrio fischeri*. *Chemosphere* 38:3291-3301.

[257] Shen LL, Mitscher LA, Sharma PN, O'Donnell TJ, Chu DWT, Cooper CS, Rosen T, Pernet AG. 1989. Mechanism of inhibition of DNA gyrase by quinolone antibacterials: A cooperative drug-DNA binding model. *Biochemistry* 28:3886-3894.

[258] Brain RA, Ramirez AJ, Fulton BA, Chambliss CK, Brooks BW. 2008. Herbicidal effects of sulfamethoxazole in *Lemna gibba*: Using p-aminobenzoic acid as a biomarker of effect. *Environ Sci Technol* 42:8965-8970.

[259] Baran W, Sochacka J, Wardas W. 2006. Toxicity and biodegradability of sulfonamides and products of their photocatalytic degradation in aqueous solutions. *Chemosphere* 65:1295-1299.

[260] Yang L-, Ying G-, Su H-, Stauber JL, Adams MS, Binet MT. 2008. Growth-inhibiting effects of 12 antibacterial agents and their mixtures on the freshwater microalga *Pseudokirchneriella subcapitata*. *Environ Toxicol Chem* 27:1201-1208.

[261] Park S, Choi K. 2008. Hazard assessment of commonly used agricultural antibiotics on aquatic ecosystems. *Ecotoxicology* 17:526-538.

[262] Kim Y, Choi K, Jung J, Park S, Kim PG, Park J. 2007. Aquatic toxicity of acetaminophen, carbamazepine, cimetidine, diltiazem and six major sulfonamides, and their potential ecological risks in Korea. *Environ Int* 33:370-375.

[263] Jung J, Kim Y, Kim J, Jeong D-, Choi K. 2008. Environmental levels of ultraviolet light potentiate the toxicity of sulfonamide antibiotics in *Daphnia magna*. *Ecotoxicology* 17:37-45.

[264] Al-Ahmad A, Daschner FD, Kümmerer K. 1999. Biodegradability of cefotiam, ciprofloxacin, meropenem, penicillin G, and sulfamethoxazole and inhibition of waste water bacteria. *Arch Environ Contam Toxicol* 37:158-163.

[265] Göbel A, Thomsen A, McArdell CS, Joss A, Giger W. 2005. Occurrence and sorption behavior of sulfonamides, macrolides, and trimethoprim in activated sludge treatment. *Environ Sci Technol* 39:3981-3989.

[266] Carballa M, Omil F, Ternes T, Lema JM. 2007. Fate of pharmaceutical and personal care products (PPCPs) during anaerobic digestion of sewage sludge. *Water Res* 41:2139-2150.

[267] Demoling LA, Bååth E, Greve G, Wouterse M, Schmitt H. 2009. Effects of sulfamethoxazole on soil microbial communities after adding substrate. *Soil Biol Biochem* 41:840-848.

[268] Mandell G, Petri W. 1996. Goodman and Gilman's the pharmacological basis of therapeutics. In Goodman L, Limbird L, Millinof P, Ruddon R, Goodman Gilman A, eds, *Antimicrobial Agents: Sulfonamides,Trimethoprim-Sulfamethoxazole, Quinolones, and Agents for Urinary Tract Infections*, 9thed. McGraw Hill, New York, pp 1057-1072.

[269] Stevenson AC, Clarke G, Patel CR, Hughes DTD. 1973. Chromosomal studies *in vivo* and *in vitro* of trimethoprim and sulfamethoxazole (co-trimoxazole). *Mutat Res-Fund Mol M* 17:255-260.

[270] Sørensen PJ, Jensen MK. 1981. Cytogenetic studies in patients treated with trimethoprim—sulfamethoxazole. *Mutat Res Genet Toxicol Environ Mutagen* 89:91-94.

[271] Abou-Eisha A, Marcos R, Creus A. 2004. Genotoxicity studies on the antimicrobial drug sulfamethoxazole in cultured human lymphocytes. *Mutat Res Genet Toxicol Environ Mutagen* 564:51-56.

[272] Daughton CG, Ternes TA. 1999. Pharmaceuticals and personal care products in the environment: Agents of subtle change? *Environ Health Perspect* 107:907-938.

[273] Scholz S, Gutzeit HO. 2000. 17-α-ethinylestradiol affects reproduction, sexual differentiation and aromatase gene expression of the medaka (*Oryzias latipes*). *Aquat Toxicol* 50:363-373.

[274] Isidori M, Lavorgna M, Nardelli A, Parrella A, Previtera L, Rubino M. 2005. Ecotoxicity of naproxen and its phototransformation products. *Sci Total Environ* 348:93-101.

[275] Sanderson H, Brain RA, Johnson DJ, Wilson CJ, Solomon KR. 2004. Toxicity classification and evaluation of four pharmaceuticals classes: antibiotics, antineoplastics, cardiovascular, and sex hormones. *Toxicology* 203:27-40.

[276] Muñoz I, Rodríguez A, Rosal R, Fernández-Alba AR. 2009. Life Cycle Assessment of urban wastewater reuse with ozonation as tertiary treatment: A focus on toxicity-related impacts. *Sci Total Environ* 407:1245-1256.

[277] Fent K, Weston AA, Caminada D. 2006. Ecotoxicology of human pharmaceuticals. *Aquat Toxicol* 76:122-159.

[278] Hapeshi E, Achilleos A, Vasquez MI, Michael C, Xekoukoulotakis NP, Mantzavinos D, Fatta-Kassinos D. 2010. Drugs degrading photocatalytically: Kinetics and mechanisms of ofloxacin and atenolol removal on titania suspensions. *Water Res* 44:1737-1746.

[279] ISO. 2000. *ISO 11843: Capability of Detection. Part 2: Methodology in the Linear Calibration Case.* International Organization for Standardization, Switzerland.

[280] National Institute of Physics and Biophysics. 2012. *Pseudokirchneriella subcapitata*. 2012.

[281] OECD. 2011. *Test no. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test.* Organisation for Economic Co-operation and Development.

[282] *Lepidium sativum*. 2012. 2012. Available at: www.wikipedia.com/

[283] OECD. 2006. *Test no. 208: Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test.* Organisation for Economic Co-operation and Development.

[284] Liber K, Solomon KR. 1994. Acute and chronic toxicity of 2,3,4,6-tetrachlorophenol and pentachlorophenol to *Daphnia* and rotifers. *Arch Environ Contam Toxicol* 26:212-221.

[285] Kato Y, Kobayashi K, Watanabe H, Iguchi T. 2011. Environmental sex determination in the branchiopod crustacean *Daphnia magna*: Deep conservation of a double sex gene in the sex-determining pathway. *PLoS Genet* 7:e1001345.

[286] OECD. 2004. *Test no. 202: Daphnia Sp. Acute Immobilisation Test.* Organisation for Economic Co-operation and Development.

[287] Léger PH, Bengtson DA, Simpson KL, Sorgeloos P. 1986. The use and nutritional value of *Artemia* as a food source. In Barnes M, ed, *Marine Biology and Oceanography: An Annual Review*. Aberdeen University Press, Aberdeen, pp 521-563.

[288] SINTEF. 2009. *Artemia salina*. 2012. Available at: www.sintef.no/

[289] MOA. 2012. Ministry of Agriculture, Natural Resources and the Environment. 2011.

[290] Ruby EG. 1996. Lessons from a cooperative, bacterial-animal association: The *Vibrio fischeri–Euprymna scolopes* light organ symbiosis. *Annu Rev Microbiol* 50:591-624.

[291] *Pseudomonas putida*. 2010. 2012. Available at: http://news.thomasnet.com/green_clean/2010/12/29/bioplastic-the-water-bottle-that-wont-live-a-thousand-years/

[292] Otenio MH, Silva, Maria Teresa Lopes da, Marques MLO, Roseiro JC, Bidoia ED. 2005. Benzene, toluene and xylene biodegradation by *Pseudomonas putida* CCMI 852. *Brazilian J Microbiol* 36:258-261.

[293] ISO. 1995. ISO 10712: Water quality-*Pseudomonas putida* growth inhibition test (*Pseudomonas* cell multiplication inhibition test). *International Organization for Standardization*.

[294] Routledge EJ, Sumpter JP. 1996. Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. *Environ Toxicol Chem* 15:241-248.

[295] Holbrook RD, Novak JT, Grizzard TJ, Love NG. 2002. Estrogen receptor agonist fate during wastewater and biosolids treatment processes:a mass balance analysis. *Environ Sci Technol* 36:4533-4539.

[296] OECD. 1992. *Test no. 301: Ready Biodegradability*. Organisation for Economic Co-operation and Development.

[297] Fenech M. 2007. Cytokinesis-block micronucleus cytome assay. *Nat Protocols* 2:1084-1104.

[298] OECD. 2010. *Test no. 487: in Vitro Mammalian Cell Micronucleus Test.* Organisation for Economic Co-operation and Development.

[299] Meriç S, Ovez S, Kaptan D, Orhon D. 2003. Operational strategies for a small wastewater treatment plant using OUR, microscopic analysis, and toxicity test. *J Environ Sci Health A Tox Hazard Subst Environ Eng* 38:2329-2338.

[300] Chapman PM. 2000. Whole effluent toxicity testing-usefulness, level of protection, and risk assessment. *Environ Toxicol Chem* 19:3-13.

[301] Persoone G, Marsalek B, Blinova I, Törökne A, Zarina D, Manusadzianas L, Nalecz-Jawecki G, Tofan L, Stepanova N, Tothova L, Kolar B. 2003. A practical and user-friendly toxicity classification system with microbiotests for natural waters and wastewaters. *Environ Toxicol* 18:395-402.

[302] Fatta-Kassinos D, Kalavrouziotis IK, Koukoulakis PH, Vasquez MI. 2011. The risks associated with wastewater reuse and xenobiotics in the agroecological environment. *Sci Total Environ* 409:3555-3563.

[303] Brain RA, Hanson ML, Solomon KR, Brooks BW. 2008. Aquatic plants exposed to pharmaceuticals: effects and risks. In Whitacre DM, ed, *Reviews of Environmental Contamination and Toxicology*, Vol192. Springer New York, pp 67-115.

