IMPACT OF PHARMACEUTICALS ON ALGAL SPECIES

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ABSTRACT

Trace amounts of activated pharmaceutical ingredients (APIs) have been reported in aquatic environments worldwide, and their toxicity to non-target organisms is of increasing concern. Algae are primary producers in aquatic food chains, and as such are very sensitive to external disturbance. The understanding of the adverse effects on the algal species such as growth and physiological effects is vital to understand the risks of APIs in the aquatic environment. This thesis therefore describes desk-based studies and a series of laboratory experiments to characterise the risk of APIs, and to investigate the effects of APIs on a wide range of algal species.

In the desk-study, a review summarising the available ecotoxicological data of APIs to algal species was initially performed, where differences in the sensitivity of the algal species towards API exposures were found. After that, an approach for prioritising APIs and associated metabolites in the UK environment was developed, where three major-use antibiotics lincomycin, tylosin and trimethoprim that pose a potential threat to algal species in the natural environment were identified for further experimental investigation. Laboratory experiments were then conducted to investigate the effects of three antibiotics on the growth and physiology of a range of algal species from chlorophytes, cyanobacteria and diatoms. Risk arising from the antibiotic mixture in the European surface waters was characterised

In conclusion three major-use antibiotics could cause inhibitory effects on both algal growth and physiology. At environmentally relevant concentrations the antibiotic mixtures can pose potential risks in European surface waters.

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AUTHOR'S DECLARATION

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Data from Chapters 2 to 6 have been written as papers for international peer-reviewed journals. These papers have been reworked, so they are presented in a consistent style for this thesis.

A publication describing the research presented in Chapter 6 is in preparation.

Table 0.1 Status of the papers presented in this thesis with respect to the publication process

Chapter	Title	Journal	Status and DOI
2	Do pharmaceuticals pose a threat to primary producers?	Critical Reviews in Environmental Science and Technology	Published 10.1080/10643389.2015.1061873
3	Toxicological and ecotoxicological risk based prioritisation of pharmaceuticals in the natural environment	Environmental Toxicology and Chemistry	Published 10.1002/etc.3319
4	Comparing the sensitivity of chlorophytes, cyanobacteria and diatoms to antibiotic exposures	Environmental Toxicology and Chemistry	Published 10.1002/etc.3430
5	Effects of veterinary antibiotics on the growth and physiology of chlorophytes, cyanobacteria and a diatom species	Environmental Toxicology and Chemistry	In preparation
6	Risks of mixtures of major-use veterinary antibiotics to blue-green algae	-	In preparation

Chapter 1

Introduction

1.1 Pharmaceuticals and Pathways into the environment

Active pharmaceutical ingredients (APIs) are used primarily to prevent or treat human and animal disease. APIs produced by manufacturers are predominantly used by households and hospitals, in aquaculture and in livestock farming (Figure 1.1). Following consumption by humans, the parent compounds APIs as well as any associated metabolites are typically discharged to wastewater treatment plants (WWTP) (Ellis, 2006, Rosi-Marshall and Royer, 2012). Effluents produced from manufacturing sites are primarily emitted to WWTPs, but in some region (e.g. in areas of India) they are emitted directly into surface waters (Monteiro and Boxall, 2010). During the wastewater treatment process, APIs may be biodegraded, adsorb to the sewage sludge and/-or survive the treatment process and be released in the wastewater effluent (Halling-Sorensen et al., 1998). APIs in effluent can then be emitted to surface waters by direct discharge or to the soil compartment where the effluent is used for irrigation purpose. APIs adsorbed onto sewage sludge can also enter the terrestrial environment when sewage sludge is spread to land as a fertiliser (Sabourin et al., 2009). APIs used in aquaculture will be directly discharged into the aquatic environment. Following use, veterinary APIs used in livestock farming will be excreted and enter soil systems when manure and slurries are applied as fertilisers. The APIs can then be transferred from the soil to the underlying groundwater, aquifers and surface water by leaching and runoff (Wu et al., 2008).

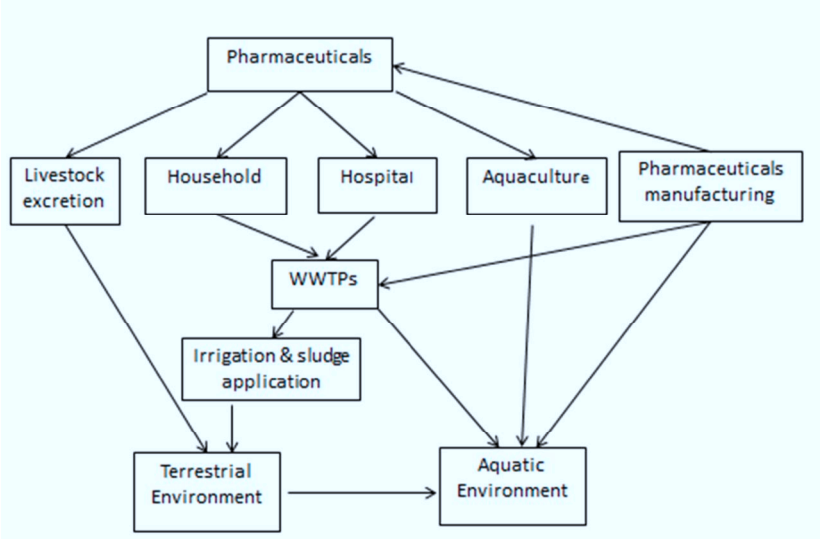


Figure 1.1 Pathways of APIs into the environment

1.2 Occurrence and side effects of APIs in the environment

The contamination of surface water with pharmaceutical residues has become an emerging environmental concern. Over the past 15 years, a large number of studies on the risk evaluation and control of APIs have been undertaken involving the determination of the occurrence, fate and effects of APIs in the environment (Boxall, 2004). The occurrence of a wide range of APIs from different therapeutic classes in surface water has been reported worldwide at concentrations ranging from ng L^{-1} to ug L^{-1} levels (Monteiro and Boxall, 2010). While the reported concentrations are generally low, many APIs have been detected throughout the year across a variety of hydrological, climatic and land-use settings and some APIs can persist in the environment from months to years (Monteiro and Boxall, 2010). APIs are biologically active molecules that are designed to either interact with the receptors in humans and animals or kill infectious organisms (e.g. bacteria, fungi and parasites) (Boxall, 2004). However, many groups of non-target organisms (i.e. invertebrates and vertebrates) which have similar receptor systems could also be affected. Effects not related to the

therapeutic mode of action of a pharmaceutical are also possible as illustrated by the effects of diclofenac on Indian vulture species. Diclofenac is a commonly used anti-inflammatory drug and is highly toxic to some vulture species. Diclofenac was used as a veterinary treatment in areas of India and Pakistan. Vultures were exposed to the diclofenac when they consumed contaminated animal carcasses resulting in mortality and, over time, a large decline in population numbers of vultures (Oaks et al., 2004).

While a wide range of standard studies (i.e. following OECD protocol) indicate that the detected concentration of APIs in the environment do not trigger evident negative effects on test organisms, APIs are continuously released to the environment and subtle side effects after a long-term exposure are therefore possible (Daughton and Ternes, 1999) For example, it is believed that continuous exposure to 17-alpha-ethinylestradio (EE2), the active ingredient in many oral contraceptives, could result in the reduction of fish reproduction (e.g. fertility of sexually maturing male rainbow trout) and the collapse of fish populations (Jobling et al., 2006, Kidd et al., 2007, Schultz et al., 2003). As a result of findings like those described above, three APIs (diclofenac, EE2 and 17-beta-estradiol (E2)) have been included in the Water Framework Directive (Directive 2013/39/EU) watch list (EC, 2013), with the goal of generating monitoring data and determining the most appropriate mitigation measures for their risk.

1.3 Algae and APIs in the environment

Algae, as a particularly sensitive class of organisms to APIs exposure, are suitable, quick and cost effective indicator organisms for environment health assessment studies (Pavlic et al., 2005). Side effects of APIs on algae could not only result in the inhibition of their growth but

also affect the entire ecosystem as a results of their important ecosystem functions such as oxygen production, nutrient cycling and food supply (DeLorenzo and Fleming, 2008). Algae often provide one of the first signals of ecosystem impact due to their short response times, which allows corrective regulatory and management actions on APIs to be taken before the occurrence of further damage occurs within the ecosystem (Pavlic et al., 2005). Algal species are therefore routinely used in the risk assessment of APIs for human and veterinary use (EMEA, 2008, EMEA, 2006).

While a wide range of investigations have previously focused on the effects of APIs on algal species, most studies have only looked at the effects on a handful of algal species, mainly on chlorophytes. Differences in the responses of algal species towards APIs have been found in some studies i.e. cyanobacteria have been shown to exhibit higher sensitivity to APIs with antibacterial properties than chlorophytes (Halling-Sorensen, 2000). Model species (i.e. chlorophytes) used for effect assessment may therefore not be the most appropriate test organisms to all API exposures. As a result of their observed sensitivity to antibacterial compounds, cyanobacteria are now incorporated into risk assessment procedures for human and veterinary medicines. However, for some classes of algae (e.g. diatoms) our understanding of sensitivity to APIs is limited as is our understanding of sensitivity of different species from the same organism class. A study systematically exploring the sensitivity of commonly used indicator algal species (i.e. species recommended in OECD 201 guideline) towards API exposures is therefore needed to ensure the natural environment is protected.

In the current ecotoxicological test protocols of APIs on algae (i.e. OECD 201, 2011) (OECD, 2011), while the cell density has commonly been used as surrogate endpoint for growth, it

might be misleading as the unviable cells, having lost their biomass, are still counted over the test period (Bellinger and Sigee, 2015). To overcome this defect, photosynthetic endpoints such as oxygen evolution rate which are directly related to viable cells might be an alternative to replace cell number. In this case, the sensitivity comparison between the endpoints of oxygen evolution rate and cell density should be initially clarified. Despite the inhibitory effects of APIs on algal growth having been extensively observed, the toxic mechanisms are still unclear. As algae are photosynthetic organisms, inhibition of growth might be due to the damage of the algal photosynthesis processes (Liu et al., 2011). Effects of APIs on the algal physiology such as light-harvesting pigment synthesis and light utilisation efficiency therefore warrant further consideration.

Surface waters are more likely to be exposed to the antibiotic mixtures than single substances (Backhaus et al., 2011), so it is vital to assess the combination effects and potential risks of antibiotic mixtures in the natural environment. Environmental risk should be assessed on the organisms that are likely to protect the broader environment.

1.4 Aims of the Thesis

The overall aim of this thesis was therefore to assess the impacts of selected APIs on a wide range of algal species. The work was performed using three major use antibiotics and seven algal species from the chlorophytes, cyanobacteria and diatom classes. The specific objectives were to:

1. Review the current knowledge regarding the effects of APIs on the growth of algal species to explore the evidence base as to whether APIs pose a threat to algae in surface waters

and to investigate the algal sensitivity towards API exposures (Chapter 2).

2. Prioritise APIs in use based on their toxicological and ecotoxicological risks in the natural environment and combine the results with findings from objective 1 to target the antibiotics for further laboratory study (Chapter 3).
3. Compare the sensitivity of chlorophytes, cyanobacteria and diatoms to major use antibiotics (Chapter 4).
4. Investigate the effects of antibiotics on the growth and physiology of four of the most sensitive species to obtain information on the underlying toxic mechanisms (Chapter 5).
5. Assess the risks of mixtures of major use antibiotics in the European Union by using the most sensitive species identified in earlier Chapters (Chapter 6).

1.5 Thesis overview

This thesis comprises seven chapters. A description of each is given below:

Chapter 2 synthesises the existing knowledge on the toxicity of APIs to algal species and communities. This Chapter explores the differences in the sensitivity of a range of algal groups to APIs and assessed the potency of commonly used APIs to algae. The data generated are combined with predicted exposure levels for APIs in order to establish the potential risks of APIs to algal populations. The importance of algae in the ecosystem, potential toxicity mechanisms, and a comparison of the risks of APIs to that of herbicides and future recommendations are also discussed.

Chapter 3 describes the development and implementation of a holistic risk-based prioritisation approach for pharmaceuticals entering the aquatic and terrestrial environment through

wastewater in the UK. The prioritisation approach considered APIs used in primary and secondary care, medicines sold over the counter and major pharmaceutical metabolites. Both aquatic and terrestrial exposure routes and acute and chronic effects on algae, invertebrates, fish, birds, model mammals and humans are considered. The approach was applied to 146 active ingredients and associated metabolites to identify APIs with high potential risk in the UK environment. Combined with the results in Chapter 2, three major use antibiotics, tylosin, lincomycin and trimethoprim, were identified for further experimental investigation.

Chapter 4 explores the sensitivity of seven algal species towards major use antibiotic exposures at EC_{50} levels. Dose-response curves of the target antibiotics were generated for seven test species from chlorophytes (*Pseudokirchneriella subcapitata, Desmodesmus subspicatus and Chlorella vulgaris*), cyanobacteria (*Synechococcus leopoliensis and Anabaena flos-aquae*) and diatoms (*Navicula pelliculosa and Phaeodactylum tricornutum*).

Chapter 5 investigates the inhibitory effects of the major use antibiotics on the physiological endpoints including oxygen evolution rate, chlorophyll a, chlorophyll b, total carotenoid content and light utilisation efficiency for the four most sensitive algal species identified in Chapter 4 (*P. subcapitata*, *D. subspicatus*, *A. flos-aquae* and *N. pelliculosa*). The endpoint sensitivity of growth and oxygen evolution rate was compared at EC_{50} levels. The information generated was used to explore the potential toxic mechanisms of APIs on algal growth.

Chapter 6 describes work to determine the combined effects of the major use antibiotics on the cyanobacterial species *A. flos-aquae*. An evaluation of the predictive capability of two mixture toxicity models, concentration addition (CA) and independent action (IA), is presented. The CA model was then used alongside predictions of exposure for different European scenarios to

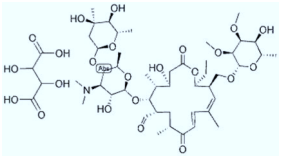
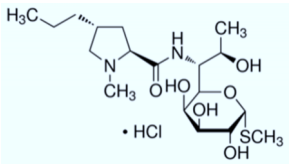
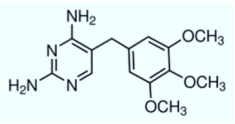
characterize the risks arising from the exposure of European surface waters to the three antibiotics.

Chapter 7 discusses the main findings of the study and the potential implications for environment risk assessment approaches. Recommendations for specific studies following on from the work in this thesis and for more general studies into API impacts in the environment are presented.

1.6 Study Compounds

In this thesis, three major use antibiotics, trimethoprim, tylosin and lincomycin are used in the experimental investigations. The antibiotics were selected using a prioritisation study based on the risk of APIs to a broad range of algal species. The substances represent different groups of antibiotics: tylosin is a macrolide; lincomycin is a lincosamide; trimethoprim is a pyrimidine. To facilitate the test (high solubility) and to be consistent with published literatures, tylosin tartrate and lincomycin hydrochloride were used as the test compounds, but in this thesis these two substances are referred to as lincomycin and tylosin. The physico-chemical properties of the antibiotics tested are shown in Table 1.1. The maximum occurrences of the three antibiotics were found in the US with concentrations of 0.05 (Kim and Carlson, 2007), 0.73 and 0.71 ug L^{-1} (Monteiro and Boxall, 2010) being found for tylosin, lincomycin and trimethoprim, respectively.

Table 1.1: Physicochemical properties of tylosin, lincomycin and trimethoprim,

	Tylosin tartrate	Lincomycin hydrochloride	Trimethoprim
CAS-no.	1405-54-5	859-18-7	738-70-5
Structure			
Molecular weight (g mol^{-1})	1066.19	443	290.32
Log Kow	1.63[a]	0.56[b]	0.91[b]
Pka	7.73[c]	7.6[c]	7.12[b]
Solubility in H_2O	Very soluble ($5X10^4$ mg L^{-1})[d]	Free Soluble [d]	Slightly soluble (400 mg L^{-1})[e]
Mode of action	Inhibit bacterial protein synthesis by binding to 50S ribosome[b]	Inhibit bacterial protein synthesis by binding to 50S ribosome[b]	Inhibit dihydrofolate reductase[d]

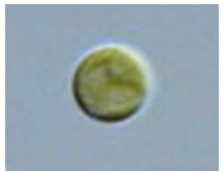
a. (Loke et al., 2002); b. (Drugbank, 2013); c. (HSDB, 2015); d. (Sigma-Aldrich, 2015); e. (EPA, 2013).

1.7 Study species

Six algal species recommended in the OECD 201 guidelines along with a widely used diatom species were chosen as study organisms. Speices included three chlorophytes *Pseudokirchneriella subcapitata*, *Desmodesmus subspicatus* and *Chlorella vulgaris*; two cyanobacteria *Anabaena flos-aquae* and *Synechococcus leopoliensis*; and two diatoms *Navicula pelliculosa* and *Phaeodactylum tricornutum*. The appearance, characteristics and

distributions of test species are shown in Table 1.2. Details of the algal culturing methodologies and test procedures are described in Chapter 4 – 6.

Table 1.2: Appearance, characteristics and distributions of the algal test species

	P. subcapitata	*D. subspicatus*	*C. vulgaris*	*A. flos-aquae*	*S. leopoliensis*	*N. pelliculosa*	*P. tricornutum*.
Strain	CCAP 278/4	CCAP 258/137	CCAP 211/11b	CCAP 1403/13A	CCAP 1405/1	CCAP 1050/9	CCAP 1052/1b
Test meidum and pH	Kuhl, 6.8	Kuhl, 6.8	Kuhl, 6.8	JM, pH 7.8	JM, pH 7.8	ESAW + f/2, 8.2	ESAW + f/2, 8.2
Picture[a]							
Appearance[b]	Curved, twisted single cells	Oval, mostly single cells	Spherical, single	Chains of oval cells	Rods	Rods	Fusiform, triradiate, and ova (paper)
Size (LXW) µm[b]	8-14 X 2-3	7-15 X 3-12	3 (diameter)[d]	4.5 X 3	6 X 1	7.1 X 3.7	n.a
Cell volume	40-60	60-80	n.a.	30-40	2.5	40-50	n.a

(μm^3 $cell^{-1}$)[b]					
Cell dry weight	2-3 X 10^{-8}	3-4 X 10^{-8}	n.a.	1-2 X 10^{-8}	2-3 X 10^{-9}
(mg $cell^{-1}$) [b]					
Freshwater/	Freshwater	Freshwater	Freshwater	Freshwater	Freshwater
marine[a]					
Distribution	Bulgarian, Denmark,	Britain, Germany,	Austria, Brazil, Britain,	Australia, Brazil,	Norway, US.
reported in the	Egypt, Estonia,	New Zealand,	Czech Republic,	Britain, China,	
literature[c]	Finland, Germany,	Romania, Russian,	Denmark, Egypt,	Denmark,	
	Italy, Nigeria,	Singapore, Spain,	France, Germany,	Germany,	
	Romania, Spain,	Taiwan, Turkey.	Ireland, Iran, Mexico,	Israel, Lithuania,	
	Thailand,		New Zealand，	Nepal,	
			Netherlands,	New Zealand	
			Pakistan,	Romania, Russia,	

	Romania, Spain,	Senegal, Spain,
	Sweden, Taiwan,	Sweden,
	Turkey, US.	Singapore, US.

a. (CCAP, 2015); b. (OECD, 2011); c. (AB, 2015); d. (Bionumber, 2015).

Chapter 2

Do pharmaceuticals pose a threat to primary producers?