[304] Pedrero F, Kalavrouziotis I, Alarcón JJ, Koukoulakis P, Asano T. 2010. Use of treated municipal wastewater in irrigated agriculture—Review of some practices in Spain and Greece. *Agric Water Manage* 97:1233-1241.

[305] Rizzo L, Meriç S, Kassinos D, Guida M, Russo F, Belgiorno V. 2009. Degradation of diclofenac by TiO_2 photocatalysis: UV absorbance kinetics and process evaluation through a set of toxicity bioassays. *Water Res* 43:979-988.

[306] Ra J, Kim H, Chang N, Kim S. 2007. Whole Effluent Toxicity (WET) tests on wastewater treatment plants with *Daphnia magna* and *Selenastrum capricornutum*. *Environ Monit Assess* 129:107-113.

[307] Rizzo L, Meriç S, Guida M, Kassinos D, Belgiorno V. 2009. Heterogenous photocatalytic degradation kinetics and detoxification of an urban wastewater treatment plant effluent contaminated with pharmaceuticals. *Water Res* 43:4070-4078.

[308] Naddeo V, Meriç S, Kassinos D, Belgiorno V, Guida M. 2009. Fate of pharmaceuticals in contaminated urban wastewater effluent under ultrasonic irradiation. *Water Res* 43:4019-4027.

[309] Mendonça E, Picado A, Paixão SM, Silva L, Cunha MA, Leitão S, Moura I, Cortez C, Brito F. 2009. Ecotoxicity tests in the environmental analysis of wastewater treatment plants: Case study in Portugal. *J Hazard Mater* 163:665-670.

[310] Chapman PM, Bailey H, Canaria E. 2000. Toxicity of total dissolved solids associated with two mine effluents to chironomid larvae and early life stages of rainbow trout. *Environ Toxicol Chem* 19:210-214.

[311] Cooman K, Gajardo M, Nieto J, Bornhardt C, Vidal G. 2003. Tannery wastewater characterization and toxicity effects on *Daphnia spp.*. *Environ Toxicol* 18:45-51.

[312] Schilirò T, Pignata C, Rovere R, Fea E, Gilli G. 2009. The endocrine disrupting activity of surface waters and of wastewater treatment plant effluents in relation to chlorination. *Chemosphere* 75:335-340.

[313] Wu Q, Hu H, Zhao X, Sun Y. 2009. Effect of chlorination on the estrogenic/antiestrogenic activities of biologically treated wastewater. *Environ Sci Technol* 43:4940-4945.

[314] Johnson AC, Williams RJ. 2004. A model to estimate influent and effluent concentrations of estradiol, estrone, and ethinylestradiol at sewage treatment works. *Environ Sci Technol* 38:3649-3658.

[315] Žegura B, Heath E, Černoša A, Filipič M. 2009. Combination of *in vitro* bioassays for the determination of cytotoxic and genotoxic potential of wastewater, surface water and drinking water samples. *Chemosphere* 75:1453-1460.

[316] ECETOC. 2004. *Whole Effluent Assessment.* 94. European Centre for Ecotoxicology and Toxicology of Chemicals, Brussels, Belgium.

[317] Asano T, Cotruvo JA. 2004. Groundwater recharge with reclaimed municipal wastewater: health and regulatory considerations. *Water Res* 38:1941-1951.

[318] Brenner A. 2012. Limitations and challenges of wastewater reuse in Israel. *Clean-Soil Air Water* 1:3-9.

[319] Naddeo V, Belgiorno V, Kassinos D, Mantzavinos D, Meriç S. 2010. Ultrasonic degradation, mineralization and detoxification of diclofenac in water: Optimization of operating parameters. *Ultrason Sonochem* 17:179-185.

[320] Sparks T. 2000. *Statistics in Ecotoxicology.* Wiley, Chichester, New York.

[321] Bliss CI. 1934. The method of probits. *Science* 79:38-39.

[322] Finney DJ. 1952. *The Probit Analysis.* Cambridge University Press, Cambridge, England.

[323] Norberg-King T. 1993. *A Linear Interpolation Method for Sublethal Toxicity: The Inhibition Concentration (IC_p) Approach.* 03-93. National Effluent Toxicity Assessment Center, Environment Research Laboratory, U.S. Environmental Protection Agency, Duluth, MN.

[324] De Liguoro M, Di Leva V, Gallina G, Faccio E, Pinto G, Pollio A. 2010. Evaluation of the aquatic toxicity of two veterinary sulfonamides using five test organisms. *Chemosphere* 81:788-793.

[325] DeLorenzo ME, Keller JM, Arthur CD, Finnegan MC, Harper HE, Winder VL, Zdankiewicz DL. 2008. Toxicity of the antimicrobial compound triclosan and formation of the metabolite methyl-triclosan in estuarine systems. *Environ Toxicol* 23:224-232.

[326] Webb SF. 2004. A data based perspective on the environmental risk assessment of human pharmaceuticals I - collation of available ecotoxicity data. In Kümmerer K, ed, *Pharmaceuticals in the Environment: Sources, Fate, Effects and Risks*, 2nded. Springer, Berlin, Germany, pp 317-342.

[327] Fent K. 2008. Effects of pharmaceuticals on aquatic organisms. In Kummerer K, ed, *Pharmaceuticals in the Environment*, 2nded. Springer, Berlin, Germany, pp 175-203.

[328] Ankley GT, Black J, Garric J, Hutchinson TH, Iguchi T. 2005. A framework for assessing the hazard of pharmaceutical materials to aquatic species. In Williams RT, ed, *Human Pharmaceuticals: Assessing the Impacts on Aquatic ecosystems*. SETAC Press, Pensacola, Florida, pp 183-238.

[329] Gunnarsson L, Jauhiainen A, Kristiansson E, Nerman O, Larsson DGJ. 2008. Evolutionary Conservation of Human Drug Targets in Organisms used for Environmental Risk Assessments. *Environ Sci Technol* 42:5807-5813.

[330] Seiler JP. 2002. Pharmacodynamic activity of drugs and ecotoxicology - Can the two be connected? *Toxicol Lett* 131:105-115.

[331] Backhaus T, Scholze M, Grimme LH. 2000. The single substance and mixture toxicity of quinolones to the bioluminescent bacterium *Vibrio fischeri. Aquat Toxicol* 49:49-61.

[332] Neuwoehner J, Escher BI. 2011. The pH-dependent toxicity of basic pharmaceuticals in the green algae *Scenedesmus vacuolatus* can be explained with a toxicokinetic ion-trapping model. *Aquat Toxicol* 101:266-275.

[333] Heckmann LH, Sibly RM, Connon R, Hooper HL, Hutchinson TH, Maund SJ, Hill CJ, Bouetard A, Callaghan A. 2008. Linking molecular and organismal stress responses in *Daphnia magna*. *Gen Biol* 9:40.

[334] Scholz S, Schirmer K, Altenburger R. 2010. Pharmaceutical contaminants in urban water cycles: a discussion of novel concepts for environmental risk assessment. In Fatta-Kassinos D, Bester K, Kümmerer K, eds, *Xenobiotics in the Urban Water Cycle*, 1sted, Vol16. Springer Netherlands, pp 227-243.

[335] Sumpter JP. 2009. Protecting aquatic organisms from chemicals: the harsh realities. *Philos T R Soc A* 367:3877-3894.

[336] de Zwart D, Dyer SD, Posthuma L, Hawkins CP. 2006. Predictive models attribute effects on fish assemblages to toxicity and habitat alteration. *Ecol Appl* 16:1295-1310.

[337] Khetan SK, Collins TJ. 2007. Human pharmaceuticals in the aquatic environment: A challenge to green chemisty. *Chem Rev* 107:2319-2364.

[338] Altenburger R, Walter H, Grote M. 2004. What contributes to the combined effect of a complex mixture? *Environ Sci Technol* 38:6353-6362.

[339] Kortenkamp A, Backhaus T, Faust M. 2009. *State of the Art Report on Mixture Toxicity.* Study Contract No. 070307/2007/485103/ETU/D.1.

[340] SCCS, SCER, SCENIHR. 2011. *Opinion on the Toxicity and Assessment of Chemical Mixtures.*

[341] Chou TC. 2010. Drug combination studies and their synergy quantification using the chou-talalay method. *Cancer Res* 70:440-446.

[342] Backhaus T, Faust M. 2012. Predictive environmental risk assessment of chemical mixtures: a conceptual framework. *Environ Sci Technol* 46:2564-2573.

[343] Carpenter DO, Arcaro K, Spink DC. 2002. Understanding the human health effects of chemical mixtures. *Environ Health Perspect* 110:25-42.

[344] Chou TC. 2006. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacol Rev* 58:621-681.

[345] Rodea-Palomares I, Petre AL, Boltes K, Leganés F, Perdigón-Melón JA, Rosal R, Fernández-Piñas F. 2010. Application of the combination index (CI)-isobologram equation to study the toxicological interactions of lipid regulators in two aquatic bioluminescent organisms. *Water Res* 44:427-438.

[346] Rosal R, Rodea-Palomares I, Boltes K, Fernández-Piñas F, Leganés F, Gonzalo S, Petre A. 2010. Ecotoxicity assessment of lipid regulators in water and biologically treated wastewater using three aquatic organisms. *Environ Sci Pollut Res* 17:135-144.

[347] Fatta-Kassinos D, Vasquez MI, Kümmerer K. 2011. Transformation products of pharmaceuticals in surface waters and wastewater formed during photolysis and advanced oxidation processes - Degradation, elucidation of byproducts and assessment of their biological potency. *Chemosphere* 85:693-709.

[348] Sortino S, Marconi G, Giuffrida S, De Guidi G, Monti S. 1999. Photophysical properties of rufloxacin in neutral aqueous solution. *Photochem Photobiol* 70:731-736.

[349] Condorelli G, De Guidi G, Giuffrida S, Sortino S, Chillemi R, Sciuto S. 1999. Molecular mechanisms of photosensitization induced by drugs XII. Photochemistry and photosensitization of rufloxacin: An unusual photodegradation path for the antibacterials containing a fluoroquinolone-like chromophore. *Photochem Photobiol* 70:280-286.

[350] Sakore S, Choudhari S, Chakraborty B. 2010. Biowaiver monograph for immediate release solid oral dosage forms: Ofloxacin. *Int J Pharmcy Pharm Sci* 2:156-161.

[351] Sunderland J, Tobin CM, Hedges AJ, MacGowan AP, White LO. 2001. Antimicrobial activity of fluoroquinolone photodegradation products determined by parallel-line bioassay and high performance liquid chromatography. *J Antimicrob Chemother* 47:271-275.

[352] Quintana JB, Weiss S, Reemtsma T. 2005. Pathways and metabolites of microbial degradation of selected acidic pharmaceutical and their occurrence in municipal wastewater treated by a membrane bioreactor. *Water Res* 39:2654-2664.

[353] Gauthier H, Yargeau V, Cooper DG. 2010. Biodegradation of pharmaceuticals by *Rhodococcus rhodochrous* and *Aspergillus niger* by co-metabolism. *Sci Total Environ* 408:1701-1706.

[354] Rizzo L. 2011. Bioassays as a tool for evaluating advanced oxidation processes in water and wastewater treatment. *Water Res* 45:4311-4340.

[355] Fasani E, Rampi M, Albini A. 1999. Photochemistry of some fluoroquinolones: Effect of pH and chloride ion. *J Chem Soc Perkin Trans 2*:1901-1907.

[356] Paul T, Miller PL, Strathmann TJ. 2007. Visible-light-mediated TiO_2 photocatalysis of fluoroquinolone antibacterial agents. *Environ Sci Technol* 41:4720-4727.

[357] Vasconcelos TG, Kümmerer K, Henriques DM, Martins AF. 2009. Ciprofloxacin in hospital effluent: Degradation by ozone and photoprocesses. *J Hazard Mater* 169:1154-1158.

[358] Albini A, Monti S. 2003. Photophysics and photochemistry of fluoroquinolones. *Chem Soc Rev* 32:238-250.

[359] Schulze T, Weiss S, Schymanski E, von der Ohe PC, Schmitt-Jansen M, Altenburger R, Streck G, Brack W. 2010. Identification of a phytotoxic photo-transformation product of diclofenac using effect-directed analysis. *Environ Pollut* 158:1461-1466.

[360] Trovó AG, Nogueira RFP, Agüera A, Sirtori C, Fernández-Alba AR. 2009. Photodegradation of sulfamethoxazole in various aqueous media: Persistence, toxicity and photoproducts assessment. *Chemosphere* 77:1292-1298.

[361] Vasquez MI, Garcia-Käufer M, Hapeshi E, Menz J, Kostarelos K, Fatta-Kassinos D, Kümmerer K. 2012. Chronic ecotoxic effects to *Pseudomonas putida* and *Vibrio fischeri*, and cytostatic and genotoxic effects to the hepatoma cell line (HepG2) of ofloxacin photo(cata)lytically treated solutions. *Sci Total Environ* http://dx.doi.org/10.1016/j.scitotenv.2012.05.096.

[362] Brendler-Schwaab S, Czich A, Epe B, Gocke E, Kaina B, Müller L, Pollet D, Utesch D. 2004. Photochemical genotoxicity: Principles and test methods: Report of a GUM task force. *Mutat Res -Rev Mutat* 566:65-91.

[363] Viola G, Facciolo L, Canton M, Vedaldi D, Dall'Acqua F, Aloisi GG, Amelia M, Barbafina A, Elisei F, Latterini L. 2004. Photophysical and phototoxic properties of the antibacterial fluoroquinolones levofloxacin and moxifloxacin. *Chem Biodivers* 1:782-801.

[364] Gocke E, Albertini S, Chételat AA, Kirchner S, Muster W. 1998. The photomutagenicity of fluoroquinolones and other drugs. *Toxicol Lett* 102-103:375-381.

[365] de Guidi G, Bracchitta G, Catalfo A. 2011. Photosensitization reactions of fluoroquinolones and their biological consequences. *Photochem Photobiol* 87:1214-1229.

[366] Sharma BN, Li R, Bernhoff E, Gutteberg TJ, Rinaldo CH. 2011. Fluoroquinolones inhibit human polyomavirus BK (BKV) replication in primary human kidney cells. *Antiviral Res* 92:115-123.

[367] Garcia-Käufer M, Haddad T, Bergheim M, Gminski R, Gupta P, Mathur N, Kümmerer K, Mersch-Sundermann V. 2012. Genotoxic effect of ciprofloxacin during photolytic decomposition monitored by the in vitro micronucleus test (MNvit) in HepG2 cells. *Environ Sci Pollut Res* 10.1007/s11356-011-0686-y.

[368] Budai M, Gróf P, Zimmer A, Pápai K, Klebovich I, Ludányi K. 2008. UV light induced photodegradation of liposome encapsulated fluoroquinolones: An MS study. *J Photochem Photobiol A* 198:268-273.

[369] Sanchez G, Hidalgo ME, Vivanco JM, Escobar J. 2005. Induced and photoinduced DNA damage by quinolones: ciprofloxacin, ofloxacin and nalidixic acid determined by comet assay. *Photochem Photobiol* 81:819-822.

[370] Vasquez MI, Hapeshi E, Fatta-Kassinos D, Kümmerer K. 2012. Biodegradation potential of ofloxacin and its resulting transformation products during photolytic and photocatalytic treatment. *Environ Sci Pollut Res* doi: 10.1007/s11356-012-1096-5:1-8.

[371] An T, Yang H, Song W, Li G, Luo H, Cooper WJ. 2010. Mechanistic considerations for the advanced oxidation treatment of fluoroquinolone pharmaceutical compounds using TiO_2 heterogeneous catalysis. *J Phys Chem A* 114:2569-2575.

[372] Zhang H, Huang C. 2005. Oxidative transformation of fluoroquinolone antibacterial agents and structurally related amines by manganese oxide. *Environ Sci Technol* 39:4474-4483.

[373] Haque MM, Muneer M. 2007. Photodegradation of norfloxacin in aqueous suspensions of titanium dioxide. *J Hazard Mater* 145:51-57.

[374] Paul T, Dodd MC, Strathmann TJ. 2010. Photolytic and photocatalytic decomposition of aqueous ciprofloxacin: Transformation products and residual antibacterial activity. *Water Res* 44:3121-3132.

[375] Vasconcelos TG, Henriques DM, König A, Martins AF, Kümmerer K. 2009. Photo-degradation of the antimicrobial ciprofloxacin at high pH: Identification and biodegradability assessment of the primary by-products. *Chemosphere* 76:487-493.

[376] Ternes TA, Joss A, Siegrist H. 2004. Scrutinizing pharmaceuticals and personal care products in wastewater treatment. *Environ Sci Technol* 38:392A-399A.

[377] Michael I, Hapeshi E, Michael C, Fatta-Kassinos D. 2010. Solar Fenton and solar TiO_2 catalytic treatment of ofloxacin in secondary treated effluents: Evaluation of operational and kinetic parameters. *Water Res* 44:5450-5462.

[378] Escher BI, Fenner K. 2011. Recent advances in environmental risk assessment of transformation products. *Environ Sci Technol* 45:3835-3847.

[379] Brack W, Burgess R. 2011. Considerations for incorporating bioavailability in effect-directed analysis and toxicity identification evaluation. In Brack W, ed, *Effect-Directed Analysis of Complex Environmental Contamination*, Vol15. Springer Berlin / Heidelberg, pp 41-68.

ANNEX I: LEGISLATION CONCERNING CHEMICALS AND PHARMACEUTICALS

Directive/ Regulation	Commentary
Commission Regulation (EU) No 196/2010	Amending Annex I to Regulation (EC) No 689/2008 concerning the export and import of dangerous chemicals
Commission Regulation (EU) No 15/2010	Amending Annex I to Regulation (EC) No 689/2008 concerning the export and import of dangerous chemicals
Regulation (EC) No 689/2008	Concerning the export and import of dangerous chemicals
Commission Regulation (EC) No 1376/2007	Amending Annex I to Regulation (EC) No 304/2003 concerning the export and import of dangerous chemicals
Commission Regulation (EC) No 1238/2007	Laying down rules on the qualifications of the members of the Board of Appeal of the European Chemicals Agency
Council Regulation (EC) No 129/2007	Providing for duty-free treatment for specified pharmaceutical active ingredients bearing an international non-proprietary name (INN) from the World Health Organisation and specified products used for the manufacture of finished pharmaceuticals and amending Annex I to Regulation (EEC) No 2658/87
Regulation (EC) No 816/2006	On compulsory licensing of patents relating to the manufacture of pharmaceutical products for export to countries with public health problems
Directive 2004/10/EC	On the harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances
Commission Regulation (EC) No 1232/2002	Replacing the Annex to Council Regulation (EEC) No 3677/90 laying down measures to be taken to discourage the diversion of certain substances to the illicit manufacture of narcotic drugs and psychotropic substances and amending Regulation (EEC) No 3769/92
Council Regulation (EC) No 988/2002	Amending Regulation (EEC) No 3677/90 laying down measures to be taken to discourage the diversion of certain substances to the illicit manufacture of narcotic drugs and psychotropic substances
Council Regulation (EC) No 1116/2001	Amending Regulation (EEC) No 3677/90 laying down measures to be taken to discourage the diversion of certain substances to the illicit manufacture of narcotic drugs and psychotropic substances
Commission Regulation (EC) No 1610/2000	Amending Regulation (EEC) No 3769/92 implementing and amending Council Regulation (EEC) No 3677/90 laying down measures to be taken to discourage the diversion of certain substances to the illicit manufacture of narcotic drugs and psychotropic substances
Commission Directive 2000/39/EC	Establishing a first list of indicative occupational exposure limit values in implementation of Council Directive 98/24/EC on the protection of the health and safety of workers from the risks related to chemical agents at work
Council Directive 98/24/EC	On the protection of the health and safety of workers from the risks related to chemical agents at work (fourteenth individual Directive within the meaning of Article 16(1) of Directive 89/391/EEC)