2.1 Introduction

Over the past ten years, our understanding of the environmental fate and effects of APIs has increased significantly and numerous published scientific papers relating to the toxicity of APIs to non-target organisms are now available (Figure 2.1). These include studies on the ecotoxicity of APIs to fish and invertebrates and a number of syntheses have discussed data on these taxonomic groups (Nentwig, 2007, Corcoran et al., 2010). However, while data are available on the toxicity of many APIs to algae (around a third of published papers out of all toxicity studies; Figure 2.1), no attempt has been made to synthesise and make sense of this information. This Chapter therefore brings together available information on the toxicity of APIs to algae and use this information to explore differences in sensitivity of a range of algal groups and also differences in potency of common API groups to algae. The data are also used alongside exposure predictions to establish the potential risks of APIs to algal populations. Finally gaps in our current knowledge are identified and recommendations provided on priorities for future research.

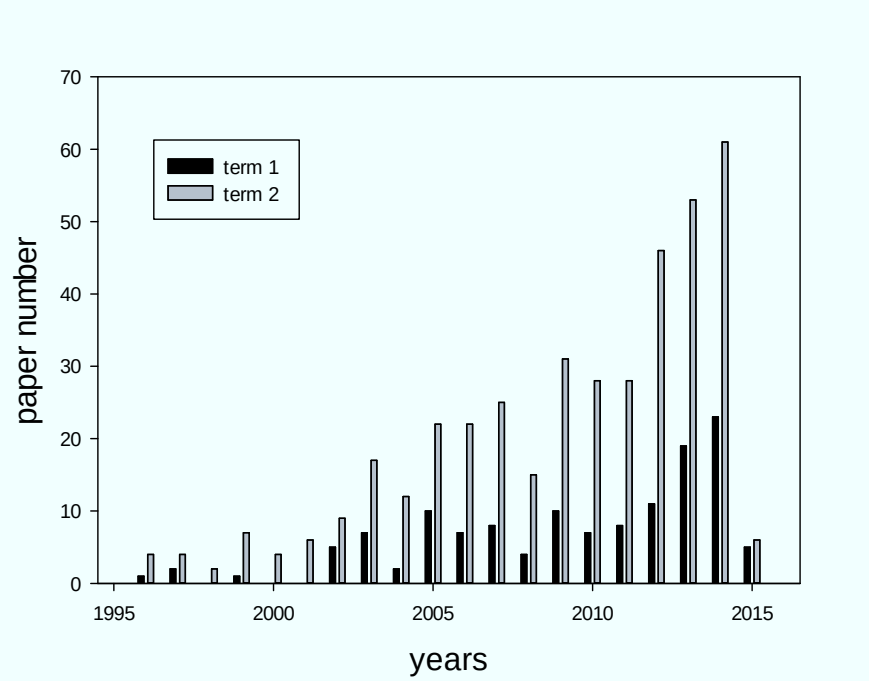


Figure 2.1 Published paper numbers of algae and all the standard toxicity organisms including algae, fish and invertebrates identified by Web of Science (2014). Term 1: (Algae AND ecotox* AND (antibiotic OR pharmaceutical OR medicine)); Term 2: (Algae OR fish OR daphni* OR invertebrate*) AND ecotox* AND (antibiotic OR pharmaceutical OR medicine)

2.2 Why are algae important?

Algae are widely distributed in aquatic ecosystems, and comprise a large proportion of the aquatic biomass. Supplying food to the early larval stages of animals such as molluscs and fish, algae are an essential component of aquatic food chains (Lai et al., 2009). Detrimental effects of a compound on algae could therefore pose a potential threat not only to algal populations but also to higher trophic levels. Algal groups also perform important ecosystem functions. For example, cyanobacteria perform a nitrogen-fixing role in the marine environment. Cyanobacteria filaments contain cells that specialize in photosynthesis and heterocysts that can fix nitrogen, and in the nitrogen cycle they convert dinitrogen gas to more easily

assimilated forms for organisms such as ammonia (Amin et al., 2012). Like other plants, algae produce a large amount of oxygen as a by-product of photosynthesis. If they are destroyed, other aquatic organisms could therefore be adversely affected due to an oxygen shortage (DeLorenzo and Fleming, 2008, Larned, 2010, Backhaus et al., 2011).

While algae play a pivotal role in nutrient cycling, they can also cause negative effects on ecosystems. Harmful algal bloom (HAB) events have been reported worldwide that negatively affect human health and the ecosystem balance (Fire et al., 2011, Laycock et al., 2012, Capper et al., 2013). Potent algal toxins produced by toxic HABs can cause mortality and morbidity in humans and aquatic organisms and the decomposition of the bloom also results in a drastic reduction in dissolved oxygen (Laycock et al., 2012).

2.3 Why might algae be vulnerable to pharmaceutical exposure?

Pharmaceuticals are designed, and used, to prevent and cure diseases and improve the quality of life of humans and animals. The principal way in which they do this is by interacting with receptors and pathways inside the human or animal or in infectious organisms such as bacteria and fungi (Boxall, 2004). Many of these receptors and pathways might be conserved in other organisms in the natural environment (Boxall, 2004). Some evidence has been presented in the literature indicating that receptor conservation will occur in algae and that therefore subtle effects could be expected. For example, Brain et al. (2008) reported that a very high degree of homology existing between the chloroplast and bacteria in terms of general translation factors and most of the ribosomal proteins (through phylogenetic analysis) infering that numerous basic processes of translation are conserved in both bacteria and the

chloroplast. As macrolide and lincosamide antibiotics hinder protein synthesis by interacting with the peptidyl transferase domain of bacterial 23S rRNA, and aminoglycosides block bacterial protein synthesis by irreversibly binding to 30S and 50S subunits of ribosomes, these might disrupt transcription/translation in the chloroplasts of photosynthetic organisms such as green algae (Brain et al., 2008b).

Statins are a class of pharmaceuticals that decrease total cholesterol and low-density lipoprotein cholesterol (LDLc). They are highly specific inhibitors competing with 3-hydroxy-3-methylglutaryl coenzyme-A reductase (HMGR), which is the rate-limiting enzyme in cholesterol biosynthesis. In plants, HMGR is also an essential enzyme that regulates the mevalonic acid (MVA) pathway of isoprenoid biosynthesis, and as in humans, statins inhibit this enzyme (Brain et al., 2008a). The MVA pathway is also present in the red alga *Cyanidium caldarum* and the diatom *Ochromonas danica* (Lange et al., 2000), and this may therefore represent one potential toxicity mechanism of statins to algae.

2.4 Indirect effects from bacteria

Algae could also be affected by a pharmaceutical indirectly as a result of toxicity of some pharmaceuticals to bacterial species. Algae (especially diatoms) and bacteria have co-existed for more than 200 million years, resulting in synergistic interactions between them (Liu et al., 2012). One such interaction between diatoms and bacteria is the way that bacteria produce and supply vitamins, such as Cobalamin, or vitamin B12, to different diatom species. Croft et al. (2005) demonstrated that more than half of diatoms investigated cannot grow in B12-limited medium, and they also confirmed that bacteria provide vitamins to most B12-auxotrophic

phytoplankton in exchange for fixed carbon (Croft et al., 2005).

In natural aquatic environments, bacteria are usually embedded in a biofilm (microbial cells immobilised in a matrix of extracellular polymers acting as an independent functioning ecosystem, homeostatically regulated) (Percival et al., 2011). In this form, bacteria obtain benefits such as the sharing of metabolic capabilities, niche separation and resistance against toxic substances. However, a variety of antibiotics (e.g. amoxicillin and erythromycin) at environmentally relevant concentrations (µg L^{-1}) can block the initial adhesion of bacteria (first step for biofilm formation), especially for amoxicillin which strongly inhibits the adhesion of *Escherichia coli* and *Aquabacterium commune* (Schreiber and Szewzyk, 2008). A range of antibiotics also show their own capacity to damage bacteria. Polymyxins alter bacterial outer membranes irreversibly by dissolving the fatty acid portion in its hydrophobic region; chloramphenicol behaves through a bacteriostatic action by inhibiting the peptidyl transferase; aureomycin inhibits bacterial protein synthesis by combining with the small (30S) subunit of the ribosomes - all these antibiotics have been shown to be toxic to luminescent bacterium (Duggar, 2011, Ji et al., 2013). Though toxicity of these antibiotics to bacteria is observed at the experimental scale, similar damage mechanisms are also likely to occur in bacteria that supply nutrients to algal species in the natural environment. Evidence for API effects on algae is presented in the next section.

2.5 Ecotoxicological effects of pharmaceuticals on algae

A wide range of data on the ecotoxicity (EC_{50}) of APIs to various algal species is now available. Table 2.1 summarises all the published ecotoxicity data covering 350 pharmaceuticals and

related products from 43 therapeutic classes for different algal species (the original toxicity data extracted from published literature and databases are shown in Tables A.1 to A.4 (Appendix 1). Most of the research summarised in Tables A.1 to A.4 (Appendix 1) was undertaken using the OECD (2011) Guidelines for alga growth inhibition tests (72h/ 96h duration, biomass yield/ growth rate endpoint; nominal/ measured concentration used for test is indicated as a footnote in Tables A.1 to A.4, Appendix 1). In the tests, nine species of chlorophytes have been used, three species of cyanobacteria, three algal communities and one diatom species (Tables A.1 to A.4, Appendix 1).

Table 2.1 Summary of ecotoxicity data of pharmaceuticals to algae

Pharmaceutical class	Mode of action for human	Example of pharmaceuticals in this class	EC_{50} range (mg L^{-1})		
			Chlorophytes	Cyanobacteria	Others (e.g. diatom)
Analgesic	Inhibit both isoforms of cyclooxygenase, COX-1, COX-2, and COX-3 enzymes involved in prostaglandin (PG) synthesis	Fentanyl Paracetamol	0.98-134		
Androgenic	Activate the androgen receptor; activate certain estrogen receptors by conversing to estradiol	Testosterone	0.5		
Anesthetic	Block the sodium-channel and decrease chances of depolarization and consequent action potentials	Prilocaine Ropivacaine,	0.045-154		
Antiarrhythmic	Inhibit voltage gated sodium (Na+) channels	Lidocaine Dronedarone	0.045-780	0.25	
	Na, K-activated myocardial adenosine triphosphatase	Amiodarone			
Antiasthmatic	Antagonize leukotriene D_4 (LTD_4) at the cysteinyl leukotriene receptor	Montelukast	100		
Antibiotic	Inhibit ptidyl transferase; inhibit amino acids	(Macrolide) Clarithromycin Erythromycin, Tylosin,	0.002-1.38	0.034	
	Inhibit cell-wall synthesis enzyme	(β-lactam) Amoxicillin, Cefradine	1.77-630	0.0022-1.38	
	N.A.	Chloramphenicol Florfenicol	0.1-1283		1.3-38[b]

		Thiamphenicol		
	Inhibit DNA gyrase	(Fluoroquinolone)	7.4	
		Levofloxacin		
	Inhibit peptide bond formation	(Lincosamide)	0.07	
		Lincomycin		
	Inhibit bacterial nucleic acid synthesis	(nitroimidazole)	39.1	
		Metronidazole		
	Inhibit water reabsorption in the nephron by blocking sodium-potassium-chloride cotransporter (NKCC2)	(Sulfonamides) Furosemide	322.2	
	Inhibit the protein synthesis by binding of tRNA to the mRNA-ribosome	(Tetracycline)	0.31	0.09-0.24
		Minocycline, Tetracycline		
	Inhibit the enzymatic conversion of pteridine and p-aminobenzoic acid (PABA) to dihydropteroic acid	(Sulfamethoxazole) Bactrim (mixture)	70	112
	Inhibit dihydrofolate reductase	Trimethoprim	9	
	Block 30S ribosomal subunit of susceptible organisms	Streptomycin	0.13-20.08	0.28
Anticholinergic	Inhibit cholinesterase	Galantamine	100	
Anticoagulant	Inhibit vitamin K reductase	Warfarin	11	
Anticonvulsant	Inhibit voltage-sensitive sodium channels and/or calcium channels	Carbamazepine	4.48-100	
		Lamotrigine, Topirmat		
Antidementia	Inhibit butyrylcholinesterase and acetylcholinesterase	Rivastigmine	83	

Antidepressant	Inhibit serotonin reuptake	Fluoxetine, Sertralin, Trimipramine	0.027-240		0.038[a]
	Inhibit serotonin-norepinephrine reuptake	Duloxetine	0.2		
	Block dopamine uptake	Bupropion	0.95		
Antidiabetic	Reduce potassium conductance and cause depolarization of membrane on the pancreatic cell surface	Glimepiride Metformin	320-1000		
	Inhibit dipeptidyl peptidase-4 (DPP-4)	Sitagliptin	39		
Antidiarrheal	Inhibit peristaltic activity of intestine and affect water and electrolyte movement through the bowel	Loperamide	54-76		
Antiemetic	Inhibit 5HT-3 receptor	Aprepitant Granisetron	0.18-22.6		
Antifungal	Block cytochrome P-450 dependent enzyme, sterol 14α-demethylase	Itraconazole Posaconazole,	0.19- 1000	1000	
	Inhibit sterol ergostol	Clotrimazole Ketoconazol	0.0032		0.15[a]
	Inhibit bacterial Fatty Acid Synthesis	Triclosan	0.0036		0.34[a]
	Disrupt membrane transport by blocking the proton pump	Zinc-Pyrithione			0.0023[a]
Antihistamine	Compete with free histamine for binding at H1-receptors in the GI tract	Fexofenadin Levocabastine	0.7-200	32	

		Loratadine		
Antihyperlipidemic	Inhibit cholesterol absorption	Ezetimibe	4	
	Inhibit 3-hydroxy-3-methylglutaryl coenzyme	Simvastatin	22.8	
Antihypertensive	Block angiotensin-receptor;	Candesartan	56-460	
		Irbesartan		
	Interfere with the binding of angiotensin II to the angiotensin II AT_1-receptor	Telmisartan	9.88	
	Antagonize Alpha1-receptor	Terazosin	160	
	Inhibit angiotensin converting enzyme (ACE)	Captopril	100-168	
		Ramipril		
	Block catecholamine stimulation of β1-adrenergic receptors	Atenolol,	5.8-620	0.084[a]
		Pindolol		
		Propranolol		
	Block alpha-adrenergic receptors in the lower urinary tract	Alfuzosin	0.7-	
			52.7	
Anti-inflammatory	Inhibit phospholipase A2 inhibitory proteins	Mometasone	3.2	
	N.A.	Budesonide	8.6	
	Inhibit leukocyte migration and the enzyme cylooxygenase (COX-1 and COX-2)	Diclofenac	10-320	7.1[c]
		Ibuprofen		
		Naproxen		
Antilipemic	Activate peroxisome proliferator activated receptor a (PPARa)	Lipanthyl	0.102	

	Inhibit hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase	Rosuvastatin	330	
Antimalarial	Interact with heme	Lariam (mixture)	0.16-	
		Mefloquine,	0.33	
Antineoplastic	Alkylate DNA and lead to single and double-strand DNA breaks and apoptotic cell death	Temozolomide	90	
	Inhibit inosine monophosphate dehydrogenase (IMPDH)	Mycophenolate mofetil	0.068	
	Inhibit Epidermal growth factor receptor (EGFR) tyrosine kinase	Gefitinib	1.02-	
		Imatinib	2.5	
	Inhibit proteasome	Bortezomib	0.3	
	Inhibit DNA synthesis and cytotoxicity	Gemcitabine	5.4-100	
		Nelarabine		
	Inhibit mitotic and interphase cellular functions	Cabazitaxel	0.013	
	Inhibit tyrosine kinase	Nilotinib	0.016	
Antiobesity	Inhibit pancreatic lipase	orlistat	1.92	
Antiparkinsonian	Stimulate dopamine receptors	Pramipexol	29.3-	
		Ropinirole	240	
Antiplatelet	Prevent binding of adenosine diphosphate (ADP) to its platelet receptor	Clopidogrel	0.85	
Antipsychotic	Block 5-HT2 receptors	clozapine	2.5-	
		Olanzapine	141	
		Paliperidon		
Antiretroviral	Inhibit reverse transcriptase	Efavirenz	0.012-	0.76

		Lamivudine	96.9
		Nevirapine	
	Inhibit protease	Darunavir	43-100
		Telzir	
Antirheumatic	Inhibit pyrimidine synthesis	Leflunomide	22.4
Antispasmodic	Block muscarinic receptors	Butylscopolamine	80
Antithrombotic	Inhibit phosphodiesterase	Dipyridamole	2.36
Antitusivo	Stimulate synthesis and release of surfactant by type Ⅱ pneumocytes	Ambroxol	25.6
Antiulcer	Block a non-imidazole histamine receptors	Esomeprazole	85-150
		Omeprazole	
		Ranitidine	
Antiviral	Inhibit viral DNA polymerase	Acyclovir	99
	Inhibit influenza virus neuraminidase	Oseltamivir	463
	Inhibit viral replication process	Entecavir	110
	Block nucleic acid synthesis	Ribavirin	100
	Inhibit nonpeptidic protease	Tipranavir	40.4
Anxiolytic	Inhibit neurotransmitter gamma-aminobutyric acid (GABA)	Midazolam	11.4
Bronchodilator	Stimulate beta2-adrenergic receptor	Terbutaline	2.8- 500
		Salmeterol	
Calcium regulator	Inhibit farnesyl pyrophosphate (FPP)enzyme	Ibandronate	0.76-15
		Zoledronic acid	
		diltiazem	

Cardiovascular	Compete with adrenergic neurotransmitters	Metoprolol	7.3-
		Seloken	58.3
Diuretic	n.a.	Furosemide	322
Iron Chelating Agents	Bind ferric iron to form a stable complex	Deferasirox	0.32
Hypnotics	Potentiate gamma-aminobutyric acid (GABA)	Zolpidem	2.2
Immunosuppressive	Inhibit calcineurin, lymphokine and interleukin	Ciclosporin	100
	Inhibit mammalian target of rapamycin (mTOR)	Everolimus	16
Psychoanaleptics	n.a.	Methylphenidate	6
Vasodilator	Activate of enzyme guanylate cyclase	Glyceryl trinitrate	0.4

N.A. not available; Bracket shows the subcategory of antibiotics.

a Natural community

b *Isochrysis galbana* (Isochrysis) (Lai et al., 2009)

c Skeletonema costatum (Diatom)

The EC_{50} values range from 0.002 mg L^{-1} (clarithromycin to chlorophyte *Pseudokirchneriella subcapitata*) (Isidori et al., 2005) to 1283 mg L^{-1} (Thiamphenicol to chlorophyte *Chlorella pyrenoidosa;* (Lai et al., 2009) with many compounds not causing any toxicity at the highest concentrations tested. Antibiotics (e.g. macrolide and β-lactam) from classes operating with different modes of action show high toxicity to algal species. Other pharmaceutical classes, including compounds from the analgesic, androgenic, anesthetic, antiarrhythmic, antidepressant, antifungal, antihypertensive, antilipemic, antimalarial, antineoplastic, antiplatelet, antiretroviral, calcium regulator, iron chelating agents and vasodilator groups also exhibit high toxicity with EC_{50} values below 1 mg L^{-1} for selected compounds and species. The toxicity data extracted from the Swedish Fass (2012) database are mainly for pharmaceutical products, and their ingredients are listed in a separate column in Tables A.1 to A.2 (Appendix 1). Some pharmaceutical products are mixtures of APIs e.g. Bactrim (sulfamethoxazole and trimethoprim) and Riamet (artemether and lumefantrine), as it is uncertain which ingredient is tested, the toxicities of these products are listed separately (Tables A.1 to A.2, Appendix 1). Therapeutic classes with more than 4 sets of toxicity data to algae were selected and compared by using EC_{50} values (Figure 2.2). Previous algal toxicity tests were mainly focused on antibiotic, antidepressant, antifungal and antineoplastic, however the values cover a wide range (e.g. antibiotic EC_{50} varies from 0.01 to 1000 mg L^{-1}). Antiretroviral, antifungal and antibiotic were all found at EC_{50} values less than 0.01, but there are also antibiotics with available data in this range (Figure 2.2). Cytochrome p450 (CYP) is primarily responsible for drug metabolism in some higher trophic levels organisms (e.g. human and fish), and occurs in some algal species (e.g. chlorophyte *Chlamydomonas reinhardtii*; (Gangl et al., 2015). While a

range of pharmaceuticals (e.g. ketoprofen and fluoxtine) were observed to inhibit the cytochrome P450 activity in human and fish liver microsomes (Jenkins et al., 2011, Smith et al., 2012), no evidence linking the traditional ecologically endpoint (e.g. growth) and this specific molecular level responses has been currently reported (Boxall et al., 2012).