Directive/ Regulation	Commentary
Council Regulation (EEC) No 900/92	Amending Regulation (EEC) No 3677/90 laying down measures to be taken to discourage the diversion of certain substances to the illicit manufacture of narcotic drugs and psychotropic substances
Commission Directive 91/322/EEC	On establishing indicative limit values by implementing Council Directive 80/1107/EEC on the protection of workers from the risks related to exposure to chemical, physical and biological agents at work
Directive 2008/105/EC	On environmental quality standards in the field of water policy, amending and subsequently repealing Council Directives 82/176/EEC, 83/513/EEC, 84/156/EEC, 84/491/EEC, 86/280/EEC and amending Directive 2000/60/EC
Directive 2008/32/EC	Amending Directive 2000/60/EC establishing a framework for Community action in the field of water policy, as regards the implementing powers conferred on the Commission
Directive 2006/7/EC	Concerning the management of bathing water quality and repealing Directive 76/160/EEC
Directive 2000/60/EC	Establishing a framework for Community action in the field of water policy
Council Directive 98/83/EC	On the quality of water intended for human consumption
Council Directive 91/271/EEC	Concerning urban wastewater treatment
Directive 2006/118/EC	On the protection of groundwater against pollution and deterioration
Council Directive 80/68/EEC	On the protection of groundwater against pollution caused by certain dangerous substances
Directive 2010/75/EU	On industrial emissions (integrated pollution prevention and control)
Directive 2008/1/EC	Concerning integrated pollution prevention and control
Directive 2006/11/EC	On pollution caused by certain dangerous substances discharged into the aquatic environment of the Community
Council Directive 80/68/EEC	On the protection of groundwater against pollution caused by certain dangerous substances
Commission Regulation (EU) No 837/2010	Amending Regulation (EC) No 1418/2007 concerning the export for recovery of certain waste to certain non-OECD countries
Directive 2008/98/EC	On waste and repealing certain Directives
Directive 2000/76/EC	On the incineration of waste
Council Directive 1999/31/EC	On the landfill of waste
Council Directive 94/31/EC	Amending Directive 91/689/EEC on hazardous waste
Council Directive 91/271/EEC	Concerning urban wastewater treatment

ANNEX II: LITERATURE REVIEW FOR THE YEARS 1998-2012

The preparation of the tables was performed in cooperation with Mrs Elena Eliadou, Mrs Evdokia Evthymiou and Dr. Evroulla Hapeshi, who are kindly thanked.

Occurrence of antibiotics in wastewater

	STP Influent (ng/L)	STP Effluent (ng/L)	Reference
atenolol	data not available	1310	[1]
	971	664	[2]
	data not available	987 med	[3]
	1442	20	[4]
	1980-2290	360-1330	[5]
	2407	725	[6]
	795	330	[7]
	2300	3600	[8]
	data not available	1700	[9]
	1880	data not available	[10]
	738-2883	210-681	[11]
	540-med	300 med	[12]
	data not available	4800-15000	[13]
	1197	1025	[14]
	11239-5113	5911-261	[15]
	data not available	2000	[16]
	data not available	411	[17]
	1445.25	397	[18]
	160	30	[19]
	data not available	1241	[20]
	data not available	420-2300	[21]
	data not available	1435	[22]
	data not available	1720	[23]
metoprolol	411	375	[2]
	data not available	244	[3]
	211	n.d.	[4]
	n.d.	n.d.	[24]
	240-260	160-240	[5]
	223	156	[6]
	1660	755	[7]
	4900	1700	[8]
	data not available	250	[9]
	953	data not available	[10]
	14-597	12-199	[11]
	810 med	640 med	[12]
	20	19	[14]
	2-6	3	[15]
	data not available	39	[16]
	data not available	161	[17]

	STP Influent (ng/L)	STP Effluent (ng/L)	Reference
	data not available	79	[25]
	data not available	61	[23]
	40-90	20-140	[26]
	data not available	730	[27]
	160	190	[19]
propranolol	data not available	130-510	[28]
	1390	400	
	700	304	[29]
	10	45	[2]
	data not available	30 med	[30]
	n.d.	n.d.	[4]
	n.d.	n.d.	[24]
	n.d.-50	30-70	[5]
	266	203	[6]
	510	180	[8]
	data not available	360	[9]
	132	data not available	[10]
	334	62	[31]
	690	135	
	1090	110	
	40 med	40 med	[12]
	36	36	[14]
	data not available	292	[16]
	data not available	130-320	[28]
	data not available	50	[17]
	data not available	44	[23]
	440-770	10-30	[32]
	data not available	170	[27]
	data not available	10-90	[33]
	50	30	[19]
	data not available	17	[20]
diclofenac	data not available	B.l.d. -381	[34]
	data not available	160-720	[28]
	<15	<15	
	1000	290	[29]
	0.4-1.9	0.4-1.9	[35]
	0.35	0.17-0.35	[36]
	3020	2510	[37]
	n.d.	n.d.	[24]
	345	155	[38]
	2000	1300	[8]
	data not available	530	[9]
	859	data not available	[10]
	397	119	[31]
	782	176	
	981	78	
	3-437	4-101	[11]
	data not available	61	[17]
	data not available	360-1700	[13]
	232	220	[14]

	STP Influent (ng/L)	STP Effluent (ng/L)	Reference
	59-243	13-49	[15]
	data not available	8.8-127	[39]
	data not available	1000-3000	[40]
	9.5-19.3	3.17-8.3	[41]
	data not available	<2-30	[42]
	data not available	n.d.-269	[43]
	data not available	280-2320	[44]
	251	46	[45]
	<280	<140	[46]
	data not available	1320	[16]
	12370000	<50000	[47]
	230	100	[48]
	2-43	0.3-78	[26]
	320	<2.5	[49]
	data not available	160-890	[28]
	data not available	204	[25]
	data not available	826	[23]
	280	10-280	[32]
	data not available	210.7-486.4	[50]
	1867.7	406.5	[18]
	data not available	4425	[20]
	data not available	78-210	[21]
	data not available	942	[22]
	data not available	810	[27]
	data not available	5-359	[51]
	470-1920	310-930	[52]
	data not available	250-5450	[33]
	160	120	[19]
	370	430	[53]
	2000	1300	[54]
ibuprofen	data not available	868-85000	[34]
	data not available	80-8020	[28]
	57 400	52 300	[55]
	2600-5700	900-2100	[56]
	28000	3000	[29]
	2-3	0.6-0.8	[35]
	13.1	0-3.8	[36]
	990-3300	≈2	[57]
	1360	13	
	2040	81	
	data not available	100	[37]
	9922	13	[4]
	<760-3204	n.d.	[24]
	7851	78	[38]
	3400	130	[8]
	data not available	370	[27]
	data not available	250	[9]
	3200	data not available	[10]
	711-17 933	313-3777	[11]
	data not available	1758	[17]
	data not available	540-4700	[13]

	STP Influent (ng/L)	STP Effluent (ng/L)	Reference
	2678	135	[14]
	1599-2853	15-75	[15]
	data not available	10-137	[39]
	data not available	<12-34	[42]
	data not available	204-476	
	data not available	4.5-48	[44]
	1966	35	[45]
	69700-115000	4130-10160	[46]
	data not available	21700	[16]
	6900	47.5	[48]
	82-3080	3-359	[26]
	840	<12	[49]
	data not available	800-8020	[28]
	data not available	2567	[23]
	1610-5990	50-690	[32]
	data not available	17.7-219	[50]
	24	n.d.	[18]
	data not available	726	[20]
	data not available	<25-99	[21]
	data not available	381	[22]
	data not available	79-1885	[51]
	data not available	20-7110	[33]
	3590	105	[19]
	8380	890	[53]
	12500	1500	[54]
erythromycin	830	620	[8]
		9 - 294	[39]
	320 – 2700		[16]
		1842*	[58]
		47.4 med	[59]
erythromycin-H_2O	226 - 1537	361 – 811	[11]
	1200*	300*	[60]
		838*	[61]
		246 – 4330	[62]
		695	[17]
	470 – 810	510 – 850	[63]
	51 – 217	38 – 96	[64]
		110 - 199	[65]
sulfamethoxazole	3000*	200*	[66]
	500	320	[67]
	14 – 261	79 – 472	[41]
	820	620	[8]
		4 - 407	[39]
	520	130 – 500	[68]
	179 – 1760	47 - 964	[11]
	1250*	370*	[60]
		871*	[61]
	390 – 1000	310	[69]
		5 – 278	[62]
	250 – 1300		[16]
		226	[17]

	STP Influent (ng/L)	STP Effluent (ng/L)	Reference
	13 – 155	4 – 39	[26]
	147 – 356	15 – 47	[64]
		242	[70]
	880	220 – 680	[71]
		2000	[72]
	450		[73]
		289	[74]
		127 ^{med}	[59]
		132*	[58]
		370 - 6000	[75]
	5450 - 7910	9460	[76]
ofloxacin	540	183	[77]
	115 - 1274	53 - 991	[11]
		506*	[61]
	400 – 1000	110	[69]
		96 – 7870	[62]
	890 – 31700		[16]
		123	[17]
	104 – 336	2 – 556	[64]
		50 – 210	[72]
		600^{med}	[59]
		548	[30]
	3520 - 5560	740 - 5700	[76]

* : maximum value

^{med} : median value

Occurrence of antibiotics in surface water, groundwater and marine water

	Surface water (ng/L)	Groundwater (ng/L)	Marine Water (ng/L)	Reference
atenolol	4.7	5.5		[78]
	971	664		[2]
	3-60			[79]
	3-22			
	182-311			[80]
	<10 -83			[5]
	318-6167			[81]
	11-1237			[82]
	1.9-334.3			[83]
	220			[84]
	<1.5			[85]
	41.5			[86]
		60.8		[18]
	2.9-271			[87]
	<2.7-6.2			
	640-800			[19]
	83-475			[88]
		4		[89]
metoprolol	0.8	0.3		[78]
	7-8			[79]
	51-155			
	<10-36			[5]
		9.3-56.3		[90]
	2.33-6.85			[82]
	25-76			[81]
	1.8-26			[83]
	50			[84]
	14			[32]
	738			[86]
	2.8-274.5			[87]
	<2-9.5			
	45 med			[27]
	180-240			[19]
		6		[89]
	411	375		[2]
propranolol	20-140			[28]
	<29			[55]
	35-107			[29]
	1.2	1.8		[78]
	4.6			[91]
	5-7			
	9-22			[80]
	<5-8			[5]
	15-178			[81]
	0.1-7.3			[83]
	30			[84]
	<1.1			[85]
	6.7			[86]

	Surface water (ng/L)	Groundwater (ng/L)	Marine Water (ng/L)	Reference
	2-12			[92]
	12 med			[93]
	20-30			[19]
			93	[94]
			169-2663	
		12		[89]
	10	45		[2]
diclofenac	5.4	9.7		
	31-484			[34]
	21-850			
	50-90			[28]
	<15			[55]
	2-34			[36]
	20-60			[95]
	n.d.-1030			[37]
	2-50			[80]
	86-1200			[96]
		60.2-219		[90]
	n.d.-140			[92]
	2 200			
	6.41-148			[82]
	313-3363			[81]
	<0.3			[85]
	1.1-68			[39]
	2.3			[91]
	24-62			[42]
	100-850			[97]
	19-55			[26]
		256		[18]
	9.4-147			[98]
	7-17.6			[99]
	0.7-156			[83]
	25-41			[32]
	1.36-33.2			[50]
	1959-2216			[100]
				[101]
	87.7			
	1.4			[102]
		n.d.		[43]
	150			[27]
	26-194			[51]
	med			
	18-50			
	<1-370			[52]
	5-410			[103]
		380		
		590		[104]
	140-180			[19]
	15			[88]
	n.d.			[38]

	Surface water (ng/L)	Groundwater (ng/L)	Marine Water (ng/L)	Reference
	11	98		[105]
	2-190			[106]
ibuprofen	3-6300			[106]
	100-2700			[34]
	16-1600			
	560-1210			[28]
	620			[55]
	144-2370			[29]
	1.5-7.8		n.d.	[57]
	10			[95]
	n.d.-55			[37]
	5.5			[78]
	8-289			[80]
	41			[96]
	2.74-541			[82]
	2234-14671			[81]
	<0.9			[85]
		10		[107]
	11-38			[39]
	<12-30			[42]
	10-2383			[26]
	4.9-477			[98]
	0-34			[108]
	8.5-64.9			[99]
	n.d.-14181			[109]
	2.2-330			[110]
	6.3-2748			[83]
	1370			[84]
	14-44			[32]
	n.d.-4.5			[50]
	7250-7791			[100]
	152.9			[86]
		185		[18]
		3		[111]
	1.2			[102]
		3110 max		[112]
		n.d.		[43]
	70			[27]
	64-141med			[51]
	8- 27			
	2-280			[103]
		200		
	520-640			[19]
	23			[88]
			<70-300	[113]
		43		[105]
	3.5-64			[36]
	n.d.			[38]
erythromycin	1.8 – 4.8			[39]
	1.4 – 15.9			[114]

	Surface water (ng/L)	Groundwater (ng/L)	Marine Water (ng/L)	Reference
	30 – 70			[115]
	620			[116]
	3.2 - 15.9			[59]
	1022*			[58]
erythromycin-H_2O			4.7 - 1900	[62]
	636		5.2	[117]
	1700*			[118]
	450			[39]
	9 - 41			[119]
			9.5 - 486	[120]
sulfamethoxazole	2 *			[66]
	1.7 - 36			[39]
	50 – 120			[68]
	56 - 450			[75]
	300*			[69]
	1 – 7			[121]
			0.6 – 47.5	[62]
	480			[116]
	193			[117]
	520*			[118]
	30 – 70			[115]
	110			[39]
	20 - 174			[119]
	69*			[122]
	1 - 22			[26]
	6.4 – 1488	9.9		[70]
	47 – 96			[123]
		1110*		[112]
		0.08 – 312.2		[124]
	<50*			[58]
	1020	220		[125]
ofloxacin	19.31 – 306.10			[126]
			8.1 – 634	[62]
	30*			[122]
	108		16.4	[117]
	33.1 – 306.1			[59]

* : maximum value

med : median value

Occurrence of antibiotics in sediments and soil

	Sediment (ng/g)	Soil (ng/g)	Reference
atenolol	0.49-36.4		[82]
	22		[127]
metoprolol	6.57		[128]
	0.59-4.10		[82]
	n.d.	0.320	[129]
propranolol	1.51		[128]
	2.60		
	n.d.	n.d.	[129]
diclofenac	5-38		[130]
		<1	[131]
		<1	
		1.16	[43]
	0.0085-0.0095	0.009-0.09	[129]
	11-144		[132]
	0.69-2.65		[82]
ibuprofen	1.76-20.6		[131]
		0.3-0.23	
		0.18-0.23	
		5.03	[43]
	0.021-0.071	0.098-0.190	[129]

		750	[127]
	n.d.		[107]
	5-28		[132]
Sulfamethoxazole	0.47 – 0.145		[121]
		2300 – 5200	[133]
		120 – 2800	[134]
		100 – 3760	[135]
		72 – 9990	[136]
Ofloxacin	8.95 – 12.03		[128]

[1] Oppenheimer J, Eaton A, Badruzzaman M, Haghani AW, Jacangelo JG. 2011. Occurrence and suitability of sucralose as an indicator compound of wastewater loading to surface waters in urbanized regions. *Water Res* 45:4019-4027.

[2] MacLeod SL, Sudhir P, Wong CS. 2007. Stereoisomer analysis of wastewater-derived β-blockers, selective serotonin re-uptake inhibitors, and salbutamol by high-performance liquid chromatography–tandem mass spectrometry. *J of Chromatogr A* 1170:23-33.

[3] Lee J, Ji K, Lim Kho Y, Kim P, Choi K. 2011. Chronic exposure to diclofenac on two freshwater cladocerans and Japanese medaka. *Ecotoxicol Environ Saf* 74:1216-1225.

[4] Conkle JL, White JR, Metcalfe CD. 2008. Reduction of pharmaceutically active compounds by a lagoon wetland wastewater treatment system in Southeast Louisiana. *Chemosphere* 73:1741-1748.

[5] Alder AC, Schaffner C, Majewsky M, Klasmeier J, Fenner K. 2010. Fate of beta-blocker human pharmaceuticals in surface water: comparison of measured and simulated concentrations in the Glatt Valley Watershed, Switzerland. *Water Res* 44:936-948.

[6] Gabet-Giraud V, Miège C, Choubert J, Ruel SM, Coquery M. 2010. Occurrence and removal of estrogens and beta blockers by various processes in wastewater treatment plants. *Sci Total Environ* 408:4257-4269.

[7] Vieno N, Tuhkanen T, Kronberg L. 2007. Elimination of pharmaceuticals in sewage treatment plants in Finland. *Water Res* 41:1001-1012.

[8] Ternes TA, Bonerz M, Herrmann N, Teiser B, Andersen HR. 2007. Irrigation of treated wastewater in Braunschweig, Germany: An option to remove pharmaceuticals and musk fragrances. *Chemosphere* 66:894-904.

[9] Benner J, Salhi E, Ternes T, von Gunten U. 2008. Ozonation of reverse osmosis concentrate: Kinetics and efficiency of beta blocker oxidation. *Water Res* 42:3003-3012.

[10] Terzić S, Senta I, Ahel M, Gros M, Petrović M, Barcelo D, Müller J, Knepper T, Martí I, Ventura F. 2008. Occurrence and fate of emerging wastewater contaminants in Western Balkan Region. *Sci Total Environ* 399:66-77.

[11] Lin AY, Yu T, Lateef SK. 2009. Removal of pharmaceuticals in secondary wastewater treatment processes in Taiwan. *J Hazard Mater* 167:1163-1169.

[12] Wick A, Fink G, Joss A, Siegrist H, Ternes TA. 2009. Fate of beta blockers and psycho-active drugs in conventional wastewater treatment. *Water Res* 43:1060-1074.

[13] Muñoz I, Gómez-Ramos MJ, Agüera A, Fernández-Alba AR, García-Reyes JF, Molina-Díaz A. 2009. Chemical evaluation of contaminants in wastewater effluents and the environmental risk of reusing effluents in agriculture. *TrAC-Trend Anal Chem* 28:676-694.

[14] Rosal R, Rodea-Palomares I, Boltes K, Fernández-Piñas F, Leganés F, Gonzalo S, Petre A. 2010. Ecotoxicity assessment of lipid regulators in water and biologically treated wastewater using three aquatic organisms. *Environ Sci Pollut Res* 17:135-144.

[15] Behera SK, Kim HW, Oh JE, Park HS. 2011. Occurrence and removal of antibiotics, hormones and several other pharmaceuticals in wastewater treatment plants of the largest industrial city of Korea. *Sci Total Environ* 409:4351-4360.

[16] Radjenović J, Petrović M, Barceló D. 2009. Fate and distribution of pharmaceuticals in wastewater and sewage sludge of the conventional activated sludge (CAS) and advanced membrane bioreactor (MBR) treatment. *Water Res* 43:831-841.

[17] Lin AYC, Yu TH, Lin CF. 2008. Pharmaceutical contamination in residential, industrial, and agricultural waste streams: Risk to aqueous environments in Taiwan. *Chemosphere* 74:131-141.

[18] Teijon G, Candela L, Tamoh K, Molina-Díaz A, Fernández-Alba A. 2010. Occurrence of emerging contaminants, priority substances (2008/105/CE) and heavy metals in treated wastewater and groundwater at Depurbaix facility (Barcelona, Spain). *Sci Total Environ* 408:3584-3595.

[19] Bendz D, Paxéus NA, Ginn TR, Loge FJ. 2005. Occurrence and fate of pharmaceutically active compounds in the environment, a case study: Höje River in Sweden. *J Hazard Mater* 122:195-204.