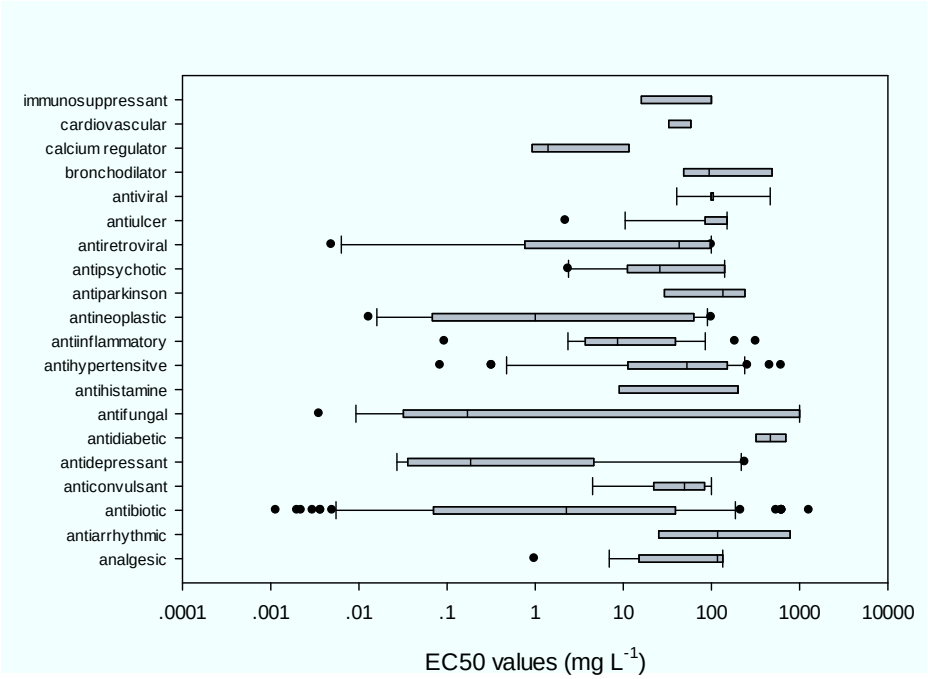


Figure 2.2 Toxicity value comparisons for selected therapeutic classes of pharmaceuticals

A large number of data were obtained on two chlorophytes *Pseudokirchneriella subcapitata* (previously known as *Selenastrum capricornutum*) and *Desmodesmus subspicatus* (previously known as *Scenedesmus subspicatus*) following the OECD (2011) Guidelines (Tables A.1 to A.2, Appendix 1). Other algal species have also been used for testing such as isochrysis (antibiotics to *Isochrysis galbana* (EC_{50} 1.38-38 mg L^{-1}; (Lai et al., 2009) and diatoms (anti-inflammatory to *Skeletonema costatum* (EC_{50} 7.1 mg L^{-1}; (Halling-Sorensen et al., 1998), but the data are few. Some data are also available on the effects of APIs (antifungals and antidepressants) on natural algal communities with EC_{50} values ranging from 0.0023 to 0.34 mg L^{-1} (Tables A.1 to A.4, Appendix 1). In terms of tested algal species, only chloramphenicol,

oxytetracycline, streptomycin, diclofenac and amoxicillin have been tested on three or more algal species with ecotoxicity values ranging from 0.007 (streptomycin to *M. aeruginosa*; (Halling-Sorensen, 2000) to 630 mg L^{-1} (amoxicillin to *P. subcapitata*; (Fass.se, 2011). While the available ecotoxicity data on chloramphenicol, oxytetracycline and diclofenac only focus on chlorophytes, their toxicity data varies considerably (e.g. chloramphenicol, EC_{50} 0.1 – 41 mg L^{-1}; (Sanchez-Fortun et al., 2009, Goncalves Ferreira et al., 2007).

2.6 Environmental risk assessment (ERA of active pharmaceutical ingredients (APIs) to algal species

From the previous section it appears that some APIs are highly toxic to algal species. Therefore in this section, to assess whether this toxicity could be realised in the natural environment under typical usage scenarios, the environmental risk assessment guidelines proposed by the European Medicines Agency (EMEA) for human and veterinary pharmaceuticals are used alongside the ecotoxicity data discussed in the previous section, to estimate the level of risk to algal communities.

Data on the amount/or sales of human pharmaceuticals were obtained from the Prescription Cost Analysis (PCA) (2011) in England (NHS, 2012). Using data on usage the potential amounts of APIs released to environment were estimated. Exposure concentrations of APIs in the aquatic environment were estimated separately for human and veterinary use compounds (EMEA, 2006, EMEA, 2008).

Predicted Environmental Concentration ($PEC_{surfacewater}$) values for human pharmaceuticals were calculated using Equation 2.1 (EMEA, 2006).

$$PEC_{SURFACEWATER} = \frac{consumption}{WASTEWinhab \times DILUTION \times inhabitants \times 365}$$ Equation 2.1

Where WASTEWinhab: Amount of wastewater per inhabitant per day, default value, 200, [L inh^{-1} d^{-1}]; DILUTION: Dilution factor, default value, 10; $PEC_{SURFACEWATER}$: Local surface water concentration, [mg L^{-1}]; Consumption: the total quantity of an active molecule consumed in a defined area, [mg $year^{-1}$]; Inhabitants: the population in UK, 62641000 in 2011. The $PEC_{surfacewater}$ results for human pharmaceuticals are listed in Tables A.1 to A.4 (Appendix 1). The $PEC_{surfacewater}$ for veterinary usage (listed in Table 2.2) were calculated using Equation 2.2 (EMEA, 2008):

$$PEC_{SURFACEWATER} = \frac{380.46 \times SOL \times D \times AD \times BW \times P \times Fh}{Ny \times H \times (VP \times MW + 2369.49 \times SOL + 355.42Koc)}$$ Equation 2.2

Where D = Daily dose of the active ingredient [$mg.kg_{bw}^{-1}.day^{-1}$]; Ad = Number of days of treatment [d]; BW = Animal body weight [kg_{bw}], calves 140kg, cattle 450kg and pig 12.5kg; P = Animal turnover rate per place per year [$place^{-1}.year^{-1}$], calves 1.8, cattle 1 and pig 6.9; Fh = Fraction of herd treated, 1 for antibiotics (feed and water medication) and 0.5 for antibiotics (injectable); Ny= Nitrogen produced in one year per place [$kg.N.place^{-1}.year^{-1}$], calves 10, cattle 35 and pig 2.25; H = housing factor, calves 1, cattle 0.5 and pig 1; VP = Vapour pressure [Pa]; MW = Molar mass [$g.mol^{-1}$]; SOL = Water solubility [$mg.L^{-1}$]; Koc = water-organic carbon distribution coefficient [1.kg]. The information on daily dose of the active ingredient and number of days of treatment were identified from Compendium of Data Sheet for Animal Medicines (NOAH, 2011). Vapour pressure, water solubility and Koc were

the Environment Protection Agency EPI suite software (4.1 version; (EPA, 2013)).

Table 2.2 Toxicity of veterinary pharmaceuticals and environmental risk assessment to algae

Species	Pharmaceuticals	Test duration	EC_{50} (mg L^{-1})	Reference	PEC (mg L^{-1})	PEC:PNEC ratio*
Chlorella pyrenoidosa	Florfenicol	72h	215	(Lai et al., 2009)[1]	0.046	0.021
Chlorella vulgaris	Oxytetracycline	48h	6.4	(Pro et al., 2003)[3]	0.00021	0.0033
Desmodesmus subspicatus	Paracetamol	72h	134	(FASS, 2012)[3]	0.09	0.067
Microcystis aeruginosa	Amoxicillin	7d	0.008	(Liu et al., 2012)[3]	0.0099	122.98
	Amoxicillin	7d	0.0037	(FASS, 2012)[3]	0.0099	266.9
	Tetracycline	72h	0.09	(Halling-Sorensen, 2000)[2]	0.00017	0.19
	Tiamulin	72h	0.003		0.0033	108.25
	Tylosin	72h	0.034		0.0035	10.42
Pseudokirchneriella subcapitata	Oxytetracyline	72h	0.6	(van der Grinten et al., 2010)[2]	0.00021	0.035
	Trimethoprim	72h	9		0.49	5.46
	Tylosin	72h	0.0089		0.0035	39.81
	Erythromycin	72h	0.02	(Isidori et al., 2005b)[3]	0.0093	46.56
	Lincomycin	72h	0.07		0.044	62.46
	Oxytetracyline	72h	0.17		0.00021	0.12
	Amoxicillin	72h	630	(FASS, 2012)[3]	0.0099	0.0016
	Chlortetracycline	72h	3.1		0.00016	0.0053
	Fentanyl	72h	15.1		5.7E-06	3.77E-05
	Tetracycline	72h	2.2		1.7E-4	0.0076
	Tiamulin	72h	0.17		0.0032	1.97
	Tylosin	72h	1.38		0.0035	0.26
Scenedesmus obliquus	Enrofloxacin	72h	45.1	(Qin et al., 2012)[3]	2.29E-05	5.09E-05
Synechococcus leopoliensis	Amoxicillin	96d	0.0022	(FASS, 2012)[3]	0.0099	444.84
Tetraselmis chuii	Florfenicol	96h	6.06	(Goncalves et al., 2007)[3]	0.046	0.76
	Oxytetracyline	96h	11.18		0.00021	0.0019

*PNEC= EC_{50}/100

1 real concentration used; 2 nominal concentration used; 3 unknown

As the PEC human pharmaceutical calculation relies on API usage data and the PEC veterinary pharmaceuticals are calculated by using daily dose and treatment days, as well as

other different factors and parameters considered in Equation 2.1 & 2.2, two PEC values are obtained. Usually the PEC value calculated for veterinary pharmaceuticals is higher than for human pharmaceuticals (e.g. trimethoprim PEC_{human} 0.00019 mg L^{-1} and $PEC_{veterinary}$ 0.49 mg L^{-1}; amoxicillin PEC_{human} 0.0022 mg L^{-1} and $PEC_{veterinary}$ 0.0099 mg L^{-1}).

Effluent from the Waste Water Treatment Plants (WWTPs) receiving sewage from pharmaceutical manufacturing sites and hospitals are another important source of APIs entering the environment. To assess the contribution of APIs emitted from each source and their potential risk, a wide range of literature sources were used to identify the measured environmental concentrations (MEC) of pharmaceuticals in WWTPs receiving sewage from municipal, hospital, manufacture and livestock. APIs with available MEC in effluent from different sources and toxicity data to algae were collated and illustrated in Table 2.3 (if more than one MEC is available, the highest value is cited).

Table 2.3 Risk assessment for pharmaceuticals in WWTP effluent receiving wastewater from municipal, hospital, manufacture and livestock

Pharmaceuticals	EC_{50}* (mg L^{-1})	Municipal MEC (ug L^{-1})	Hospital MEC (ug L^{-1})	Manufacture (ug L^{-1})	Livestock (ug L^{-1})	Municipal RQ	Hospital RQ	Manufacture RQ	Livestock RQ
Trimethoprim	9	0.6[1]	29[5]	9.03[5]	23.6[5]	0.0067	0.32	0.1	0.26
Carbamazepine	49.4	1.52[1]	3.56[5]	51.7[5]	n.a	0.0031	0.0072	0.1	n.a
Erythromycin	0.02	0.05[2]	0.94[6]	5[5]	0.1[5]	0.25	4.69	25	0.51
Ibuprofen	7.1	0.14[3]	0.28[7]	45.87[6]	n.a	0.0019	0.004	0.65	n.a
Naproxen	31.82	0.35[2]	0.7[6]	50[5]	1.77[7]	0.0011	0.0022	0.16	0.0056
Diclofenac	10	0.35[4]	0.33[6]	50[5]	0.19[5]	0.0035	0.0033	0.5	0.0019
Tetracycline	0.09	n.a	0.089[6]	0.025[6]	1.13[7]	n.a	0.099	0.028	1.25
Enrofloxacin	45.1	0.17[4]	0.026[5]	5[5]	0.59[5]	3.7E-4	5.7E-5	0.011	0.0013
Chlortetracycline	0.05	n.a	0.22[5]	0.68[5]	2.82[5]	n.a	0.44	1.36	5.64
Florfenicol	6.06	n.a	n.a	5[5]	18.8[5]	n.a	n.a	0.083	0.31
Lincomycin	0.07	n.a	29.8[5]	14.83[5]	615[5]	n.a	42.57	21.19	878.57
Penicillin G	0.006	n.a	n.a	1[5]	13.5[5]	n.a	n.a	16.67	225

1. (McEneff et al., 2014), Ireland; 2. (Moreno-Gonzalez et al., 2014), Spain; 3. (Ortiz de Garcia et al., 2013), Spain; 4. (Collado et al., 2014), Spain; 5. (Sim et al., 2011), Korea; 6. (Lin and Tsai, 2009), Taiwan; 7. (Lin et al., 2008), Taiwan;

* EC_{50} is the lowest value of pharmaceuticals derived from Table A1.1 to A1.4, Appendix 1. PNEC= EC_{50}/100

Predicted no-effect concentration (PNEC) (for both human and veterinary) is defined as the level of concentration at which no negative effects are observed (NOEC), added to an assessment factor (AF) (Equation 2.3). Here, NOEC is replaced by EC_{50} (50% of the tested organisms are affected); An AF is used to reduce the level of uncertainty, a default value of 100 was applied by considering inter-species variations of differences in sensitivity (10) and laboratory data to field impact extrapolation (10) (EMEA, 2006). While the OECD 201 Guidelines are followed by most studies, the statistic toxicity value EC_{50} still varies due to different testing conditions, devices and models used to fit dose-response curves. In this case, the lowest EC_{50} values were used for conservative risk assessment.

$$PNEC = EC_{50}/AF \qquad \text{Equation 2.3}$$

The environmental risk of pharmaceuticals to algal species is characterised through a risk quotient (RQ; equation 2.4; MEC was used to replace PEC to assess risk for other emission sources). The results are listed in the Tables A.1 to A.4, Appendix 1).

$$RQ = PEC/PNEC \qquad \text{Equation 2.4}$$

The RQ value will be compared against a value of one, with a value less than one predicting that no toxicity of APIs to algae in aquatic environments is observed (EMEA, 2006, EMEA, 2008). Those compounds identified as having potential risks were considered to be high priority for investigation of their impact on algal species.

The ERA results of pharmaceuticals for human use show that the risk characterisation ratios (RQ) for clarithromycin, erythromycin and amoxicillin are above one. The high RQ values of the first two APIs are due to their high ecotoxicity to the chlorophyte *P. subcapitata* (EC_{50} 0.002 mg L^{-1} and 0.02 mg L^{-1}; (Isidori et al., 2005). The high RQ value for amoxicillin is due to the sensitivity of the cyanobacteria *M. aeruginosa* (EC_{50} 0.008 mg L^{-1}; (Liu et al., 2012). Tiamulin and amoxicillin are the two veterinary pharmaceuticals with the highest RQ values, 108.25 and 444.84, respectively (Table 2.2), followed by lincomycin (62.46), erythromycin (46.56), tylosin (39.81) and trimethoprim (5.46) all with RQ values above one. The RQ values of pharmaceuticals for human and veterinary use are synthesised and compared in Table 2.4. The high RQ values (>1) are only seen for three human pharmaceuticals; whereas six pharmaceuticals for veterinary use show high RQ values (>1), five of which have RQ values greater than 10. When comparing RQ values with other published pharmaceutical ranking studies, agreement can be found for some pharmaceuticals such as ibuprofen with a PEC: PNEC ratio 0.06 (Escher et al., 2011), 0.0018 (this study). However, in some cases large discrepancies are observed e.g. clarithromycin 0.035 (Escher et al., 2011) and 12.33 (this study). While Escher et al. (2011) used the lowest QSAR-based EC_{50} values from either fish, daphnia or algae for the PNEC calculation, the real environment risk would be vastly underestimated due to the predicted toxicity data.

Table 2.4 Classification of risk quotients of pharmaceuticals for human and veterinary use

PEC:PNEC ratio range	Human pharmaceuticals	Veterinary pharmaceuticals
>10	Clarithromycin, Amoxicillin	Tylosin, Erythromycin
1 - 10	Erythromycin	Lincomycin, Tiamulin, Amoxicillin Trimethoprim
0.1 - 1	Oxytetracycline, Mycophenolate mofetil, Fluoxetine, Propranolol	Florfenicol, Oxytetracyline, Tetracycline
0.01 – 0.1	Ibuprofen, Clotrimazole, Diclofenac, Dronedarone, Duac (mixture), Tetracycline, Ketoconazole, Lincomycin, Dipyridamole, Paracetamol, Benzoyl peroxide, Duloxetine, Fusidic acid,	Paracetamol
< 0.01	Minocycline, Metformin, Simvastatin, naproxen, Asasantin Retard, Felodipine, Penicillin G, Trimethoprim, Cefradine, Carbamazepine, Ceftazidim, Testosterone, ceftazidim, Metoprolol, Alfuzosin, Metronidazole, Ranitidine, Bupropion, Mefloquine, Clobetasol, Irbesartan, Mometasone, Omeprazol, Ezetimibe, Lamotrigine, Risedronic acid, Gluceryl trinitrate, Ofloxacin, Telmisartan, Atenolol, Bisoprolol, Sitagliptin, Warfarin, Deferasirox, Tadalafil, Zolpidem, Ibandronate, Furosemide, Ramipril, Lidocaine, Fexofenadine, Irbesartan, Amiodarone, Sertralin, Eprosartan, Candesartan, Econazole, Orlistat, Chloramphenicol, Budesonid, Naproxen, Sumatriptan, Lamotrigine, Carvedilol, Trimipramine, Esomeprazole, Levofloxacin, Riluzol, Posaconazole, Methylphenidate, Butylscopolamine, Etravirine, Fusidic acid, Levofloxacin, Noretisteron, Streptomycin, Triclosan, Montelukast, Valaciclovir, Loperamide, Leflunomide, Sumatriptan, Risperidone, Olanzapine, Captopril, Ropinirole, Zolpidem, Olanzapine, Omeprazole, Ropinirole, Fexofenadine, Galantamin, Loratadine, Acyclovir, Midazolam, Cefuroxime, Flagyl, Budesonide, Aprepitant, Rivastigmine, Furadantin, Pindolol, Mometasone, Valaciclovir, Pindolol, Xyloproct (mixture), Lamivudine, Atazanavir, Metronidazole, Terazosin, Amiodarone, Risperidone, Qlaira (mixture), Budesonide, Cefuroxime, Glimepirid, Symbicort (mixture), Foradil (mixture), Ribavirin, Ceftriaxone, Imatinib, Riamet (mixture), Leflunomide, Fentanyl, Kivexa (mixture), Itraconazole, Ciclosporin, Naratriptan, Oseltamivir, Salmeterol, Nevirapine, Pramipexol, Moxonidine, Lamivudine, Abacavir, Darunavir, Yasmin (mixture), Cefuroxime, Terbutaline, Paliperidon, Atacand Plus (mixture), Ertapenem, Bambuterol, Telzir, Granisetron, Lidocaine, Glimepirid, Granisetron, Paliperidon, Moxonidine, Rivastigmine, Entecavir, Tipranavir, Xylocain (mixture), Zoledronic acid, Formoterol, Prilocaine, Glibenclamide, Sorafenib,	Chlortetracycline, Enrofloxacin, Fentanyl

Nelarabine, Livocabastine, Temozolomide

The RQ values obtained from four diverse sources using MEC of pharmaceuticals in WWTPs effluent receiving sewage from municipal, hospital, manufacture and livestock are synthesised and contrasted in Figure 2.3. It can be seen that pharmaceuticals measured in livestock and manufacturing, are the two main sources exhibiting high RQ values. For lincomycin and penicillin G RQ values are even above 10. In some cases hospital effluent exhibits high RQ values (e.g. erythromycin 4.69), no evident difference for hospital and municipal effluent were observed.

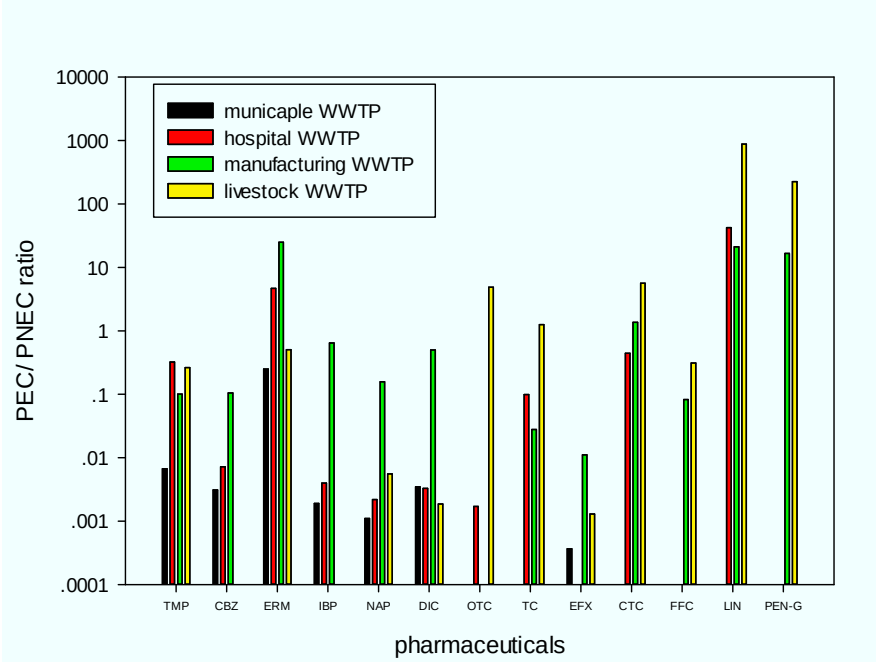


Figure 2.3 risk assessments for pharmaceuticals with available measured concentrations in WWTP effluent receiving wastewater from municipal, hospital, manufacturing and livestock. Trimethoprim (TMP), carbamazepine (CBZ), erythromycin (ERM), ibuprofen (IBP), naproxen (NAP), diclofenac (DIC), tetracycline (TC), enrofloxacin (EFX), chlortetracycline (CTC), florfenicol (FFC), lincomycin (LIN), Penicillin G (PEN-G).

In this section exposure assessment (PEC) for human pharmaceutical is considered using a total residue approach. This is a conservative estimation without considering the removal of

pharmaceuticals from the system by the individual process of patient metabolism and degradation in wastewater treatment plants. Metcalfe et al. (2008) compared the MEC and PEC by using ibuprofen as an example. They found that PEC values calculated by this approach are always very conservative relative to the MEC data within a factor of less than 100 from the 90^{th} percentile (Metcalfe et al., 2008). However, a reasonable agreement between MEC and PEC data calculated by using the EMEA guidelines (2008) for veterinary pharmaceuticals was found for the four environmental compartments (soil, dung, surface and sediment; Metcalfe et al., 2008). A wide range of API residues were reported in surface water worldwide, especially data available for different classes of antibiotics (e.g. macrolide and sulfonamide with maximum ug L^{-1} levels in the USA; Monteiro and Boxall, 2010). However, to enable a better risk assessment, more data covering wider spatial and temporal scales are required. Risk assessment methods from different geographical regions, climates, demographics, and cultural background should be further developed (Boxall et al., 2012).

2.7 Comparison of the risks of pharmaceuticals to that of herbicides

From the previous section it appears that the occurrence of pharmaceuticals in WWTP effluent produced by municipal (general human pharmaceutical use), hospital, manufacture and livestock use could pose a risk to algal communities. However, an important question is how important is the risk posed by pharmaceuticals compared to other stressors in the natural environment (Boxall et al., 2012). One group of other chemicals that are known to have high potency to algae are the herbicides. Herbicides are the most widely used agricultural chemicals. Following their application, herbicide residues can enter the aquatic environment

and they have been detected worldwide (Boutin et al., 2014). In the section below, we therefore explore the relative risks posed by pharmaceuticals compared to herbicides.

Data for herbicides and trace organics in surface water were obtained from a wide range of literature sources (highest reported data were cited if more than one data was available). EC_{50} data for each of the herbicide for algal species were obtained from the Pesticides Properties Database (PPDB, 2014). Nineteen herbicides which are currently authorised with available monitoring data and toxicity data were screened and targeted. The highest reported monitoring data for pharmaceuticals in surface water were collated and these with available toxicity data to algal species were targeted. Seventeen pharmaceuticals were therefore selected.

A simple assessment of risk was then performed by calculating measured environmental concentration (MEC): algal EC_{50} ratios for each herbicide and pharmaceutical (Table A1.5; Appendix 1). Herbicides and pharmaceuticals ranked top 10 by MEC: EC_{50} ratio in each group were contrasted and plotted in Figure 2.4. While only 19 herbicides and 17 pharmaceuticals were compared, a similar distribution of MEC: EC_{50} for herbicides and pharmaceuticals was observed (herbicides range from 1.5E-5 to 0.14, pharmaceuticals range from 1.6E-5 to 1.2; Figure 2.4), and in some cases pharmaceuticals even exhibit higher ratio (e.g. top two items in each group, clarithromycin 1.2 and diquat 0.14). This evidence therefore suggests that the risk posed by pharmaceuticals at environmentally relevant concentrations to algae is as high as that of herbicides. In the following section the current knowledge gaps are discussed and recommendations for further research are presented.

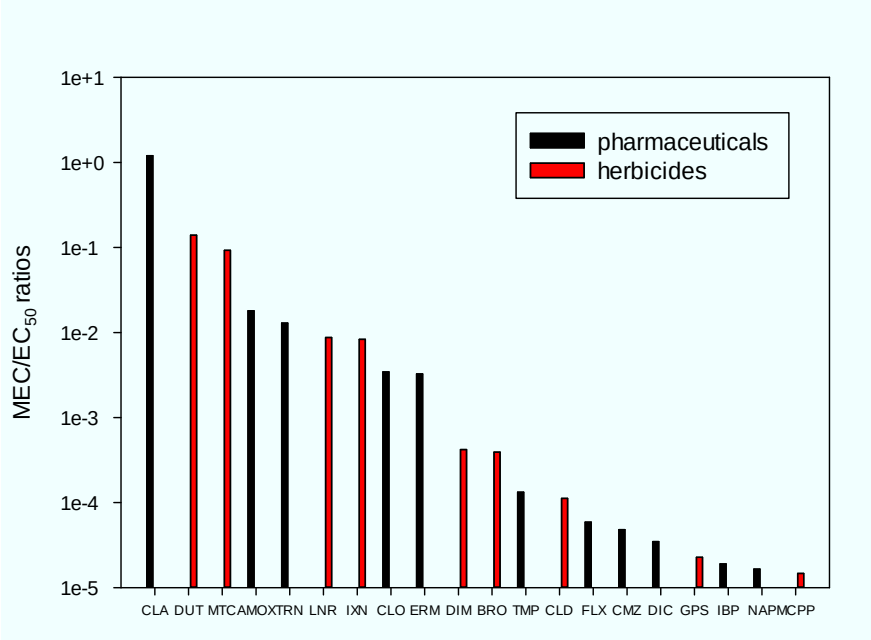


Figure 2.4 Risk comparisons between selected pharmaceuticals and herbicides. Clarithromycin (CLA), diquat (DUT), metazachlor (MTC), amoxicillin (AMOX), triclosan (TRN), linuron (LNR), ioxynil (IXN), clozapine (CLO), erythromycin (ERM), dicamba (DIM), bromoxynil (BRO), trimethoprim (TMP), chloridazon (CLD), fluoxetine (FLX), carbamazepine (CMZ), diclofenac (DIC), glyphosate (GPS), ibuprofen (IBP), naproxen (NAP), mecoprop-P (MCPP),

2.8. Recommendations for future work

While a range of toxicity data of pharmaceuticals (around 350) to algae have been published, information is still only available for a small proportion of 1500 pharmaceutical active ingredients that are currently on market and for a few species. The relationship between effects that will occur in the real environment is also unclear. It is therefore very difficult to get a real understanding of how pharmaceuticals are impacting primary production. In the future, we therefore recommend that research focuses on the following, interrelated areas:

Identification of APIs of most concern - The large number and variety of pharmaceuticals

available mean that it is unlikely that we will be able to monitor and test all the substances and all algal groups, so it is sensible to target effects of compounds that are likely to have the greatest potential to cause adverse impact on environment. One approach to identify these substances is to use prioritisation schemes that bring together information on likely exposure alongside mode of action and property information and algal biochemistry (Roos et al., 2012) to identify substances of greatest concern. Targeted monitoring and testing of these compounds would then be performed.

Better understanding of emission pathways and amounts - A key data requirement for determining the likely impacts is information on the amount of API used in different regions. In some countries (e.g. UK), good data are readily available on amounts of pharmaceuticals prescribed. However, for some regions these data are not available. Pharmaceuticals can also purchase 'over-the-counter' at retail outlets and information on amounts distributed via this route are typically not available. A better understanding of API use and emission pathways for different regions of the world is therefore needed.

Development of predictive models for effects - Instead of employing a testing approach, quantitative structure-activity relationship (QSAR) modelling and read across methods may be a valuable tools for screening APIs in terms of ecotoxicity (Sanderson and Thomsen, 2009, Cassani et al., 2013). While a handful of research has attempted to use QSAR modelling to estimate the environmental effect of chemicals, mainly on fish and daphnia (Yuan et al., 2007, Kar and Roy, 2010), an accurate and well-designed QSAR model for predicting the ecotoxicity of APIs to algal species is still required (Sanderson et al., 2004).

Better understanding of sensitivity of different algal species to APIs - Currently most toxicity

tests are performed according to the OECD 201 guideline using two freshwater algal species (*P. subcapitata and D. subspicatus*) as representatives for the ecotoxicity test, whereas in different cases other non-standard species might be selected (e.g. marine algal species should be tested to investigate the potential hazards of APIs to marine and estuarine environment) (DeLorenzo and Fleming, 2008). Some endpoints such as physiological responses (e.g. effects on photosynthesis) rather than biomass and growth rate may be adopted to provide more information on damage processes.

Understanding why different species respond the way they do - Evolutionary conservation of pharmaceuticals targets across species and life stages might explain the sensitivity among species. Pharmaceuticals are designed to deliver the desired therapeutic effect in human and animals, whereas there is evidence that the same targets and/or pathways may also be present in algal species in the natural environment. Exposure to these pharmaceuticals might elicit effects in those species (Boxall et al., 2012). Our understanding of target conservation in algae is however extremely limited. Efforts should therefore be made to develop gene sequences for key algal species and to explore the presence/absence of drug receptors these species (e.g. using approaches similar to that of Gunnarsson et al., 2008, JGI, 2014). By combining these analyses with targeted ecotoxicological testing it may be possible to develop approaches for identifying the vulnerability of different algal species to API exposure. These approaches would be invaluable for more intelligent environmental risk assessments.

Understanding effects of transformation products - In reality before being emitted to the environment, although some APIs remain unchanged in humans, a wide range of APIs will be transformed and metabolised to corresponding metabolites or transformation products (e.g.

atorvastatin is >98% metabolised to ortho-hydroxyatorvastatin and para-hydroxyatorvastatin) (Drugbank, 2013). To obtain more realistic exposure concentrations, the predicted PEC should be refined by considering the unchanged percentage of pharmaceuticals in human metabolism process (Metcalfe et al., 2008). Also for those compounds with a high metabolised percentage, the potential risk assessment of corresponding metabolites or transformation products is required. A more detailed and complete risk assessment collating current available metabolism percentage of APIs is therefore needed.

Effects of API mixtures - Drug residues detected in the aquatic environment usually occur as mixtures and not as single compounds. However, empirical knowledge of the ecotoxicology of pharmaceutical mixtures is still limited (Backhaus et al., 2011). This risk assessment has considered single pharmaceuticals. However many compounds will have the same mode of action and some compounds are known to interact toxicologically in patients (i.e. they are contraindicated; (Juurlink et al., 2003). The same mechanisms may occur in algae. A logical extension to this assessment exercise would be to consider the potential interactions of high priority compounds which have the same mode of action or those which are contraindicated. One potential method might be by fitting models. Two concepts have been well developed to explain the combination effects of APIs: concentration addition and independent action. Concentration addition is suitable for the prediction of the toxicity of mixtures of similarly acting chemicals; Independent action mode fits the compounds of a given mixture acting on different physiological systems within the exposed organisms (Backhaus et al., 2000, Backhaus et al., 2011). Application of these two concepts to the toxicity of pharmaceutical mixtures may help to identify the interactions between the chosen APIs (synergistic, antagonistic or no interaction).

Effects of APIs on communities - In natural aquatic environments algal communities occur more frequently than single species (Porsbring et al., 2009), and the sensitivity of communities to a range of APIs may vary due to competition between the composition of algal species. Instead of using single algal species for ecotoxicity test, evaluation the effects of APIs on community level and investigation in structure change might be more realistic methods (Backhaus et al., 2011). Currently standard algal tests use cell number as a surrogate to identify algal biomass. If multi-species are tested, it is necessary to recognise different cells using a microscope with a counting chamber instead of other measurements derived from instruments such as spectrometer and fluorimeter.

2.9. Conclusions

This review has summarised the ecotoxicological effects of APIs on algal species, and synthesised the available toxicity data of APIs to algal species. A risk assessment approach has been used together with information on consumption and physico-chemical properties to estimate the effects to algal species in the environment. The main conclusions of our review are as follows:

1. Over the past decade, studies investigating the ecotoxicology of APIs to aquatic organisms have increased, especially the large amount of data on the direct effects of APIs to algal species in the environment. This dataset provides strong evidence that a range of algal species are very vulnerable.
2. Algal species are an essential element of food webs and nutrient-cycling processes in natural environment and therefore only impact from APIs to algae might cause damage to

the whole ecosystem.

3. Pharmaceutically active substances can inhibit the algal species by indirectly affecting the co-existing system between algae and bacteria. The nutrient produced and supplied by bacteria to algae is vital for algae growth. APIs especially antibiotics might disrupt the relationship by inhibiting the bacterial activity and structure.
4. An assessment method applied to rank APIs on the basis of their environment risk, identified a series of antibiotics which pose a potential threat to algal species at predicted environmental exposure levels. Risk assessment methods adopted by pharmaceuticals for veterinary use might obtain higher predicted environmental concentrations (PEC) than human pharmaceuticals due to different parameters and factors considered in each scenario. A higher risk was therefore observed by using the veterinary pharmaceuticals scenario.
5. A similar environment risk to algal species was observed for APIs and herbicide by using the measured occurrence data. Pharmaceuticals, as an emerging contaminant with continuous high consumption worldwide, more concerns might be therefore raised on the fate and behaviour in the natural environment following their pathways (e.g. WWTPs, surface water and terrestrial environment).
6. While a number of toxicity data are available for single compound, few data on the mixture of APIs and their interactions exist. Current studies mainly focus on short-term tests, and therefore long-term effects of API residues environmentally relevant concentration levels to algal species are still uncertain (e.g. selection of antimicrobial resistant microorganisms, resistance development).

While a range of antibiotics were shown to be the highly ranked substances that could inhibit the growth of algae in Chapter 2, it is still difficult to target the compounds for future experimental investigations. The studies in Chapter 2 only considered the APIs with available toxicity data of algae. However, these APIs are a small proportion of all the APIs licenced on the market. The ranking results in this Chapter cannot guarantee that they are the APIs with the highest priority for future algal toxicological studies. Therefore, in the next Chapter, a prioritisation method is developed and applied to try to identify which APIs in use are likely to pose the greatest risk to the environment.

Chapter 3

Toxicological and ecotoxicological risk based prioritisation of pharmaceuticals in the natural environment

3.1 Introduction

While a large amount of data has been published in the past decade on different aspects of APIs in the environment, information is still only available for a very small proportion of the 1500 or so active pharmaceutical ingredients that are currently in use. It is possible, therefore, that monitoring and effects-based studies are missing substances that could be causing adverse impacts in the environment. It would be impossible to experimentally assess the hazards and risks for all the pharmaceuticals in use in a timely manner. However, prioritisation approaches can be used to focus monitoring, testing and research resources and to identity those compounds that are likely to pose the greatest risk in a particular situation. A number of prioritisation methods have already been proposed, and applied to, human and veterinary APIs (Boxall et al., 2003, Capleton et al., 2006, Roberts and Thomas, 2006, Kostich et al., 2010, Sanderson et al., 2004). Prioritisation approaches are also available for other classes of emerging contaminant such as pesticide metabolites (Sinclair et al., 2006). Many of these approaches use exposure and toxicological predictions or information on API potency in humans so they can be readily applied to large numbers of compounds. Until now, prioritisation methods for APIs have tended to focus on risks of parent compounds in surface waters to aquatic organisms and risks to humans *via* drinking water consumption and tended

to focus on single use categories (e.g. prescription or hospital use). Less emphasis has been placed on risks to other environmental compartments such as soils, sediments and ground waters, risks to top predators or on the risks of metabolites of APIs.

This Chapter therefore, describe a holistic risk-based prioritisation approach for identifying APIs of concern in aquatic and terrestrial systems. The use of the prioritisation approach is illustrated using a subset of APIs used in primary and secondary care in the United Kingdom as well as those distributed by pharmacists 'over the counter' and major metabolites of these. The approach considers aquatic and terrestrial exposure routes and acute and chronic effects on algae, invertebrates, fish, birds and mammals, including humans. Effects relating to the therapeutic mode of action are also considered. The approach is illustrated using 146 active ingredients that were either high usage in the UK or where experts indicated that they might be of environmental concern. While the approach has been applied to the UK situation, there is no reason why it cannot be applied to prioritise APIs in use in other regions of the World.

3.2 Methods

The prioritisation approach used risk scores (RS) as the primary parameter to rank the APIs in terms of their potential environmental risk (Figure 3.1 A, B). Risk score values were calculated by comparing predictions of exposure of APIs in different environmental compartments to measures of potential hazard towards different organisms from different trophic levels. The prioritisation process considered aquatic and terrestrial organisms as well as humans, acute and chronic apical ecotoxicological effects and potential effects related to the mode of action of an API (Figure 3.1 A, B). In the next sections we describe how the exposure concentrations

and hazard paramaters were derived. Specific equations are provided in the Appendix 2.