[20] Prieto-Rodriguez L, Miralles-Cuevas S, Oller I, Agüera A, Puma GL, Malato S. 2012. Treatment of emerging contaminants in wastewater treatment plants (WWTP) effluents by solar photocatalysis using low TiO_2 concentrations. *J Hazard Mater* 211:131-137.

[21] Wert EC, Gonzales S, Dong MM, Rosario-Ortiz FL. 2011. Evaluation of enhanced coagulation pretreatment to improve ozone oxidation efficiency in wastewater. *Water Res* 45:5191-5199.

[22] Acero JL, Benitez FJ, Leal AI, Real FJ, Teva F. 2010. Membrane filtration technologies applied to municipal secondary effluents for potential reuse. *J Hazard Mater* 177:390-398.

[23] Bueno MJM, Agüera A, Gómez MJ, Hernando MD, García-Reyes JF, Fernández-Alba AR. 2007. Application of liquid chromatography/quadrupole-linear ion trap mass spectrometry and time-of-flight mass spectrometry to the determination of pharmaceuticals and related contaminants in wastewater. *Anal Chem* 79:9372-9384.

[24] Lacey C, McMahon G, Bones J, Barron L, Morrissey A, Tobin J. 2008. An LC-MS method for the determination of pharmaceutical compounds in wastewater treatment plant influent and effluent samples. *Talanta* 75:1089.

[25] Sui Q, Huang J, Deng S, Yu G, Fan Q. 2010. Occurrence and removal of pharmaceuticals, caffeine and DEET in wastewater treatment plants of Beijing, China. *Water Res* 44:417-426.

[26] Pailler JY, Krein A, Pfister L, Hoffmann L, Guignard C. 2009. Solid phase extraction coupled to liquid chromatography-tandem mass spectrometry analysis of sulfonamides, tetracyclines, analgesics and hormones in surface water and wastewater in Luxembourg. *Sci Total Environ* 407:4736-4743.

[27] Ternes TA. 1998. Occurrence of drugs in German sewage treatment plants and rivers. *Water Res* 32:3245-3260.

[28] Camacho-Muñoz D, Martín J, Santos JL, Aparicio I, Alonso E. 2010. Occurrence, temporal evolution and risk assessment of pharmaceutically active compounds in Doñana Park (Spain). *J Hazard Mater* 183:602-608.

[29] Roberts PH, Thomas KV. 2006. The occurrence of selected pharmaceuticals in wastewater effluent and surface waters of the lower Tyne catchment. *Sci Total Environ* 356:143-153.

[30] Lee HB, Peart TE, Svoboda ML. 2007. Determination of ofloxacin, norfloxacin, and ciprofloxacin in sewage by selective solid-phase extraction, liquid chromatography with fluorescence detection, and liquid chromatography tandem mass spectrometry. *J Chromatogr A* 1139:45-52.

[31] Zhou JL, Zhang ZL, Banks E, Grover D, Jiang JQ. 2009. Pharmaceutical residues in wastewater treatment works effluents and their impact on receiving river water. *J Hazard Mater* 166:655-661.

[32] Pedrouzo M, Reverte S, Borrull F, Pocurull E, Marce RM. 2007. Pharmaceutical determination in surface and wastewaters using high-performance liquid chromatography-(electrospray)-mass spectrometry. *J Sep Sci* 30:297-303.

[33] Andreozzi R, Raffaele M, Nicklas P. 2003. Pharmaceuticals in STP effluents and their solar photodegradation in aquatic environment. *Chemosphere* 50:1319-1330.

[34] Farré M, Ferrer I, Ginebreda A, Figueras M, Olivella L, Tirapu L, Vilanova M, Barceló D. 2001. Determination of drugs in surface water and wastewater samples by liquid chromatography-mass spectrometry: Methods and preliminary results including toxicity studies with *Vibrio fischeri*. *J Chromatogr A* 938:187-197.

[35] Tauxe-Wuersch A, De Alencastro LF, Grandjean D, Tarradellas J. 2005. Occurrence of several acidic drugs in sewage treatment plants in Switzerland and risk assessment. *Water Res* 39:1761-1772.

[36] Lindqvist N, Tuhkanen T, Kronberg L. 2005. Occurrence of acidic pharmaceuticals in raw and treated sewages and in receiving waters. *Water Res* 39:2219.

[37] Heberer T. 2002. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: A review of recent research data. *Toxicol Lett* 131:5-17.

[38] Quintana JB, Rodil R, Muniategui-Lorenzo S, López-Mahía P, Prada-Rodríguez D. 2007. Multiresidue analysis of acidic and polar organic contaminants in water samples by stir-bar sorptive extraction-liquid desorption-gas chromatography-mass spectrometry. *J Chromatogr A* 1174:27-39.

[39] Kim SD, Cho J, Kim IS, Vanderford BJ, Snyder SA. 2007. Occurrence and removal of pharmaceuticals and endocrine disruptors in South Korean surface, drinking, and waste waters. *Water Res* 41:1013.

[40] Stülten D, Zühlke S, Lamshöft M, Spiteller M. 2008. Occurrence of diclofenac and selected metabolites in sewage effluents. *Sci Total Environ* 405:310-316.

[41] Spongberg AL, Witter JD. 2008. Pharmaceutical compounds in the wastewater process stream in Northwest Ohio. *Sci Total Environ* 397:148-157.

[42] Chen HC, Wang PL, Ding WH. 2008. Using liquid chromatography–ion trap mass spectrometry to determine pharmaceutical residues in Taiwanese rivers and wastewaters. *Chemosphere* 72:863-869.

[43] Chen F, Ying GG, Kong LX, Wang L, Zhao JL, Zhou LJ, Zhang LJ. 2011. Distribution and accumulation of endocrine-disrupting chemicals and pharmaceuticals in wastewater irrigated soils in Hebei, China. *Environ Pollut* 159:1490-1498.

[44] Brown JN, Paxéus N, Förlin L, Larsson DGJ. 2007. Variations in bioconcentration of human pharmaceuticals from sewage effluents into fish blood plasma. *Environ Toxicol Pharmacol* 24:267-274.

[45] Kimura K, Hara H, Watanabe Y. 2007. Elimination of selected acidic pharmaceuticals from municipal wastewater by an activated sludge system and membrane bioreactors. *Environ Sci Technol* 41:3708-3714.

[46] Santos J, Aparicio I, Callejón M, Alonso E. 2009. Occurrence of pharmaceutically active compounds during 1-year period in wastewaters from four wastewater treatment plants in Seville (Spain). *J Hazard Mater* 164:1509-1516.

[47] Badawy MI, Wahaab RA, El-Kalliny A. 2009. Fenton-biological treatment processes for the removal of some pharmaceuticals from industrial wastewater. *J Hazard Mater* 167:567-574.

[48] Zorita S, Mårtensson L, Mathiasson L. 2009. Occurrence and removal of pharmaceuticals in a municipal sewage treatment system in the south of Sweden. *Sci Total Environ* 407:2760-2770.

[49] Busetti F, Linge KL, Heitz A. 2009. Analysis of pharmaceuticals in indirect potable reuse systems using solid-phase extraction and liquid chromatography—tandem mass spectrometry. *J Chromatogr* 1216:5807-5818.

[50] Togola A, Budzinski H. 2008. Multi-residue analysis of pharmaceutical compounds in aqueous samples. *J Chromatogr A* 1177:150-158.

[51] Metcalfe CD, Koenig BG, Bennie DT, Servos M, Ternes TA, Hirsch R. 2009. Occurrence of neutral and acidic drugs in the effluents of Canadian sewage treatment plants. *Environ Toxicol Chem* 22:2872-2880.

[52] Buser H, Poiger T, Muller MD. 1998. Occurrence and fate of the pharmaceutical drug diclofenac in surface waters: rapid photodegradation in a lake. *Environ Sci Technol* 32:3449-3456.

[53] Hijosa-Valsero M, Matamoros V, Martín-Villacorta J, Bécares E, Bayona JM. 2010. Assessment of full-scale natural systems for the removal of PPCPs from wastewater in small communities. *Water Res* 44:1429-1439.

[54] Kosma CI, Lambropoulou DA, Albanis TA. 2010. Occurrence and removal of PPCPs in municipal and hospital wastewaters in Greece. *J Hazard Mater* 179:804-817.

[55] Camacho-Muñoz D, Martín J, Santos JL, Aparicio I, Alonso E. 2009. An affordable method for the simultaneous determination of the most studied pharmaceutical compounds as wastewater and surface water pollutants. *J Separ Sci* 32:3064-3073.

[56] Carballa M, Omil F, Lema JM, Llompart M, García-Jares C, Rodríguez I, Gómez M, Ternes T. 2004. Behavior of pharmaceuticals, cosmetics and hormones in a sewage treatment plant. *Water Res* 38:2918-2926.

[57] Buser H, Poiger T, Muller MD. 1999. Occurrence and environmental behavior of the chiral pharmaceutical drug ibuprofen in surface waters and in wastewater. *Environ Sci Technol* 33:2529-2535.

[58] Ashton D, Hilton M, Thomas KV. 2004. Investigating the environmental transport of human pharmaceuticals to streams in the United Kingdom. *Sci Total Environ* 333:167-184.

[59] Zuccato E, Castiglioni S, Fanelli R. 2005. Identification of the pharmaceuticals for human use contaminating the Italian aquatic environment. *J Hazard Mater* 122:205-209.

[60] Karthikeyan K, Meyer MT. 2006. Occurrence of antibiotics in wastewater treatment facilities in Wisconsin, USA. *Sci Total Environ* 361:196-207.

[61] Miao XS, Bishay F, Chen M, Metcalfe CD. 2004. Occurrence of antimicrobials in the final effluents of wastewater treatment plants in Canada. *Environ Sci Technol* 38:3533-3541.

[62] Minh TB, Leung HW, Loi IH, Chan WH, So MK, Mao JQ, Choi D, Lam JCW, Zheng G, Martin M, Lee JHW, Lam PKS, Richardson BJ. 2009. Antibiotics in the Hong Kong metropolitan area: Ubiquitous distribution and fate in Victoria Harbour. *Mar Pollut Bull* 58:1052-1062.