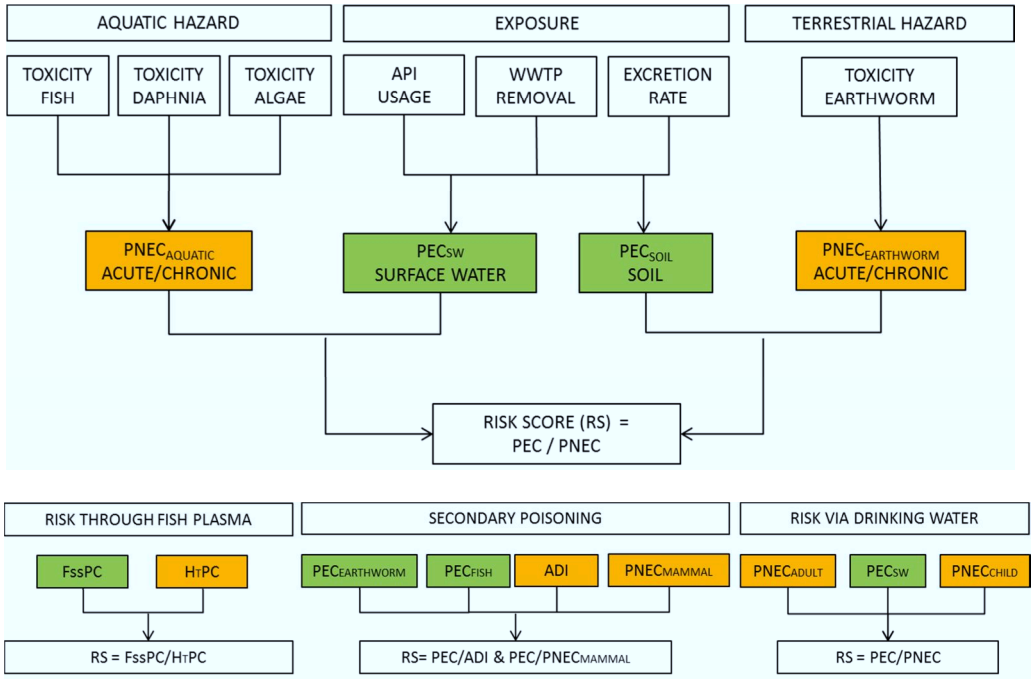


Figure 3.1: The overall approach for prioritisation of activated pharmaceutical ingredients (APIs). Risk scores on (A) standard end-point effect; (B) non-standard end-point effects. Green: estimated exposure; Orange: estimated effect. $PNEC_{AQUATIC}$: predicted no effect concentration for aquatic organisms, including fish, daphnia and algae; PEC_{SW}: predicted environmental concentration in surface water; PEC_{SOIL}: predicted environmental concentration in soil; $PNEC_{EARTHWORM}$: predicted no effect concentration in earthworm; $F_{SS}PC$: fish steady state plasma concentration; H_TPC: human therapeutic plasma concentration; $PEC_{EARTHWORM}$: predicted environmental concentration in earthworm; PEC_{FISH}: predicted environmental concentration in fish; ADI: acceptable daily intake for human; $PNEC_{MAMMAL}$: predicted no effect concentration in mammal; $PNEC_{ADULT}$: predicted no effect concentration for adult; $PNEC_{CHILD}$: predicted no effect concentration for child.

3.2.1 Identification of substances for prioritisation

In the United Kingdom (UK), the main ways that pharmaceuticals are made available to patients are through the fulfilment of primary care prescriptions by pharmacies and dispensing in secondary care (including hospitals). Some can also be purchased 'over-the-counter' at retail outlets. It would be a mammoth task to determine the usage of all compounds in the UK. We therefore, developed a substance list for prioritisation that included the top usage compounds in these different categories. To ensure that the list caught compounds of low use but very high potency, we also used expert opinion to identify potent compounds that might be of concern. Forty international experts from academia, industry and Government agencies based in North America, Europe and Asia were contacted via email. These experts were selected based on their track record in the area of ecotoxicology and environmental risks of pharmaceuticals. Many of them had participated in the Society of Environmental Toxicology and Chemistry 'Big Questions' exercise on pharmaceuticals and personal care products in the environment (Boxall et al., 2012). Their responses were used to collate a list of substances of high perceived concern.

Annual pharmaceutical usage data for the top most prescribed pharmaceuticals in primary care (by active ingredient mass) in the UK were collated from Prescription Cost Analysis (PCA) data available for England (NHS, 2012), Scotland (Scotland, 2012) and Wales (Welsh, 2011). The available PCA data obtained from Northern Ireland was not sufficient to calculate pharmaceutical usage. To reduce the time required to collate the data, the usage of all pharmaceuticals present on the PCA data for Wales was calculated (approximately 1000 active ingredients). Usage data were then obtained for England and Scotland for the top 300

compounds in use in Wales. These data were then used to generate a list of the top 100 pharmaceuticals by mass for Great Britain. Twelve substances with high usage but considered by the project team to fall outside the scope of this project were excluded from further prioritisation. These compounds were aliginic acid compound preparations, calcium carbonate, co-magaldrox (magnesium/aluminium hydroxide), ergocalciferol, ferrous fumarate, ferrous sulphate, glucose, lithium carbonate, omega-3 marine triglycerides, potassium chloride, sodium bicarbonate and sodium valproate.

Data on pharmaceutical usage in secondary care in 2012 was provided to the project team by the British Generic Manufacturers Association (BGMA). Data were provided on the usage, by mass, of the top twenty most used pharmaceuticals in secondary care. Three compounds (paracetamol, amoxicillin and codeine) that were also present on the primary usage lists had their primary and secondary care usage combined. The identity of pharmaceutical active ingredients present in pharmaceutical products available over-the-counter were obtained from information available on online retailer websites (e.g. the Boots Company website)

As some compounds will be extensively metabolised in the body, for these substances, the environment will be exposed to the metabolite and not the parent compound. Data were therefore also obtained on the extent of metabolism of the high use compounds and on the identity of the major metabolites. The recent Chemical Investigation Program (CIP) in the UK has monitored 12 pharmaceuticals in wastewater treatment plant (WWTP) effluent (Gardner, 2013). Compounds monitored in CIP but which were not in the top usage compound list or which were not identified by the experts were also added to the list for prioritisation. Overall,

146 compounds were identified for further quantitative prioritisation. An additional 23 compounds were identified that are available over-the-counter which were ranked using a more simple chemical classification approach due to the absence of quantitative usage data.

3.2.2 Environmental exposure estimation

Predicted environmental concentrations of selected pharmaceuticals in surface waters (PEC_{SW}) and terrestrial systems (PEC_{SOIL}) were estimated using standard algorithms that are described in existing regulatory guidance documents (Appendix 2, Equations 3.1 – 3.7) (TGD, 2003). The algorithms assume that pharmaceutical usage by the population is distributed evenly both temporally and spatially. The property data for APIs, collated to aid the determination of environmental exposure, included the acid dissociation constant (pKa); octanol-water partition coefficient (K_{OW}); solid-water distribution coefficient (K_d) and organic carbon partition coefficient (K_{OC}). These data were collated from a number of sources including the peer-reviewed literature, grey literature and available online databases (e.g. drugbank (Drugbank, 2013)). Where experimentally determined data were unavailable, estimation tools, such as Quantitative Structure-Property Relationships (TGD, 2003, Franco and Trapp, 2008, Drillia et al., 2005) were used to fill the data gaps. For example, K_{oc} was predicted using an estimation model developed for ionisable organic chemicals (Appendix 2, Equations 3.8 - 3.11). Default values of pH of soil recommended by the model developers (Franco and Trapp, 2008) were used in the Koc estimation (i.e. 5.8 for acids and pH 4.5 for bases).

The fish steady state plasma concentration ($F_{SS}PC$) resulting from exposure via surface water

was predicted based on estimates of the partitioning of an API between the aqueous phase and arterial blood in the fish ($P_{blood:water}$) (Fick et al., 2010). This partition coefficient was initially estimated based on the Log K_{OW} of the API, and this was subsequently combined with the PEC_{SW} to estimate the $F_{SS}PC$ (Appendix 2, Equations 3.12 – 3.15).

To estimate concentrations in fish, the Bioconcentration factor for fish (BCF_{FISH}) was estimated according to the approach of Fu *et al*. (Fu et al., 2009) assuming a pH of surface water of 7.0. The predicted environmental concentration in fish as food (PEC_{FISH}) was then calculated from the BCF and the predicted surface water concentration (Appendix 2, Equations 3.16 – 3.20).

To estimate the concentration of an API in earthworms ($PEC_{EARTHWORM}$), the concentration in the earthworms on a wet weight basis ($C_{EARTHWORM}$) was calculated using an estimate of the concentration in porewater ($C_{porewater}$) and the BCF for earthworms calculated according to the approach in the Technical guideline Document (TGD; Appendix 2, Equations 3.21 – 3.23) (TGD, 2003).

3.2.3 Hazard characterisation

Predicted no effect concentrations (PNEC) of pharmaceuticals were derived based on either experimental or estimated ecotoxicity data, using appropriate safety factors from the Technical Guideline Document (TGD) (TGD, 2003) (Appendix 2, Equations 3.24). Where multiple ecotoxicological values were available, the most sensitive end-point was used for the generation of the PNEC.

Chronic and acute aquatic and terrestrial ecotoxicity data for standard test taxa (e.g. earthworm, green algae, daphnia and fish), together with non-standard taxa and end-points,

were collated for the 146 pharmaceuticals (and relevant metabolites) under consideration (e.g. from the Fass (Fass.se, 2011) and ECOTOX (EPA, 2015) databases). A number of the compounds under consideration had no available experimentally derived ecotoxicological aquatic data. Therefore, for these compounds estimation techniques were used to fill the data gaps. A read-across approach using the OECD QSAR Toolbox was used for pharmaceuticals, and the estimation approach of Escher et al. (Sinclair and Boxall, 2009) was used for metabolites. The database present in the OECD QSAR Toolbox was used to identify experimental data for molecules deemed 'similar' to each of the individual pharmaceutical with no data. Then within the software a relationship was built to allow an estimation of the ecotoxicological endpoint for the query molecule. The approach adopted for the identification of similar compounds was to combine the protein-binding profile with endpoint specific ones, as suggested by the Toolbox instruction manual (OECD, 2013). The main procedures in the software were as follows: protein binding profile was selected as a group method to define the category. Subcategories where then established based on the classification system used by ECOSAR (US EPA). The results were then followed by a refinement for structural similarity (70 - 90% similar). The identified chemicals were then used to read across and estimate ecotoxicity data for the query pharmaceutical. Metabolite aquatic ecotoxicty data gaps were filled using the estimation approach for pharmaceutical metabolites proposed by Escher et al. (Sinclair and Boxall, 2009) which uses the principle of the toxic ratio and parent ecotoxicological data to estimate the toxic range for the metabolite. For compounds with no experimentally determined earthworm ecotoxicity data, the terrestrial toxicity (14 day LC50 in mM kg^{-1} dry soil) was predicted using the Quantitative structure-activity relationship (QSAR)

available in ECOSAR (US EPA; Appendix 2, Equations 3.25).

All human plasma therapeutic concentrations (H_TPC) were obtained from published work. Limited data are available on the toxicology of APIs to birds. Therefore, acceptable daily intakes (ADI) for humans and mammalian toxicity data (rat/mouse) were collated as surrogates to determine the potential hazards of APIs for top predators (obtained from several databases e.g. MEDSAFE (MEDSAFE, 2013), Drugs (Drugs, 2014). A PNEC for mammalian data ($PNEC_{MAMMAL}$) was generated from the median lethal dose (LD_{50}) for rat/mouse, by dividing by an assessment factor of 100. The potential hazard from drinking water was quantified by calculating the predicted no effect concentration of APIs for an adult ($PNEC_{ADULT}$) and a child ($PNEC_{CHILD}$) based on ADIs for each API using the model of Schwab *et al* (Schwab et al., 2005) (Appendix 2, Equations 3.26).

3.2.4 Ranking scenarios

To prioritise substances a risk score was calculated for the different exposure pathway/toxicity endpoint combinations by dividing the relevant exposure concentration by the relevant hazard concentration (Figure 3.1 A, B). For example, to calculate the risk score for subtle effects on fish the $F_{SS}PC$ was divided by the H_TPC. Compounds were then ranked based on their risk score with substances towards the top of the ranking deemed to be of most interest for that particular pathway and endpoint.

Due to a lack of quantitative usage data, the over-the-counter (OTC) pharmaceuticals were classified based on their hazards to the aquatic environment using a classification system proposed by European Chemicals Agency (ECHA) (ECHA, 2015). Following these criterion,

substances without adequate chronic toxicity data were categorised as either chronic 1, chronic 2 and chronic 3, on the basis of the lowest acute aquatic toxicity data from 96 h half maximal lethal concentration (LC50) for fish, 48 h half maximal effective concentration (EC50) for crustacean or 72/ 96 h EC50 for algae (Table 3.1).

Table 3.1 Classification categories for chemicals without adequate available chronic aquatic toxicity data

Category	Concentration range (mg L^{-1})
Chronic 1	<=1
Chronic 2	>1 to <=10
Chronic 3	>10 to <=100

3.3 Results

3.3.1 Target APIs and collation of pharmaceutical effect data

Overall 146 compounds were identified for further quantitative prioritisation, these were distributed as follows: 88 were used in primary care; 20 were used in secondary care; 12 were identified as 'high hazard' concern, based on expert opinion; 25 major metabolites; and 4 from the previous Chemical Investigation Program (CIP1; Table 3.2). Twenty three compounds, sold as OTC medicines, were also identified in addition to the 146 compounds for quantitative prioritisation – these underwent a qualitative assessment. A summary of the available experimental toxicological data for 146 study compounds is provided in Table 3.2. Some high profile compounds had excellent multi-species/multi-endpoint datasets. However, the majority of the compounds under consideration had limited ecotoxicological data available. For the

standard aquatic endpoints, 82 compounds had at least one experimentally derived acute or chronic ecotoxicity endpoint available. In terms of data on mammalian safety, data were available on the toxicity of 65 compounds, 139 had an acceptable daily intake and 113 had a human therapeutic plasma concentration (H_TPC) (Table 3.2). Toxicological data were not available for any of the identified metabolites.

Table 3.2 Summary of the numbers of compounds selected for prioritisation from each compound identification method and availability of experimental ecotoxicological data collated for the 146 compounds under consideration

Prioritisation type	Compound identification methodology	Number of compounds	Parameter	Number of compounds
Quantitative prioritisation	Primary care usage [a]	88[a]	Acute Fish LC50	89
	Secondary care usage [a]	20[a]	Daphnia EC50	76
	High hazard concern	12	Algae EC50	74
	Metabolites	25		
	CIP1	4	Chronic Fish LC50	13
	TOTAL	146	Daphnia EC50	40
Qualitative prioritisation	Over-the-counter	23		
			Bioconcentration factor in fish	3
			Therapeutic plasma concentration	113
			Acceptable daily intake	139
			Mammalian toxicity	65

[a] – three compounds, paracetamol, codeine and amoxicillin, identified as high usage in primary and secondary care

3.3.2 Ranking list development

The top 20 compounds derived from the different prioritisations for the aquatic and terrestrial

environments are provided in Tables 3.3 and 3.4. The prioritisation based on apical acute aquatic effects at lower trophic levels indicated that amoxicillin, clarithromycin, ciprofloxacin, azithromycin and mesalazine had the highest risk scores (RS>1). For the aquatic apical chronic prioritisation process, diclofenac, atorvastatin, estradiol, mesalazine and omeprazole demonstrated the greatest risk score (RS>1). The highest ranked compounds based on apical acute effects in soil organisms were orlistat, carbamazepine and the carbamazepine metabolite, 10,11-epoxycarbamazepine (RS 1-10; Table 3.4).

When the potential impact of subtle pharmacological effects were considered by comparing the human therapeutic concentration in plasma to estimated levels in fish, the atorvastatin metabolites ortho-hydroxyatorvastatin and para-hydroxyatorvastatin were ranked highest (RS>10) with atorvastatin, estradiol and amitriptyline just below these substances(RS 1-10; Table 3.3).

In the prioritisation based on potential of secondary poisoning in the aquatic environment (i.e. fish-eating birds and mammals), diazepam was ranked the highest (RS between 0.1-1), while in terrestrial environments (i.e. earthworm-eating birds and mammals) the highest ranked API was orlistat (RS 0.1-1). All other pharmaceuticals had a RS <0.1 (Table 3.4). The risk scores of APIs prioritised according to human consumption in drinking water for all compounds were less than $1x10^{-5}$. The top ranked compounds were phenytoin, metformin and simvastatin (Table 3.3).

Table 3.3 Top 20 compounds from each prioritisation approach for exposure via water.

Risk Score	Low trophic levels		Higher trophic levels				
			Mammalian predator		Human (uptake from drinking water)		
	Acute aquatic (PEC_{SW}/ acute $PNEC_{AQUATIC}$)	Chronic aquatic (PEC_{SW}/ chronic $PNEC_{AQUATIC}$)	PEC_{FISH}: $PNEC_{MAMMAL}$	PEC_{FISH}: ADI	Adult (PEC_{SW}: $PNEC_{ADULT}$)	Child (PEC_{SW}: $PNEC_{CHILD}$)	$F_{SS}PC$: H_TPC ratio
>10	1 amoxicillin	1 diclofenac	n.d.	n.d.	n.d.	n.d.	1 ortho-hydroxy atorvastatin 2 para-hydroxy atorvastatin
1 - 10	2 clarithromycin 3 ciprofloxacin 4 azithromycin 5 metformin 6 mesalazine	2 atorvastatin 3 estradiol 4 mesalazine 5 omeprazole	n.d.	n.d.	n.d.	n.d.	3 atorvastatin 4 estradiol 5 amitriptyline
0.1 - 1	7 paracetamol 8 phenytoin 9 n-acetyl-5-aminosalicylic acid 10 omeprazole 11 iminoquinone 12 mycophenolic acid 13 norsertraline 14 sulfasalazine 15 ranitidine 16 oxytetracycline 17 homovanillic acid 18 carbocisteine 19 mebeverine 20 propanolol	6 paracetamol 7 mebeverine 8 sulfasalazine	1 diazepam	n.d.	n.d.	n.d.	6 tamoxifen 7 propranolol 8 norsertraline 9 terbinafine
<0.1	n.d.	9 codeine 10 fluoxetine 11 azithromycin 12 diltiazem 13 mefenamic acid 14 ranitidine	2 miconazole 3 paracetamol 4 propanolol 5 tramadol 6 naproxen 7 quinine 8 trazodone 9 diltiazem	1 miconazole 2 phenytoin 3 ortho-hydroxyatorvastatin 4 estradiol 5 para-hydroxyatorvast	1 phenytoin 2 metformin 3 simvastatin 4 estradiol 5 codeine 6 omeprazole sulfone 7 lisinopril	1 phenytoin 2 metformin 3 simvastatin 4 estradiol 5 codeine 6 omeprazole sulfone[d] 7 lisinopril	10 simvastatin 11 ethinylestradiol 12 amlodipine 13 diltiazem 14 fenofibrate 15 quetiapine 16 miconazole

15 clarithromycin	10 ibuprofen	atin	8 paracetamol	8 paracetamol	17 ibuprofen
16 terbinafine	11 ranitidine	6 simvastatin	9 para-hydroxy atorvastatin	9 para-hydroxy atorvastatin	18 azithromycin
17 metformin	12 cyclophosphamide	7 omeprazole sulfone	10 citalopram	10 citalopram	19 tramadol
18 etodolac	13 carbamazepine-o-quinone	8 2-oxoclopidogrel	11 ortho-hydroxy atorvastatin	11 ortho-hydroxy atorvastatin	20 donepezil
19 carbocisteine	14 iminoquinone	9 omeprazole	12 5'-o-desmethyl omeprazole	12 5'-o-desmethyl omeprazole	
20 atenolol	15 phenytoin	10 propanolol	13 naproxen	13 naproxen	
	16 2-oxoclopidogrel	11 diltiazem	14 gliclazide	14 gliclazide	
	17 lidocaine	12 norsertraline	15 3-hydroxy omeprazole	15 3-hydroxy omeprazole	
	18 2-hydroxyiminostilbene	13 tramadol	16 5-hydroxy omeprazole	16 5-hydroxy omeprazole	
	19 mycophenolic acid	14 irbesartan	17 2-oxoclopidogrel	17 2-oxoclopidogrel	
	20 carbamazepine diol	15 terbinafine	18 omeprazole	18 omeprazole	
		16 quetiapine	19 pancreatin	19 pancreatin	
		17 tamoxifen	20 diltiazem	20 diltiazem	
		18 citalopram			
		19 5'-o-desmethyl omeprazole			
		20 codeine			

n.d. no data

Table 3.4 Top 20 compounds from each prioritisation approach considered, according to the predicted concentrations in soil (PECsoil)

Risk score	Low trophic levels	Higher trophic levels	
		Mammalian predator	
	PEC_{SOIL}: $PNEC_{WORM}$	$PEC_{EARTHWORM}$: $PNEC_{MAMMAL}$	$PEC_{EARTHWORM}$: ADI
>10	n.d.	n.d.	n.d.
1 – 10	1 orlistat 2 10,11-epoxycarbamazepine 3 carbamazepine 4 venlafaxine 5 dipyridamole 6 progesterone	n.d.	n.d. 1 orlistat
0.1 – 1	7 3-hydroxyquinine 8 2-hydroxyiminostilbene 9 norsertraline 10 terbinafine 11 cyproterone 12 norerythromycin 13 3-hydroxycarbamazepine 14 2-hydroxycarbamazepine 15 metoprolol 16 atorvastatin 17 levetiracetam 18 methocarbamol 19 bisoprolol 20 amitriptyline	1 phenytoin 2 bisoprolol 3 progesterone 4 3-hydroxyquinine 5 diazepam 6 10,11-epoxycarbamazepine 7 carbamazepine 8 quinine 9 normorphine	2 atorvastatin 3 ortho-hydroxyatorvastatin 4 tamoxifen 5 estradiol 5 terbinafine 6 para-hydroxyatorvastatin 7 bisoprolol 8 phenytoin 9 norsertraline 10
<0.1		10 fluoxetine 11 isosorbide 12 amitriptyline 13 miconazole 14 ranitidine 15 dipyridamole 16 3-hydroxyomeprazole 17 5-hydroxyomeprazole 18 5'-O-desmethyl omeprazole 19 2-hydroxyiminostilbene 20 ibuprofen	10,11-epoxycarbamazepine 11 dipyridamole 12 fenofibrate 13 venlafaxine 14 miconazole 15 carbamazepine 16 isosorbide 17 progesterone 18 aripiprazole 19 3-hydroxyomeprazole 20 5-hydroxyomeprazole

n.d. no data

For over-the-counter (OTC) pharmaceuticals, amorolfine, benzalkonium chloride, cetylpyridinium chloride, dextromethorphan, dimethicone, loratadine and xylometazoline hydrochloride were assigned to category chronic 1. The category chronic 2 included cetrimide, chlorphenamine maleate, guaifenesin, hexylresorcinol and mepyramine maleate, phenylephrine and pseudoephedrine. Beclometasone dipropionate, cetirizine hydrochloride, clotrimazole, dexpanthenol, fluticasone propionate, loperamide hydrochloride and pholcodine were assigned to category chronic 3 (Table 3.5). Acrivastine and sodium cromoglicate were not classified as no toxicity data was available and the estimation approaches did not work for these substances.