[63] Gulkowska A, Leung H, So M, Taniyasu S, Yamashita N, Yeung LWY, Richardson BJ, Lei A, Giesy J, Lam PKS. 2008. Removal of antibiotics from wastewater by sewage treatment facilities in Hong Kong and Shenzhen, China. *Water Res* 42:395-403.

[64] Li B, Zhang T, Xu Z, Fang HHP. 2009. Rapid analysis of 21 antibiotics of multiple classes in municipal wastewater using ultra performance liquid chromatography-tandem mass spectrometry. *Anal Chim Acta* 645:64-72.

[65] McArdell CS, Molnar E, Suter MJF, Giger W. 2003. Occurrence and fate of macrolide antibiotics in wastewater treatment plants and in the Glatt Valley Watershed, Switzerland. *Environ Sci Technol* 37:5479-5486.

[66] Watkinson A, Murby E, Kolpin D, Costanzo S. 2009. The occurrence of antibiotics in an urban watershed: from wastewater to drinking water. *Sci Total Environ* 407:2711.

[67] Watkinson A, Murby E, Costanzo S. 2007. Removal of antibiotics in conventional and advanced wastewater treatment: Implications for environmental discharge and wastewater recycling. *Water Res* 41:4164-4176.

[68] Yang S, Carlson K. 2004. Solid-phase extraction–high-performance liquid chromatography–ion trap mass spectrometry for analysis of trace concentrations of macrolide antibiotics in natural and waste water matrices. *J Chromatogr A* 1038:141-155.

[69] Brown KD, Kulis J, Thomson B, Chapman TH, Mawhinney DB. 2006. Occurrence of antibiotics in hospital, residential, and dairy effluent, municipal wastewater, and the Rio Grande in New Mexico. *Sci Total Environ* 366:772-783.

[70] Díaz-Cruz MS, García-Galán MJ, Barceló D. 2008. Highly sensitive simultaneous determination of sulfonamide antibiotics and one metabolite in environmental waters by liquid chromatography–quadrupole linear ion trap–mass spectrometry. *J Chromatogr A* 1193:50-59.

[71] Batt AL, Kim S, Aga DS. 2007. Comparison of the occurrence of antibiotics in four full-scale wastewater treatment plants with varying designs and operations. *Chemosphere* 68:428-435.

[72] Renew JE, Huang CH. 2004. Simultaneous determination of fluoroquinolone, sulfonamide, and trimethoprim antibiotics in wastewater using tandem solid phase extraction and liquid chromatography–electrospray mass spectrometry. *J Chromatogr A* 1042:113-121.

[73] Choi KJ, Kim SG, Kim C, Kim SH. 2007. Determination of antibiotic compounds in water by on-line SPE-LC/MSD. *Chemosphere* 66:977-984.

[74] Segura PA, Gagnon C, Sauvé S. 2007. A fully automated on-line preconcentration and liquid chromatography–tandem mass spectrometry method for the analysis of anti-infectives in wastewaters. *Anal Chim Acta* 604:147-157.

[75] Batt AL, Bruce IB, Aga DS. 2006. Evaluating the vulnerability of surface waters to antibiotic contamination from varying wastewater treatment plant discharges. *Environ Pollut* 142:295-302.

[76] Peng X, Wang Z, Kuang W, Tan J, Li K. 2006. A preliminary study on the occurrence and behavior of sulfonamides, ofloxacin and chloramphenicol antimicrobials in wastewaters of two sewage treatment plants in Guangzhou, China. *Sci Total Environ* 371:314-322.

[77] Castiglioni S, Fanelli R, Calamari D, Bagnati R, Zuccato E. 2004. Methodological approaches for studying pharmaceuticals in the environment by comparing predicted and measured concentrations in River Po, Italy. *Regul Toxicol and Pharmacol* 39:25-32.

[78] Vulliet E, Cren-Olivé C. 2011. Screening of pharmaceuticals and hormones at the regional scale, in surface and groundwaters intended to human consumption. *Environ Pollut* 159:2929-2934.

[79] Kasprzyk-Hordern B, Dinsdale RM, Guwy AJ. 2007. Multi-residue method for the determination of basic/neutral pharmaceuticals and illicit drugs in surface water by solid-phase extraction and ultra performance liquid chromatography–positive electrospray ionisation tandem mass spectrometry. *J Chromatogr A* 1161:132-145.

[80] Gros M, Petrović M, Barceló D. 2009. Wastewater treatment plants as a pathway for aquatic contamination by pharmaceuticals in the Ebro river basin (northeast Spain). *Environ Toxicol Chem* 26:1553-1562.

[81] Valcárcel Y, Alonso SG, Rodríguez-Gil J, Maroto RR, Gil A, Catalá M. 2011. Analysis of the presence of cardiovascular and analgesic/anti-inflammatory/antipyretic pharmaceuticals in river-and drinking-water of the Madrid Region in Spain. *Chemosphere* 82:1062-1071.

[82] Silva BF, Jelic A, López-Serna R, Mozeto AA, Petrovic M, Barceló D. 2011. Occurrence and distribution of pharmaceuticals in surface water, suspended solids and sediments of the Ebro river basin, Spain. *Chemosphere* 85:1331-1339.

[83] Fernández C, González-Doncel M, Pro J, Carbonell G, Tarazona J. 2010. Occurrence of pharmaceutically active compounds in surface waters of the Henares-Jarama-Tajo River system (Madrid, Spain) and a potential risk characterization. *Sci Total Environ* 408:543.

[84] Ginebreda A, Muñoz I, de Alda ML, Brix R, López-Doval J, Barceló D. 2010. Environmental risk assessment of pharmaceuticals in rivers: Relationships between hazard indexes and aquatic macroinvertebrate diversity indexes in the Llobregat River (NE Spain). *Environ Int* 36:153-162.

[85] Rodríguez-Gil JL, Catalá M, Alonso SG, Maroto RR, Valcárcel Y, Segura Y, Molina R, Melero JA, Martínez F. 2010. Heterogeneous photo-Fenton treatment for the reduction of pharmaceutical contamination in Madrid rivers and ecotoxicological evaluation by a miniaturized fern spores bioassay. *Chemosphere* 80:381-388.

[86] Lopez-Roldan R, de Alda ML, Gros M, Petrovic M, Martin-Alonso J, Barcelo D. 2010. Advanced monitoring of pharmaceuticals and estrogens in the Llobregat River basin (Spain) by liquid chromatography-triple quadrupole-tandem mass spectrometry in combination with ultra performance liquid chromatography-time of flight-mass spectrometry. *Chemosphere* 80:1337-1344.

[87] Daneshvar A, Svanfelt J, Kronberg L, Prévost M, Weyhenmeyer GA. 2010. Seasonal variations in the occurrence and fate of basic and neutral pharmaceuticals in a Swedish river–lake system. *Chemosphere* 80:301-309.

[88] Yoon Y, Ryu J, Oh J, Choi BG, Snyder SA. 2010. Occurrence of endocrine disrupting compounds, pharmaceuticals, and personal care products in the Han River (Seoul, South Korea). *Sci Total Environ* 408:636.

[89] Huerta-Fontela M, Galceran MT, Ventura F. 2010. Occurrence and removal of pharmaceuticals and hormones through drinking water treatment. *Water Res*. 45:1432-1442.

[90] Radjenović J, Pérez S, Petrović M, Barceló D. 2008. Identification and structural characterization of biodegradation products of atenolol and glibenclamide by liquid chromatography coupled to hybrid quadrupole time-of-flight and quadrupole ion trap mass spectrometry. *J Chromatogr A* 1210:142-153.

[91] Yu Z, Peldszus S, Huck PM. 2007. Optimizing gas chromatographic-mass spectrometric analysis of selected pharmaceuticals and endocrine-disrupting substances in water using factorial experimental design. *J Chromatogr A* 1148:65-77.

[92] Letzel M, Metzner G, Letzel T. 2009. Exposure assessment of the pharmaceutical diclofenac based on long-term measurements of the aquatic input. *Environ Int* 35:363-368.

[93] Ternes TA, Kreckel P, Mueller J. 1999. Behaviour and occurrence of estrogens in municipal sewage treatment plants — II. Aerobic batch experiments with activated sludge. *Sci Total Environ* 225:91-99.

[94] Wille K, Claessens M, Rappé K, Monteyne E, Janssen CR, De Brabander HF, Vanhaecke L. 2011. Rapid quantification of pharmaceuticals and pesticides in passive samplers using ultra high performance liquid chromatography coupled to high resolution mass spectrometry. *J Chromatogr A* 1218:9162-9173.

[95] Stumpf M, Ternes TA, Wilken RD, Rodrigues SV, Baumann W. 1999. Polar drug residues in sewage and natural waters in the state of Rio de Janeiro, Brazil. *Sci Total Environ* 225:135-141.

[96] Küster A, Alder AC, Escher BI, Duis K, Fenner K, Garric J, Hutchinson TH, Lapen DR, Péry A, Römbke J. 2009. Environmental risk assessment of human pharmaceuticals in the European Union: A case study with the β-blocker atenolol. *Integr Environ Assess Manag* 6:514-523.

[97] Scheurell M, Franke S, Shah R, Hühnerfuss H. 2009. Occurrence of diclofenac and its metabolites in surface water and effluent samples from Karachi, Pakistan. *Chemosphere* 77:870-876.

[98] Zhao JL, Ying GG, Wang L, Yang JF, Yang XB, Yang LH, Li X. 2009. Determination of phenolic endocrine disrupting chemicals and acidic pharmaceuticals in surface water of the Pearl Rivers in South China by gas chromatography/negative chemical ionization/mass spectrometry. *Sci Total Environ* 407:962-974.

[99] Wang L, Ying GG, Zhao JL, Yang XB, Chen F, Tao R, Liu S, Zhou LJ. 2010. Occurrence and risk assessment of acidic pharmaceuticals in the Yellow River, Hai River and Liao River of north China. *Sci Total Environ* 408:3139-3147.