Table 3.5 Classification of over the counter pharmaceuticals based on potential hazard to the aquatic environment

Pharmaceutical	Acute aquatic ecotoxicity ($mg\ L^{-1}$)			Chronic ecotoxicity ($mg\ L^{-1}$)		Classification Category
	Algae	Daphnia	Fish	Daphnia	Fish	
Acrivastine	n.a.	n.a.	n.a.	n.a.	n.a.	Not classified
Amorolfine	0.69[a]	0.68[a]	>500[b]	n.a.	n.a.	Chronic 1
Beclometasone dipropionate	n.a.	n.a.	23.7[a]	n.a.	n.a.	Chronic 3
Benzalkonium chloride	0.056[b]	0.037[b]	0.28[b]	0.04[b]	0.032[b]	Chronic 1
Cetirizine hydrochloride	102[a]	29.6[a]	n.a.	15.2[a]	n.a.	Chronic 3
Cetrimide	1.03[a]	1.38[a]	4.63[a]	n.a.	n.a.	Chronic 2
Cetylpyridinium chloride	1.26[a]	0.0032[b]	0.11[b]	0.44[a]	n.a.	Chronic 1
Chlorphenamine maleate	5.05[a]	n.a	n.a	n.a	n.a	Chronic 2
Clotrimazole	n.a.	n.a.	30[b]	n.a.	n.a.	Chronic 3
Dexpanthenol	n.a.	76.5[a]	1220[a]	n.a.	n.a.	Chronic 3
Dextromethorphan	2.6[a]	0.95[a]	5.81[a]	2.04[a]	n.a.	Chronic 1
Dimethicone	n.a.	0.36[a]	5.83[a]	0.096[a]	n.a.	Chronic 1
Fluticasone propionate	n.a.	n.a.	39.4[a]	n.a.	n.a.	Chronic 3
Guaifenesin	9.26[a]	292[a]	n.a.	6.08[a]	n.a.	Chronic 2
Hexylresorcinol	2.19[a]	11.7[a]	2.89[a]	3.6[a]	n.a.	Chronic 2
Loperamide hydrochloride	>54[c]	>56[c]	>52.3[c]	n.a	n.a	Chronic 3
Loratadine	0.7[c]	0.83[c]	0.38[c]	n.a	n.a	Chronic 1
Mepyramine maleate	8.12[a]	181[a]	20.4[a]	10.7[a]	n.a	Chronic 2
Phenylephrine	78.1[a]	40.8[a]	210[a]	8.19[a]	n.a	Chronic 2
Pholcodine	83.4[a]	401[a]	855[a]	54.2[a]	n.a	Chronic 3
Pseudoephedrine	15.7[a]	95.7[a]	331[a]	7.23[a]	n.a	Chronic 2.
Sodium cromoglicate	n.a	n.a	n.a	n.a	n.a	Not classified
Xylometazoline hydrochloride	2.17[a]	n.a	0.66[a]	0.49[a]	n.a	Chronic 1

[a] estimated by QSAR toolbox; [b] EPA ecotox; [c] FASS.

3.4 Discussion

3.4.1 Results comparisons

A final list of 16 substances including 13 parent compounds (amitriptyline, amoxicillin, atorvastatin, azithromycin, carbamazepine, ciprofloxacin, clarithromycin, diclofenac, estradiol,

mesalazine, metformin, omeprazole, orlistat) and 3 metabolites (ortho-hydroxyatovastatin, para-hydroxyatovastatin and 10,11-epoxycarbamazepine) were identified that had a risk score > 1 for one or more of the risk comparisons. A substance with RS more than 1 indicates that the estimated exposure is higher than the predicted no effect concentration, so more attention should be paid as the hazards might occur in the different environment compartments.

The ranking results for parent compounds agree with some of the previous prioritisation studies. Amitriptyline, atorvastatin, carbamazepine, diclofenac, estradiol, mesalazine and orlistat were identified as priority substances in use in the Swedish market by Roos et al. (Roos et al., 2012), with the ranking at 12th, 22nd, 16th, 5th, 4th, 10th and 11th, respectively. The risk score of diclofenac (Ashton et al., 2004) was also reported with a low RS value of 0.01 in a UK stream case study. Amoxicillin has been ranked the top in several veterinary medicine prioritisation studies, where it was classified as a substance with high hazard to aquatic environments in the UK (Boxall et al., 2003, Capleton et al., 2006), Korea (Kim et al., 2008), US (Dong et al., 2013) and China (Wang et al., 2014). Azithromycin and metformin were identified in a US surface water exercise, being ranked 12th and 5th, respectively (Dong et al., 2013). Clarithromycin has been identified in a prioritisation study in Germany and ranked 34th (Webb et al., 2003). Ciprofloxacin was classified as a substance with a high ranking (8th) in the aquatic environment in US (Dong et al., 2013), besides, it was assigned to categories with a high and medium toxicity in China (Wang et al., 2014) and Korea (Kim et al., 2008), respectively. Omeprazole was considered in the prioritisation studies in the US and Sweden, ranking 18th and 22nd, respectively (Roos et al., 2012, Dong et al., 2013).

Previously published work considering the prioritisation of pharmaceuticals has only focused

on parent compounds (Roberts and Thomas, 2006, Roos et al., 2012), whereas in reality following consumption by patients, compounds may be metabolised and excreted as metabolites, partly or completely (Boxall et al., 2003). This project is the first study that considered the impact that metabolism may have on the ranking of APIs. The ranking results demonstrated that it is important to consider these compounds, particularly the metabolites of atorvastatin (ortho-hydroxyatorvastatin and para-hydroxyatorvastatin) which were highly ranked using a number of the prioritisation indices. The classification of 'over-the-counter' APIs is a novel method applied in a prioritisation exercise, and therefore, no published works are available with which to compare our findings.

3.4.2 Potential risk of highly ranked substances in the environment

A number of the compounds we identified as high priority are receiving increasing regulatory scrutiny. For example, as part of Directive 2013/39/EU) (EC, 2013), which relates to priority substances in water, three APIs: diclofenac and two hormones 17-beta-estradiol (E2) and 17-alpha-ethinylestradiol (EE2) have been added to EU's pollutant watch list, two of these (diclofenac and E2) appear in our top 16 list. While EE2 did not fall in the top 16, it was still ranked highly using the plasma therapeutic concentration approach (number 11), even though the amounts of this compound used in the UK are small. Side effects of diclofenac on the fish kidneys (histopathological damages) have been documented (Schwaiger et al., 2004, Triebskorn et al., 2004). Diclofenac is also considered to have threatened some sensitive organisms (e.g. vultures from the *Gyps* genus) through secondary poisoning (SCHER, 2011). E2 and EE2 are the two APIs for which the toxicity have been determined at environmental

relevant concentrations. E2 is a natural estrogen with endocrine disrupting properties. Potent effects of E2 on gamete quality and maturation in two salmonid species (rainbow trout *Oncorhynchus mykiss* and grayling *Thymallus thymallus*) have been reported, even at ng L^{-1} exposure concentration levels (Lahnsteiner et al., 2006). 17-alpha-ethinylestradiol (EE2) has been ranked in the top 20 list (Table 3.3). There is widespread evidence that exposure of male fish to EE2 at ng L^{-1} levels can result in feminzation of male fish (Zha et al., 2008) and that chronic exposure of fish (i.e. fathead minnow *Pimephales promelas*) to EE2 could ultimately result in a the collapse of fathead minnow populations in surface waters (Kidd et al., 2007).

The watch list has been further developed in the European Environmental Quality Standards Directive (JRC, 2015), where four antibiotics including erythromycin, clarithromycin, azithromycin and ciprofloxacin have been added. The inclusion of antibiotics in the watch list is mainly due to their potential toxic effects to algal species. Three of these antibiotics (clarithromycin, azithromycin and ciprofloxacin) were identified as top priority in the current study. The 72/96 h acute EC50 values with growth as the endpoint for these free antibiotics are 0.002 mg L^{-1} (*Pseudokirchneriella subcapitata*) (Santos et al., 2010), 0.001 ug L^{-1} (unreported blue-green algae) (Fass.se, 2011) and 0.005 mg L^{-1} (*Microcystis aeruginosa*) (Halling-Sorensen, 2000), respectively.

The occurrence of some of the highly ranked parent APIs in aquatic the environment has been reported with concentrations at ng L^{-1} in surface waters and at up to µg L^{-1} levels in WWTP effluents (Monteiro and Boxall, 2010). Amitriptyline was reported to inhibit the growth of the macrophyte *Lemna minor* with 7 d EC50 1.69 mg L^{-1} (Agerstrand and Ruden, 2010) and cause inhibition of crustacea *Daphnia magna* with an EC50 of 5 mg L^{-1} (NCCOS, 2013). Atorvastatin

and metformin were reported to inhibit the growth of a wide range of organisms such as macrophyte (e.g. lemna) and vertebrate (e.g. fish), where the lowest 14 d NOEC 0.013 ug L^{-1} of atorvastatin with genetic endpoint was documented for Zebrafish (*Danio rerio*) (EPA, 2015) and 48 h LC50 1.35 mg L^{-1} of metformin for a crustacea *Daphnia magna* (Crane et al., 2006). While currently no experimental toxicity data were recorded for mesalazine and omeprazole, in the present study a read-cross approach was used to predict their hazards to aquatic organisms. The lowest predictive chronic toxicity data of mesalazine and omeprazole each was 0.031 mg L^{-1} and 0.009 mg L^{-1}, both of these being for crustacea *Daphnia magna*. Hazards of five classified OTC APIs to three aquatic trophic levels have been illustrated in Table 3.5. Of the three highly ranked metabolites, only the occurrence of 10,11-epoxycarbamazepine has been reported, with a mean value of 19.1 ng L^{-1} in the WWTP effluent (Monteiro and Boxall, 2010).

Except for the impacts of prioritised APIs on organism and population levels of non-target organisms in the environment, side effects of some targeted APIs (Table 3.6) on the cellular and genomic levels have also been documented. Hepatocyte cytotoxicity of the antibiotic amoxicillin has been reported in rainbow trout (*Oncorhynchus mykiss*) with a 24 h EC50 >182.7 mg L^{-1} (Laville et al., 2004). Detrimental effects of carbamazepine on the liver and kidney cytopathology of rainbow trout (*Oncorhynchus mykiss*) has been observed with LOECs >0.1 and 0.001 mg L^{-1}, respectively (Triebskorn et al., 2007). Carbamazepine and diclofenac have been reported to significantly affect the genomic template stability in Zebrafish, at concentrations of 310 ng L^{-1} and 810 ng L^{-1}, respectively (Rocco et al., 2013). Niemuth *et al*. (Niemuth et al., 2015) found that 4 wk metformin exposure at the concentration of 40 ng L^{-1}

causes potential endocrine disruption in adult male fathead minnows (*Pimephales promelas*), through inducing significant up-regulation of messenger ribonucleic acid (mRNA) encoding the protein vitellogenin.

Table 3.6 Data gaps for the highly ranked substances

Compound	Priority scheme	Comments
Amitriptyline,	Subtle pharmacological effect	Predicted $F_{SS}PC$
Amoxicillin,	Acute aquatic low trophic level	Predicted K_{OC},
Atorvastatin,	Chronic aquatic low trophic level	Predicted K_{OC}
	Subtle pharmacological effect	Predicted $F_{SS}PC$
Azithromycin,	Acute aquatic low trophic level	Predicted K_{OC}
Carbamazepine,	Terrestrial low trophic level	Predicted K_{OC}, LC_{50} earthworm
Ciprofloxacin,	Acute aquatic low trophic level	Predicted K_{OC}
Clarithromycin,	Acute aquatic low trophic level	Predicted K_{OC}
Diclofenac,	Chronic aquatic low trophic level	Predicted $K_{OC,}$
Estradiol	Subtle pharmacological effect	Predicted $F_{SS}PC$
Metformin,	Acute aquatic low trophic level	Predicted $K_{OC,}$
Mesalazine	Acute aquatic low trophic level	Predicted $K_{OC,}$ acute daphnia LC50
	Chronic aquatic low trophic level	Predicted $K_{OC,}$ chronic daphnia NOEC
Omeprazole,	Chronic aquatic low trophic level	Predicted $K_{OC,}$ chronic daphnia NOEC
Orlistat	Terrestrial low trophic level	Predicted K_{OC}, LC_{50} earthworm

In terrestrial environments, the antiepileptic carbamazepine and antiobesity orlistat were the two highest ranked substances. The occurrence of carbamazepine in soil was reported at concentrations up to 6.85×10^{-3} mg kg^{-1}, and the QSAR based 14 d LC50 toxicity to earthworm was 1060 mg kg^{-1}. While the detection of orlistat in the terrestrial environment has not been reported, a relatively high experimental BCF of 51.1 for the orlistat treated earthworm has been documented (Carter et al., 2014) and the predictive 14 d LC50 toxicity to earthworm was 28.28 mg kg^{-1}. It should be recognised that prioritisation of several substances was based on the predicted properties and/ or toxicity data (Table 3.6), especially for K_{OC} values that were absent for all compounds. For some prioritised substances selected from subtle

pharmacological effect scenario, exposures ($F_{SS}PC$) were all estimated from log K_{OW} on the basis of QSAR.

3.4.3 Limitation of methods and future improvement

Approaches for exposure estimations of APIs used in the present study rely heavily on the annual usage information for individual pharmaceutical active ingredients. However it is well recognised that as well as the primary and secondary care pharmaceutical usage, for a limited number of compounds 'over-the-counter' sales through retail outlets such as supermarkets and pharmacies may add a significant contribution to the overall usage (Cooper, 2013). Attempts were made to obtain quantitative usage data for OTC compounds during the present study but these were unsuccessful. A previous study has estimated that in Germany OTC usage can contribute up to 50% of the total usage of some pharmaceuticals. However, this can vary on a compound by compound basis, and usage through this route could not be included in the quantitative risk score based element of this project. An accurate quantification approach of OTC usage should be further established.

The exposure of APIs in the terrestrial environment was estimated by only considering a simple input pathway: APIs adsorbed to sludge in WWTP and a this sludge was then applied to the land (CHMP, 2006). Experimentally determined biodegradation data of APIs were not available. PECs and therefore, the risk scores of APIs that were susceptible to biodegradation during wastewater treatment will therefore have been significantly overestimated. Limited information on experimental physical-chemical properties such as soil-water partition coefficients (Koc) was available for some listed APIs. To fill in the data gaps, an empirical

estimation model developed by Franco and Trapp (2008) was used to estimate adsorption during wastewater treatment. This model was developed for soils and its applicability to estimating sorption in sludge is not known. The model also omits selected sorption processes, such as complexation, which may be important for some pharmaceuticals (Franco and Trapp, 2008).

In the secondary poisoning assessment of APIs in the terrestrial compartment, as very limited experimental data was available on bioconcentration factors for worms (BCF_{worm}), this parameter was predicted using the regression equation outlined in TGD (TGD, 2003). This regression can well describe uptake by worms kept in water. However, evaulation of the model against real data indicate that the estimated BCF_{worm} in the soil are usually higher than the experimental BCFs (TGD, 2003). Higher $PEC_{ORAL,\ PREDATOR(earthworm)}$ values than those that occur in reality could therefore have been obtained in the current study, and secondary poisoning effects of APIs in terrestrial environments on earthworm-eating birds may well be overestimated. Therefore, an improvement in the accuracy of BCF_{worm} estimation in soil warrants further consideration.

To target the metabolites for prioritisation, metabolic rates and metabolites of a wide range of APIs in human have been identified from the literature (e.g. Drugbank 2013). However for substances without metabolism information, we assumed that no biodegradation and biotransformation occurred in the body to implement a conservative risk score estimation (Kim et al., 2008). In this case, the exposures of these parent compounds in aquatic and terrestrial compartments may have been overestimated, and their metabolites will have been missed in our prioritisation list. For the highly ranked compounds without available metabolism data, it is

recommended that information on the properties such as the excretion rate of parent compounds and the properties and toxicities of related metabolites should be produced.

3.5 Conclusions

A holistic methodology has been developed and implemented to prioritise pharmaceuticals of concern that are released into the environment through wastewater. Pharmaceutical usage data in the UK has been used, together with information on the physical-chemical properties, patient metabolism and wastewater treatment removal to estimate concentrations in the aquatic and terrestrial environments. To rank the APIs, these concentrations have been compared to a range of hazard end-points. A series of end-points have been considered, including traditional risk assessment PEC/PNEC ratios for the aquatic and terrestrial compartments as well as non-standard endpoints such as the potential for subtle pharmacological effects and the impact on animals consuming fish and earthworms.

Sixteen substances, including parent compounds from the therapeutic classes of antibiotic, antidiabetic, anti-inflammatory, antidepressant, antiobesity, antisecretory, lipid modifying agents, antiepileptics, estrogens and three metabolites have been highly ranked. Due to significant data gaps, the rankings of some compounds were based on data generated from predictive methods. A targeted monitoring study for these compounds, therefore, needs to be performed at a few treatment works to identify whether or not these high priority substances do occur in wastewater effluents and sludge.

While, the approach has been illustrated for the UK, there is no reason why the concept cannot be applied to identify APIs of priority in other regions of the World. In doing this, the risk

ranking algorithms may need to be refined to reflect regionally relevant pathways of exposure. We believe that the broader application of the approach would be highly beneficial in focusing monitoring and testing on substances that really matter which should ultimately result in better protection of the natural environment and of human health.

In this Chapter, based on the ecotoxicological data of algae, compounds identified with high priorities were all antibiotics. This result agreed with the findings in Chapter 2. Taking into account the higher estimated exposure for APIs in veterinary usage in surface water compared to human usage, and their high ranking (Table 2.4; Chapter 2), three veterinary antibiotics tylosin, lincomycin and trimethoprim were identified for further laboratory investigation. The effects of three antibiotics on the growth and physiology of a range of algal species including chlorophyte, cyanobacteria and diatoms were then systematically evaluated (Chapter 4 & 5), and these toxicological data were used to assess the risk of a mixture including three compounds in European Community (EC, Chapter 6).

Chapter 4

Comparing the sensitivity of chlorophytes, cyanobacteria and diatoms to major use antibiotics

4.1 Introduction

Of all the Active Pharmaceutical Ingredients (API) considered in Chapter 2 & 3, algae were found to be particularly sensitive to antibiotic exposure. Available data on toxicity of antibiotics to chlorophytes (primarily *P. subcapitata* and *D. subspicatus*) show EC_{50} values generally occur at the mg L^{-1} level (Guo et al., 2015). Effects of antibiotics on cyanobacteria have also been reported and these organisms have been found to be particularly sensitive to antibiotics with EC_{50} values reported at the µg L^{-1} level (Guo et al., 2015). A limited amount of data are also available on toxicity of antibiotics to diatoms with reported EC_{50} values in the mg L^{-1} range. As a consequence of the observed high sensitivity of cyanobacteria to antibiotics, blue green algal species are recommended as one of the test species that should be used in the environmental risk assessment of antibiotics as part of the marketing authorisation process (EMEA, 2008).

In instances where data are available on the toxicity of a single antibiotic to a range of algal and cyanobacterial species, large differences can be observed in the EC_{50} values for the different species tested. These differences could be attributed to four potential reasons: 1) differences in antibiotic bioavailability, which is related to the pKa of the chemical and pH

values in the test medium during the test period (Halling-Sorensen, 2000); 2) the characteristics of binding sites in the primary targets, where highly conserved antibiotic ligand-binding pockets in some algal species may result in a higher sensitivity (McRobb et al., 2014); 3) Elimination process (enzymatic inactivation) in the various algal species that could reduce the impacts of different antibiotics by direct degradation or modification of their structure (Wright, 2005); or 4) the presence of efflux pumps, which are the transport proteins used to extrude intracellular toxic substrates, including antbiotics. Differences in efflux pumps present in the various algal species could contribute to their different responses to antibiotic exposures (Webber and Piddock, 2003).

While the differences in sensitivity of algae to antibiotics are recognised, our understanding of these differences is limited with data being available for only a handful of species and groups (Halling-Sorensen, 2000, Luetzhoft et al., 1999, Eguchi et al., 2004, DeLorenzo and Fleming, 2008). There is therefore a need for investigations examining the sensitivity of a battery of algal species, from a range of groups (e.g. chlorophytes, cyanobacteria and diatoms) to a range of antibiotics. Data from these types of studies could be invaluable in informing the development of more intelligent environmental risk assessment strategies for antibiotic compounds.

In this study, therefore we present the results of a systematic study into the sensitivity of algal/ cyanobacterial species to three major-use antibiotics, tylosin, lincomycin and trimethoprim, with contrasting mechanisms of action. These substances have been highly ranked in a recent prioritisation study of pharmaceuticals in the natural environment where they all demonstrated

risk scores greater than one, based on ecotoxicity to algae (Guo et al., 2015). Tylosin is an antibiotic administrated as a veterinary prophylactic (intestinal and respiration infections) and growth enhancer (Hagenbuch and Pinckney, 2012, De Liguoro et al., 2003). The primary mode of action is inhibiting bacterial protein synthesis by binding to the 50S ribosome. Lincomycin is a veterinary lincosamide antibiotic and its side effect on algae is thought to occur through the inhibition of the synthesis of the D1 protein in photosystem Ⅱ, which handles the algal recovery ability from light-inhibition (Hagenbuch and Pinckney, 2012). Trimethoprim is used for the treatment of urinary tract infections, uncomplicated pyelonephritis and mild acute prostatitis (Drugbank, 2013). It is a dihydrofolate reductace inhibitor, binding to susceptible bacteria and influencing folate synthesis (Table 1.1). The three antibiotics have beendetected in the surface waters of the US and elsewhere with concentrations ranging from 0.05 to 0.7 µg L^{-1} (Table 1.1).

Six algal species recommended in the OECD 201 guideline (OECD, 2011) including chlorophytes (*Pseudokirchneriella subcapitata*, *Desmodesmus subspicatus* and *Chlorella vulgaris*), cyanobacteria (*Synechococcus leopoliensis* and *Anabaena flos-aquae)* and a diatom (*Navicula pelliculosa*and *Phaeodactylum tricornutum*) were chosen for use in the ecotoxicity studies. All these seven species are ecologically relevant and their distribution have been widely reported in five continents (Asia, Europe, Africa, North America and Oceania) (AB, 2015). The hypothesis for this study was that cyanobacteria would be more sensitive than chlorophytes and diatoms, and that the two cyanobacterial species would exhibit similar sensitivities.

4.2. Materials and methods

4.2.1. Chemicals

Tylosin tartrate (referred to as tylosin, 86.4%) (CAS-no. 1405-54-5), lincomycin hydrochloride (referred to as lincomycin, ≥95%) (CAS-no. 859-18-7), trimethoprim (≥98%) (CAS-no. 738-70-5) and potassium dichromate (≥99.8%; used as reference substance) were purchased from Sigma-Aldrich. Ammonium acetate and formic acid (≥95%) as analytical reagent grade were purchased from Fisher Scientific UK and Sigma-Aldrich, respectively. Acetonitrile, methanol and water (HPLC Gradient grade) were purchased from Fisher Scientific UK.

4.2.2 Algal cultures

Algal toxicity tests were conducted using three chlorophytes: *P. subcapitata* (CCAP 278/4), *D. subspicatus* (CCAP 258/137) and *C. vulgaris* (CCAP 211/11b); two cyanobacteria: *S. leopoliensis* (CCAP 1405/1) and *A. flos-aquae* (CCAP 1403/13A); two diatoms *N. pelliculosa* (CCAP 1050/9) and *P. tricornutum* (CCAP 1052/1b) obtained from the Institute of Freshwater Ecology (Culture Collection of Algae and Protozoa, UK). *P. subcapitata*, *D. subspicatus* and *C. vulgaris* were cultured in Kuhl medium, pH 6.8 (Kuhl and Lorenzen, 1964); S. *leopoliensis* and *A. flos-aquae* were grown in Jaworski's Medium (JM), pH 7.8 (CCAP, 2014); *N. pelliculosa* and *P. tricornutum* were grown in Enriched Seawater-Artificial Water (ESAW) plus f/2 medium, pH 8.2 (Berges et al., 2004).

Cultures of algae were grown at 20 °C under gentle and continuous shaking (100 cycles per minute (cpm)) in a culture chamber, with a controlled temperature (20 ± 2 °C) and a constant

illumination (76 µmol $m^{-2}s^{-1}$). Triplicate cultures were prepared in conical flasks (250ml) containing 100 ml of medium and 1 ml algal cells. To avoid contamination, the flasks were washed in Decon, rinsed with hydrochloric acid (50mM) and then autoclaved (at 121 °C for 30 min) before use. The algal numbers for the cultivation phase were counted daily with a hemacytometer under a microscope, and growth curves (cell numbers over time) were plotted to identify the logarithmic phases (usually over 2-4 days cultivation). The algal stocks were subcultured on a weekly basis.

4.2.3 Procedures for the growth inhibition test

Growth inhibition tests were undertaken following the OECD 201 Guideline for freshwater alga and cyanobacteria, growth inhibition tests (OECD, 2011) for the study antibiotics and the reference toxicant (potassium dichromate). The inhibition experiments were conducted in two steps: range-finding and EC_{50} determination. Range-finding was used to estimate the $EC_{50,}$ and then at least six selected concentrations ((maximum 93.79, 225.73 and 344.45 µmol L^{-1} for tylosin, lincomycin and trimethoprim, respectively) of samples (triplicates each) around the predicted EC_{50} in geometric series were used for the definitive EC_{50} test. The concentration-response curve based on growth (cell density) over t days (t=1, 2, 3, 4) was then generated based on the definitive data.

Prior to use, all glassware and stoppers used in the tests were autoclaved at 121 °C for 30 min. The antibiotics in the media were prepared and filtered into a 25 ml vial, using a 0.2 µm sterilized syringe filter. The pre-cultured algal inocula, taken from logarithmic growing cultures, were diluted to 15 ml with the prepared antibiotic solutions in a 25 ml vial. The initial algal concentrations for *P. subcapitata* and *D. subspicatus* were set at 5000 cells ml^{-1}, 2× 10^4 cells

ml^{-1} for *C. vulgaris* and *A. flos-aquae,* 1×10^4 cells ml^{-1} for *N. pelliculosa* and *P. tricornutum* and 5×10^5 cells ml^{-1} for *S. leopoliensis.* The test vials were then capped with air-permeable stoppers made of cotton and muslin. All the operations were performed on a sterilized bench.

The prepared vials were put in the culture chamber under the same conditions as used for the culturing. Bioassays lasted for 96 h, and the cell numbers were measured every 24 h using UV-Vis spectrophotometry. Cell density was calculated from a calibration curve of known cell density counted by a haemocytometer against adsorption (turbidity) measured by an ultraviolet and visible (UV-Vis) spectrophotometry for each species (R^2>0.999).Measurement of turbidity (adsorption) using a spectrophotometer with an appropriate selected wavelength is a reliable method to determine cell density (ABO, 2013). Each algal culture was diluted and scanned between the 600-800 nm ranges. The wavelengths with the highest absorbance were selected for experiments. The wavelength for absorption measurement was 750 nm for *P. subcapitata*, 720 nm for *C. vulgaris*, 682 nm for *D. subspicatus*, *N. pelliculosa*, *P. tricornutum*, *A. flos-aquae* and *S. leopoliensis*.

The prepared concentration of tested samples before the test was confirmed by chemical analysis. Samples with the highest and lowest concentrations were analysed again after the test to determine the antibiotic stability. In several algal toxicity tests, the recoveries of antibiotics in the highest and lowest test concentrations were less than 80% after 4d test. In these cases, the first-order degradation reaction (Equation 4.1) was used to estimate a dissipation rate constant (k). The k was then applied in Equation 4.2 to estimate the time-weighted average concentration (TWAC) over t days (where t=1, 2, 3, 4). By comparing the TWAC with the nominal concentration, a correction factor was then obtained for use in the

concentration response analyses. Observations from the low concentration recovery tests were used for correcting the three lowest concentrations used in the ecotoxicity study while concentrations for the high concentration recovery were used for correction of the three highest concentrations.

$C_t = C_0 \text{ X } e^{-kt}$ Equation 4.1

$C_{avet} = C_0 \text{ X } (1- e^{-kt}) / kt$ Equation 4.2

Where C_0: initial concentration (µmol L^{-1}); Ct: concentration at the t day (µmol L^{-1}); C_{avet}: average concentration over t days (µmol L^{-1}); k: rate constant (day^{-1}); t: time (day) (Boesten et al., 1997).

4.2.4 Antibiotic analyses

Samples were analysed by High Performance Liquid Chromatography (HPLC) using an Agilent 1100 with C18 Supelco Discovery column (15 cm × 4.6 mm × 5 µm). Tylosin and trimethoprim were analysed using a 24 min gradient method. The mobile phase consisted of methanol (A) and a buffer (B) (50 mM ammonium acetate plus 0.01% formic acid, pH 6.5 adjusted with 2.5% ammonium solution). The gradient was as follows: 5 minute equilibration at a 10:90 ratio (A:B); 2 minutes at 50:50; 20 minutes at 90:10; and 2 minutes at 10:90. A retention time of 13 min with a flow rate of 1 ml min^{-1} and detection wavelength of 280 nm was used for tylosin and 6.4 min, 1 ml min^{-1}, 238 nm was used for trimethoprim. Lincomycin was analysed by an isocratic method using 0.1% formic acid plus acetonitrile at a ratio 75:25 with a retention time of 4 min, flow rate of 1.2 ml min^{-1} and a detection wavelength of 196 nm. A range of antibiotic standards was prepared to derive calibration curves for each of the analytical methods. A linear relationship between concentrations and peak areas was obtained for each

analyte (R^2> 0.999); the mean recovery was more than 98% for tylosin and trimethoprim and 95% for lincomycin. The limit of detection (LOD) of tylosin, trimethoprim and lincomycin in the nutrient medium were 0.44, 0.55 and 1.15 µmol L^{-1}, respectively. The limit of quantification (LOQ) value of three above antibiotics was each 1.41, 1.86 and 3.86 µmol L^{-1}.

For measuring low concentration solutions (less than 0.28 µmol L^{-1}) of tylosin and lincomycin (less than 0.68 µmol L^{-1}) for the cynobacterial tests, solid phase extract (SPE) was used to concentrate the samples prior to analysis. Oasis HLC 3cc extraction cartridges were used purchased from Waters (UK). The SPE procedures were as follows: cartridge conditioning was undertaken by adding 6 ml methanol followed by 6 ml water. The sample (100 ml) was then loaded onto the SPE. The cartridges were then rinsed with 6 ml water and eluted using 6 ml methanol. Eluates were then concentrated, by evaporation with nitrogen in a fume hood, to dryness before being taken up in 1 ml methanol. The mean SPE recovery for tylosin and lincomycin were 119% and 138%, respectively.

4.2.5 Statistical methods

The data were analysed with Sigma-plot software. The concentration response curve was obtained by fitting regression analysis of sigmoidal functions (sigmoid, logistic, weibull, gompertz, hill and chapman equations) embedded in the Sigma plot software version 12.0. The best fitting model (highest coefficient of determination (R^2)) was used for EC50, EC10 and EC5 calculation. Significant differences between inhibition percentages calculated based on the cell density in treatments and controls were determined using the Dunnett test with a p value <0.05 taken as being statistically significant. NOEC, LOEC values were derived from this

statistic analysis.

To explore whether pH in the three different algal media (Kuhl, 6.8; JM, 7.8; ESAW+f/2, 8.2) were significantly different, pH values of controls (n=3) in each algal test were compared using Tukey's test (p value <0.05).

4.3 Results and discussion

4.3.1 Chemical analyses

At the high test concentrations, decreases in antibiotic levels over the 4 d study period were observed for tylosin (*C. vulgaris* 74.4%, *A. flos-aquae* 74.8%, *S. leopoliensis* 53.14%) and trimethoprim (*P. subcapitata* 37%). Measured concentrations of unaltered antibiotics for most other antibiotic/algal combinations remained within 80 - 120% of the initial concentration (Figure 4.1). For the low test levels, decreases in concentration were observed for tylosin (*A. flos-aquae* 27.2%, *S. leopoliensis* 15.54%), lincomycin (*N. pelliculosa* 66.86%, *P. tricornutum* 64.18%) and trimethoprim (*P. subcapitata* 48.11%, *A. flos-aquae* 43.55%, *S. leopoliensis* 42.83%; Figure 4.1). The reductions in concentrations could be due to a range of processes including abiotic (photolysis, hydrolysis) or biotic (i.e. metabolism by the algae) degradation or due to sorption or uptake to/into the algal cells.

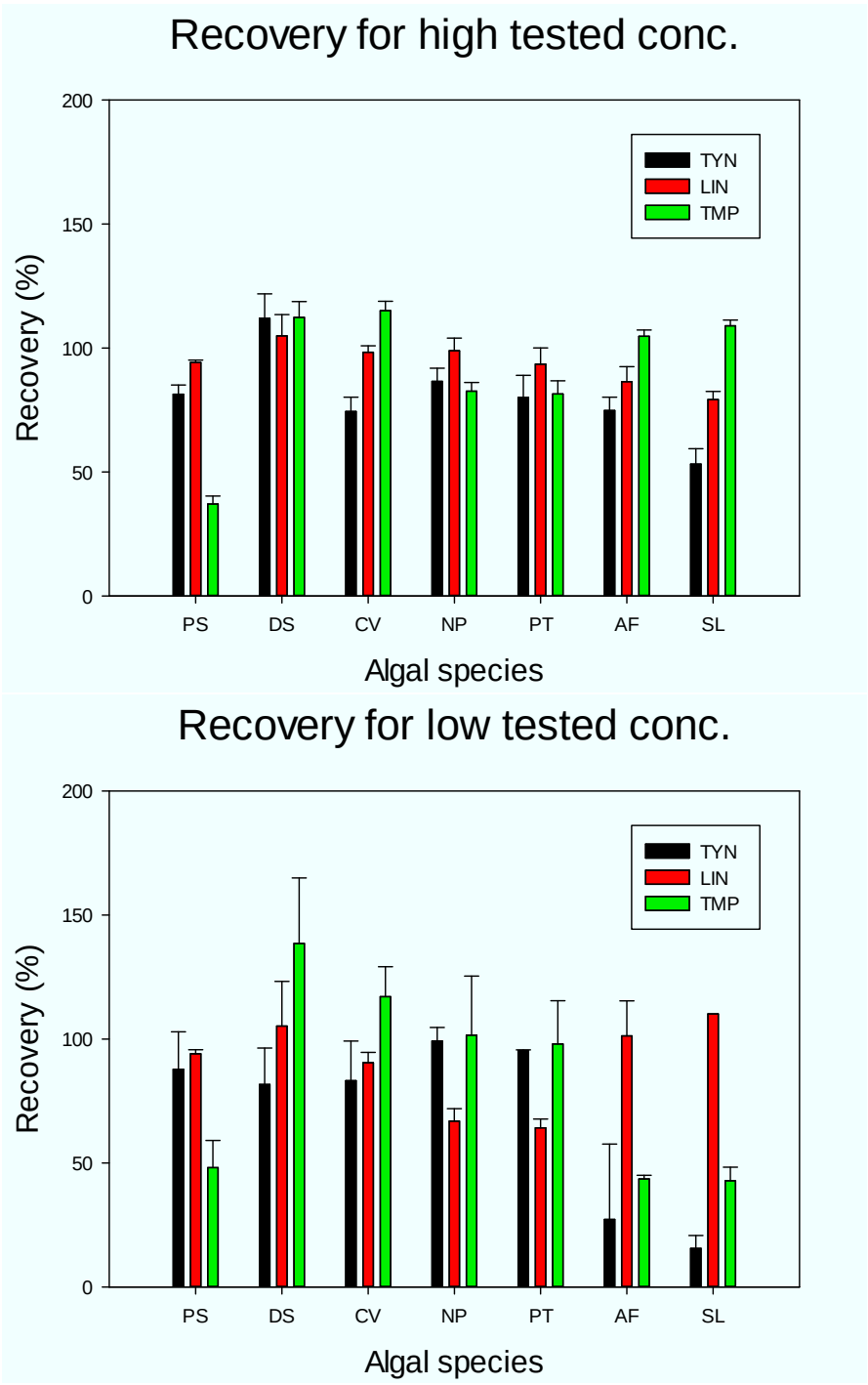


Figure 4.1: The residual percentage (%) of the three antibiotics in growth inhibition cultures of the seven algal species (samples in lowest and highest concentration for each biotest). Data represent mean ± standard deviation (n=3). PS, *P. subcapitata*; DS, *D. subspicatus*; CV, *C. vulgaris*; NP, *N. pelliculosa*; PT, *P. tricornutum*; AF, *A. flos-aquae*; SL, *S. leopoliensis*. LIN, lincomycin; TYN, tylosin; TMP, trimethoprim.

The three study compounds are known to be hydrolytically stable (Lam et al., 2004, Loftin et al., 2008, Mitchell et al., 2015). However, the photolysis of the three antibiotics has demonstrated previously. The photolysis of tylosin under simulated sunlight has been reported by Werner et al. (Werner et al., 2007), where tylosin underwent a rapid decrease in the first 4 min of the study followed by photochemical loss at a slower time scale over 120 min. Tylosin equilibrated to approximately one-half of the original concentration for over 48 h and importantly, photochemical equilibrium was independent of initial concentration and pH value. In a photolysis study of trimethoprim in two matrices (distilled water and sea water) under simulated sunlight, 50% of the original trimethoprim concentration disappeared after 780 min of exposure (Sirtori et al., 2010). However, a longer half-life was observed in the sea water solution due to the influence of salt content (Sirtori et al., 2010). Direct photolysis of lincomycin has been studied by Paola et al. (Di Paola et al., 2006), They found that parent compound with initial concentration 49.2 µmol L^{-1} dropped 40% after 5h exposure to UV light. This evidence indicated that photolysis of antibiotics may occur in algal tests during the 4d study period but this degradation is dependent on media type and the concentration of the antibiotic.