[100] Heath E, Kosjek T, Farre M, Quintana J, De Alencastro L, Castiglioni S, Gans O, Langford K, Loos R, Radjenović J. 2010. Second interlaboratory exercise on non-steroidal anti-inflammatory drug analysis in environmental aqueous samples. *Talanta* 81:1189-1196.

[101] Stanley JK, Ramirez AJ, Mottaleb M, Chambliss CK, Brooks BW. 2006. Enantiospecific toxicity of the β-blocker propranolol to *Daphnia magna* and *Pimephales promelas*. *Environ Toxicol Chem* 25:1780-1786.

[102] Loos R, Wollgast J, Huber T, Hanke G. 2007. Polar herbicides, pharmaceutical products, perfluorooctanesulfonate (PFOS), perfluorooctanoate (PFOA), and nonylphenol and its carboxylates and ethoxylates in surface and tap waters around Lake Maggiore in Northern Italy. *Anal Bioanal Chem* 387:1469-1478.

[103] Grundwasser KBO, Heberer T, Schmidt-Bäumler K, Stan H. 1998. Occurrence and distribution of organic contaminants in the aquatic system in Berlin. Part I: Drug residues and other polar contaminants in Berlin surface and groundwater. *Acta Hydrochim Hydrobiol* 26:272-278.

[104] Sacher F, Lange FT, Brauch HJ, Blankenhorn I. 2001. Pharmaceuticals in groundwaters: Analytical methods and results of a monitoring program in Baden-Württemberg, Germany. *J Chromatogr A* 938:199-210.

[105] Wu C, Spongberg AL, Witter JD. 2008. Use of solid phase extraction and liquid chromatography-tandem mass spectrometry for simultaneous determination of various pharmaceuticals in surface water. *Int J Environ Anal Chem* 88:1033-1048.

[106] Comeau F, Surette C, Brun GL, Losier R. 2008. The occurrence of acidic drugs and caffeine in sewage effluents and receiving waters from three coastal watersheds in Atlantic Canada. *Sci Total Environ* 396:132-146.

[107] Gottschall N, Topp E, Metcalfe C, Edwards M, Payne M, Kleywegt S, Russell P, Lapen D. 2012. Pharmaceutical and personal care products in groundwater, subsurface drainage, soil, and wheat grain, following a high single application of municipal biosolids to a field. *Chemosphere* 87:194-203.

[108] Zhang S, Zhang Q, Darisaw S, Ehie O, Wang G. 2007. Simultaneous quantification of polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and pharmaceuticals and personal care products (PPCPs) in Mississippi river water, in New Orleans, Louisiana, USA. *Chemosphere* 66:1057-1069.

[109] Peng X, Yu Y, Tang C, Tan J, Huang Q, Wang Z. 2008. Occurrence of steroid estrogens, endocrine-disrupting phenols, and acid pharmaceutical residues in urban riverine water of the Pearl River Delta, South China. *Sci Total Environ* 397:158-166.

[110] Crouse BA, Ghoshdastidar AJ, Tong AZ. 2011. The presence of acidic and neutral drugs in treated sewage effluents and receiving waters in the Cornwallis and Annapolis River watersheds and the Mill CoveSewage Treatment Plant in Nova Scotia, Canada. *Environ Res*. 112:92-99.

[111] Loos R, Locoro G, Comero S, Contini S, Schwesig D, Werres F, Balsaa P, Gans O, Weiss S, Blaha L. 2010. Pan-European survey on the occurrence of selected polar organic persistent pollutants in ground water. *Water Res* 44:4115-4126.

[112] Barnes KK, Kolpin DW, Furlong ET, Zaugg SD, Meyer MT, Barber LB. 2008. A national reconnaissance of pharmaceuticals and other organic wastewater contaminants in the United States--I) groundwater. *Sci Total Environ* 402:192-200.

[113] Weigel S, Berger U, Jensen E, Kallenborn R, Thoresen H, Hühnerfuss H. 2004. Determination of selected pharmaceuticals and caffeine in sewage and seawater from Tromsø/Norway with emphasis on ibuprofen and its metabolites. *Chemosphere* 56:583-592.

[114] Calamari D, Zuccato E, Castiglioni S, Bagnati R, Fanelli R. 2003. Strategic survey of therapeutic drugs in the rivers Po and Lambro in northern Italy. *Environ Sci Technol* 37:1241-1248.

[115] Wiegel S, Aulinger A, Brockmeyer R, Harms H, Löffler J, Reincke H, Schmidt R, Stachel B, Von Tümpling W, Wanke A. 2004. Pharmaceuticals in the river Elbe and its tributaries. *Chemosphere* 57:107-126.

[116] Hirsch R, Ternes TA, Haberer K, Mehlich A, Ballwanz F, Kratz KL. 1998. Determination of antibiotics in different water compartments via liquid chromatography–electrospray tandem mass spectrometry. *J Chromatogr A* 815:213-223.

[117] Xu W, Zhang G, Zou S, Li X, Liu Y. 2007. Determination of selected antibiotics in the Victoria Harbour and the Pearl River, South China using high-performance liquid chromatography-electrospray ionization tandem mass spectrometry. *Environ Pollut* 145:672-679.

[118] Kolpin DW, Furlong ET, Meyer MT, Thurman EM, Zaugg SD, Barber LB, Buxton HT. 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: A national reconnaissance. *Environ Sci Technol* 36:1202-1211.

[119] Managaki S, Murata A, Takada H, Tuyen BC, Chiem NH. 2007. Distribution of macrolides, sulfonamides, and trimethoprim in tropical waters: Ubiquitous occurrence of veterinary antibiotics in the Mekong Delta. *Environ Sci Technol* 41:8004-8010.

[120] Gulkowska A, He Y, So MK, Yeung LWY, Leung HW, Giesy JP, Lam PKS, Martin M, Richardson BJ. 2007. The occurrence of selected antibiotics in Hong Kong coastal waters. *Mar Pollut Bull* 54:1287-1293.

[121] Arikan OA, Rice C, Codling E. 2008. Occurrence of antibiotics and hormones in a major agricultural watershed. *Desalination* 226:121-133.

[122] Tamtam F, Mercier F, Le Bot B, Eurin J, Tuc Dinh Q, Clément M, Chevreuil M. 2008. Occurrence and fate of antibiotics in the Seine River in various hydrological conditions. *Sci Total Environ* 393:84-95.

[123] Nageswara Rao R, Venkateswarlu N, Narsimha R. 2008. Determination of antibiotics in aquatic environment by solid-phase extraction followed by liquid chromatography–electrospray ionization mass spectrometry. *J Chromatogr A* 1187:151-164.

[124] García-Galán MJ, Garrido T, Fraile J, Ginebreda A, Díaz-Cruz MS, Barceló D. 2010. Simultaneous occurrence of nitrates and sulfonamide antibiotics in two ground water bodies of Catalonia (Spain). *J Hydrol* 383:93-101.

[125] Lindsey ME, Meyer M, Thurman E. 2001. Analysis of trace levels of sulfonamide and tetracycline antimicrobials in groundwater and surface water using solid-phase extraction and liquid chromatography/mass spectrometry. *Anal Chem* 73:4640-4646.

[126] Castiglioni S, Pomati F, Miller K, Burns BP, Zuccato E, Calamari D, Neilan BA. 2008. Novel homologs of the multiple resistance regulator marA in antibiotic-contaminated environments. *Water Res* 42:4271-4280.

[127] Edwards M, Topp E, Metcalfe C, Li H, Gottschall N, Bolton P, Curnoe W, Payne M, Beck A, Kleywegt S. 2009. Pharmaceutical and personal care products in tile drainage following surface spreading and injection of dewatered municipal biosolids to an agricultural field. *Sci Total Environ* 407:4220-4230.

[128] Vazquez-Roig P, Segarra R, Blasco C, Andreu V, Picó Y. 2010. Determination of pharmaceuticals in soils and sediments by pressurized liquid extraction and liquid chromatography tandem mass spectrometry. *J Chromatogr A* 1217:2471-2483.

[129] Azzouz A, Ballesteros E. 2012. Combined microwave-assisted extraction and continuous solid-phase extraction prior to gas chromatography–mass spectrometry determination of pharmaceuticals, personal care products and hormones in soils, sediments and sludge. *Sci Total Environ*. 419:208-215.

[130] Varga M, Dobor J, Helenkár A, Jurecska L, Yao J, Záray G. 2010. Investigation of acidic pharmaceuticals in river water and sediment by microwave-assisted extraction and gas chromatography–mass spectrometry. *Microchem J* 95:353-358.

[131] Gibson R, Duran-Alvarez JC, Estrada KL, Chavez A, Jimenez Cisneros B. 2010. Accumulation and leaching potential of some pharmaceuticals and potential endocrine disruptors in soils irrigated with wastewater in the Tula Valley, Mexico. *Chemosphere* 81:1437-1445.

[132] Dobor J, Varga M, Záray G. 2011. Biofilm controlled sorption of selected acidic drugs on river sediments characterized by different organic carbon content. *Chemosphere*. 87: 105–110.

[133] Hu X-, Luo Y, Zhou Q-, Xu L. 2008. Determination of thirteen antibiotics residues in manure by solid phase extraction and high performance liquid chromatography. *Fenxi Huaxue/ Chinese J Anal Chem* 36:1162-1166.

[134] Zhao L, Dong YH, Wang H. 2010. Residues of veterinary antibiotics in manures from feedlot livestock in eight provinces of China. *Sci Total Environ* 408:1069-1075.

[135] Karcı A, Balcıoğlu IA. 2009. Investigation of the tetracycline, sulfonamide, and fluoroquinolone antimicrobial compounds in animal manure and agricultural soils in Turkey. *Sci Total Environ* 407:4652-4664.

[136] Aust MO, Godlinski F, Travis GR, Hao X, McAllister TA, Leinweber P, Thiele-Bruhn S. 2008. Distribution of sulfamethazine, chlortetracycline and tylosin in manure and soil of Canadian feedlots after subtherapeutic use in cattle. *Environ Pollut* 156:1243-1251.