While studies on biodegradation of three antibiotics in algal species are rare, information on their biodegradation in activated sludge have been well established. All three antibiotics show a high resistance to biodegradation in activated sludge in several studies, and they were classified as non-biodegradable compounds (Prado et al., 2009, Kim et al., 2013, Halling-Sorensen et al., 2000). The losses of antibiotics in our studies were therefore unlikely due to biodegradation in algae.

While no significant difference in pH values in JM's (7.8) and ESAW+f/2 (8.2) media used for

culturing cyanobacteria and diatoms was found, the pH in Kuhl medium used for culturing chlorophytes (6.8) was significantly different from others. The pH of the exposure medium for all the treatments varied slightly over the study period (Figure 4.2). For chlorophytes *D. subspicatus* and *C. vulgaris* the rise of pH was within 1 unit and for diatoms *N. pelliculosa* and *P. tricornutum* the variation of pH values were within 0.9 units. These variances were within the scope of OECD 201 guideline. However, no evident pH increases were observed for the tests on *P. subcapitata*, *A. flos-aquae* and *S. leopoliensis* with changes < 0.2 units. The low pH increases for these species is believed to be due to their relative low growth rates compared to other species (Luetzhoft et al., 1999). The pH variations agreed with published work e.g. Halling-Sorensen *et al*. (Halling-Sorensen, 2000) investigated the effects of eight antibiotics including tylosin on the growh of cyanobacteria *Microcystis aeruginosa* with a initial pH 8.1–8.3, where almost no increase in pH was observed for *M. aeruginosa* due to a lower growth rate. Kolar *et al*. (Kolar et al., 2014) explored the influence of trimethoprim on chlorophyte *P. subcapitata* and cyanobacteria *A. flos-aquae*, where the pH values were in the range of 7.6-8.3 and 7.1-7.4, respectively.

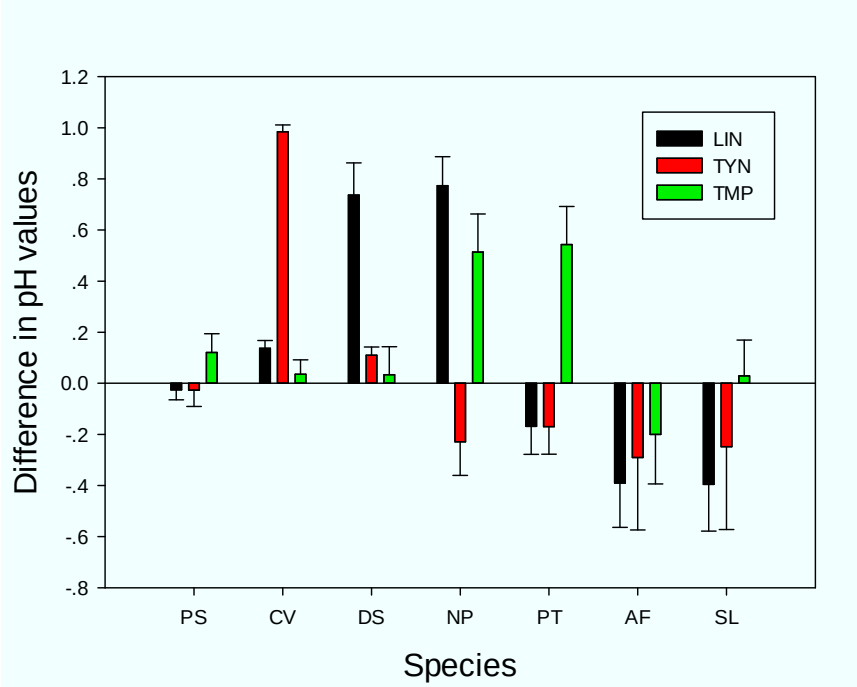


Figure 4.2 Changes in pH during 4 days of exposure to antibiotics. Data represent mean ± standard deviation (n=21). PS, *P. subcapitata*; DS, *D. subspicatus*; CV, *C. vulgaris*; NP, *N. pelliculosa*; PT, *P. tricornutum*; AF, *A. flos-aquae*; SL, *S. leopoliensis*. LIN, lincomycin; TYN, tylosin; TMP, trimethoprim.

The reference substance, potassium dichromate, has previously been tested on the three chlorophytes with the EC_{50} values in the range of 1.33-4.86 µmol L^{-1} for *D. subspicatus* (Pattard, 2009), 0.54-2 µmol L^{-1} for *C. vulgaris* (ECB, 2005) and 1.29-8.87 µmol L^{-1} for *P. subcapitata* (Pattard, 2009). In this study, EC_{50} values for *D. subspicatus* and *P. subcapitata* were 4.59 and 5.23 µmol L^{-1} respectively. For *C. vulgaris* a higher EC_{50} value 8.29 µmol L^{-1} was obtained, the discrepancy might be due to the differences in the selection of algal strain. No toxicity data of potassium dichromate on cyanobacteria and diatoms have been reported with which to compare our data. In this study EC_{50}s were found within the range from 15.94 to 33.99 µmol L^{-1} and greater than 33.99 µmol L^{-1} for cyanobacteria and diatom species, respectively.

4.3.2 Toxicity tests analysis

All three antibiotics were found to inhibit the growth of selected algal species after 4 day exposure (Table 4.1; Figure 4.3). Lincomycin inhibited the growth of all seven test species with EC_{50} values ranging from 0.095 to 225.73 µmol L^{-1}; Tylosin inhibited the growth of selected species with EC_{50} values ranging from 0.09 to 81.2 µmol L^{-1}; The EC_{50} values of seven species exposed to trimethoprim ranged from 7.36 to 344.45 µmol L^{-1} (Table 4.1). Here a wide range of algal toxicity values (as much as 4 orders of magnitude) was found for these compounds. While clear stimulation effects (hormesis) in the lower range of test concentrations were observed in some algal tests such as *N. pelliculosa*/ tylosin and *P. triconutum* for trimethoprim, most of the negative growth inhibition observed in this study were around 20% or less. Low dose stimulation effects were therefore ignored in EC_{50} calculation (OECD, 2011).

Table 4.1: Summary of the effects of tested antibiotics in 4d ecotoxicological biotests. Toxicity data derived from testing (A) lincomycin and potassium dichromate; (B) tylosin; (C) trimethoprim. All toxicity values are in µmol L^{-1} (values in brackets are the range of 95% confidence limits). Seven algal species are *P. subcapitata* (PS), *D. subspicatus* (DS), *C. vulgaris* (CV), *N. pelliculosa* (NP), *P. tricornutum* (PT), *A. flos-aquae* (AF) and *S. leopoliensis* (SL)

(A)

Spe.	Lincomycin								Potassium dichromate
	EC_{50}	EC_{10}	EC_5	NOEC	LOEC	Slope (EC50/EC5)	Model, R^2	Neutral fraction (%)	EC_{50}
PS	7.36 (4.88-11.98)	0.88	0.57	1.35	4.06	12.91	Weibull, 0.93	86.32	5.23 (3.37-n.a.)

DS	16.07 (11.2-23.72)	0.19 (n.a.-0.77)	0.13	<1.35	1.35	123.62	Weibull 0.93	86.32	4.59 (3.84-5.88)
CV	>225.73	n.a	n.a	225.73	>225.73	n.a.	n.a.	86.32	8.29 (n.a.-12.92)
NP	>225.73	35.66 (13.77-66.78)	16.07 (n.a-41.43)	121.89	180.59	14.05	Gompertz 0.64	20.08	>34
PT	>225.73	n.a.	n.a	121.9	180.59	n.a.	n.a	20.08	>34
AF	0.13 (0.11-0.15)	0.03	0.017	0.045	0.14	7.65	Weibull 0.971	38.69	15.94 (13.05-19.61)
SL	0.095 (0.076-0.13)	0.02	0.013	<0.14	0.14	7.31	Hill 0.93	38.69	>34

n.a. not available; Spe., species.

(B)

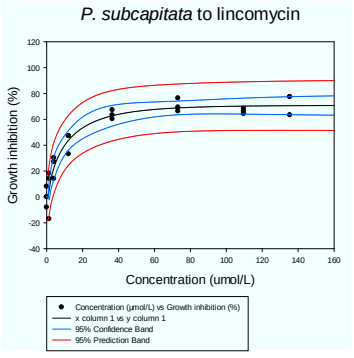
Spe.	Tylosin							
	EC_{50}	EC_{10}	EC_5	NOEC	LOEC	Slope (EC50/EC5)	model	Neutral fraction (%)
PS	4.14 (3.4-5.06)	0.91 (0.45-1.37)	0.4	0.56	1.69	10.35	Gompertz 0.963	89.49
DS	12.19 (10.57-15.42)	4.05 (1.95.-7.33)	3	<9.38	9.38	4.06	Chapman 0.955	89.49
CV	>81.2	n.a.	n.a	>81.2	>81.2	n.a	n.a.	89.49
NP	1.33 (1.14-1.76)	0.83 (0.6-1.06)	0.75	0.56	1.13	1.77	Chapman 0.916	25.31
PT	5.7 (3.67-9.6)	0.21 (n.a-0.43)	0.08	0.28	0.56	71.25	Hill 0.89	25.31
AF	0.092 (0.073-0.12)	0.02	0.012	0.037	0.074	7.67	Hill 0.96	45.98
SL	0.09 (0.068-0.13)	0.011	0.005	0.009	0.026	18	Chapman 0.95	45.98

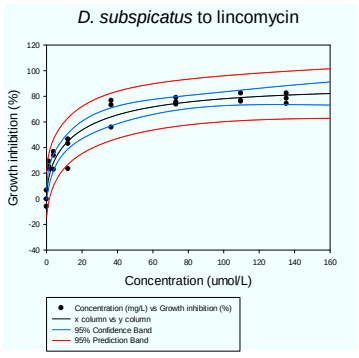
n.a. not available; Spe., species.

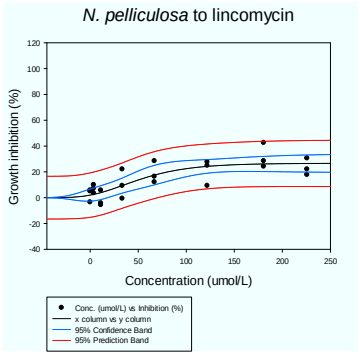
(C)

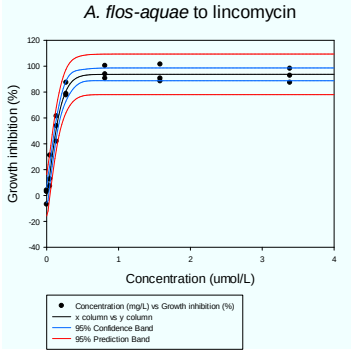
Spe.	Trimethoprim							
	EC_{50}	EC_{10}	EC_5	NOEC	LOEC	Slope (EC50/EC5)	model	Neutral fraction (%)
PS	>218.28	n.a	n.a	218.28	>218.28	n.a	n.a.	32.37
DS	>344.45	n.a	n.a	>344.45	>344.45	n.a	n.a.	32.37
CV	>344.45	n.a	n.a	>344.45	>344.45	n.a	n.a.	32.37
NP	7.36 (6.74-8.28)	4.55 (3.65-5.5)	4	4.13	6.89	1.84	Chapman 0.96	92.32
PT	74.61 (55.47-105.23)	17.19 (7.62-30.59)	11.44	20.67	62	6.52	Chapman 0.894	92.32
AF	315.78 (285.16-n.a.)	63.13	32.5	46.79	137.78	9.72	logistic 0.9	82.72
SL	>344.45	97.58	28.67	206.67	275.56	12	Sigmoid 0.74	82.72

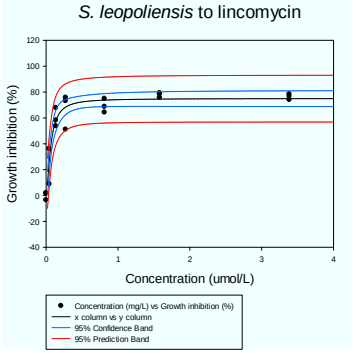
n.a. not available

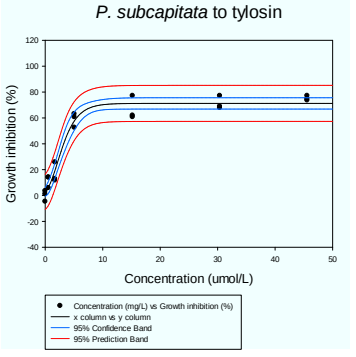












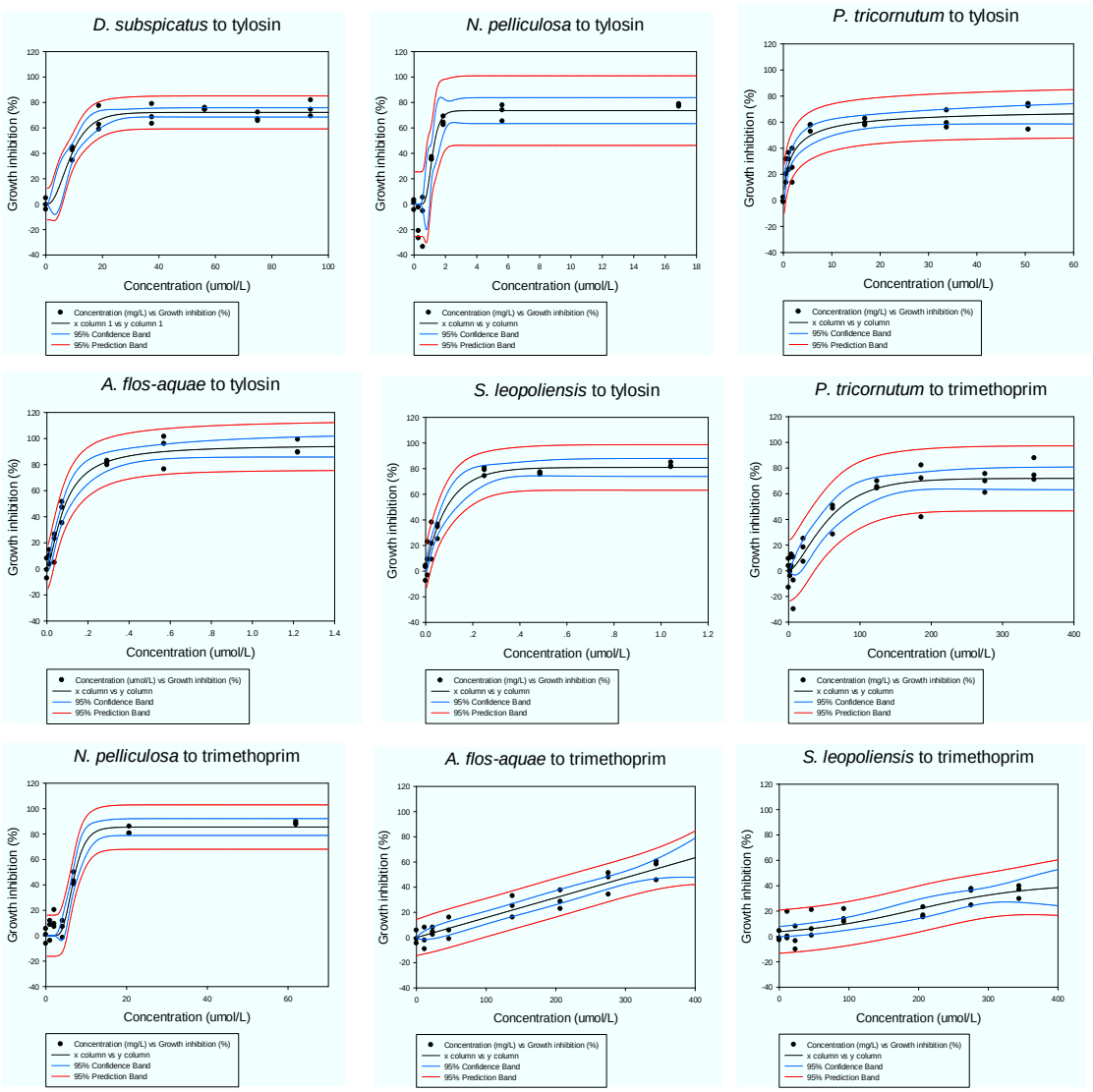


Figure 4.3: The 4d concentration-response curves for seven algal species towards single exposure

Slopes of the concentration-effect curves are of importance in algal tests. It is assumed that chemicals with the same “mode of action” have a comparable slope for a particular species (Smit et al., 2001). While no universal measure for slope of a concentration-response curve exists, it can be defined as a ratio between two EC values (e.g. the EC50/EC05 ratio), which has been reported in a range of literatures (Brosche and Backhaus, 2010). Most of the EC50/EC05 ratios in this study ranged from 1.77 to 18, which agreed with the average value (7.2) in bioassay of algae (Smit et al., 2001). However, no clear trend in slope variance was

observed for chlorophytes, cyanobacteria and diatoms (Table 4.1).The toxicological effects of the test antibiotics on selected algal species have been reported previously (Table 4.2). For tylosin, three studies have been reported on *P. subcapitata* with 72h EC_{50} ranging from 0.0083 to 1.51 µmol L^{-1} (Halling-Sorensen, 2000, van der Grinten et al., 2010). EC_{50} values for two of the studies are within an order of magnitude of the EC_{50} of 4.14 µmol L^{-1} we obtained for tylosin. The EC_{50} of 0.0083 µmol L^{-1} reported by van den Grinten et al. (van der Grinten et al., 2010) is surprisingly low in comparison to our study. Halling-Sorensen (Halling-Sorensen, 2000) reported the effects of tylosin on the cyanobacteria *Microcystis aeruginosa* with a 72h EC_{50} value of 0.037 µmol L^{-1} (Table 4.2). This value is lower than the EC_{50}s for *A. flos-aquae* and *S. leopoliensis* in the current study of 0.092and 0.09 µmol L^{-1} respectively.

For lincomycin, 72h EC_{50} previously reported EC_{50}s for *P. subspicata* are within an order of magnitude of the value we obtained (Table 4.2). Data are also available for toxicity to *S. leopoliensis* and a diatom species (Andreozzi et al., 2006). Our 96h EC_{50} 0.095 µmol L^{-1} for *S. leopoliensis* was around a factor of 4 lower than the previously reported value. For diatoms we saw no inhibition effects for either diatom species (EC_{50} >225.73 µmol L^{-1}), for *N. pelliculosa* and *P. tricornutum*) whereas Andreozzi *et al*. (Andreozzi et al., 2006), obtained an EC_{50} of 4 µmol L^{-1} although it is important to recognize this was a different species *Cyclotella meneghiniana* than we used.

For trimethoprim previously reported EC_{50}s for chlorophytes ranged from > 31 to 444.34 µmol L^{-1} (Table 4.2), whereas we obtained an EC_{50} >344.45 µmol L^{-1}. For blue green algae, our lowest 96h EC_{50} value was 315.78 µmol L^{-1} for *A. flos-aquae* which is similar to a previously reported value for this species of 871.45 µmol L^{-1}.

Toxicity data for three earlier time points are summarized in Tables A3.2-3.4 (Appendix 3). In most cases no evident algal toxicities were observed at the maximum test concentration over the first 2 days of the exposure. While the toxicity effects of antibiotics to algal species were continuously increasing from 3d to 4d exposure, the EC_{50} values were very similar. For example, over 3d and 4d exposure of *N. pelliculosa* to trimethoprim, EC_{50} values only decreased from 9.4 to 7.36 µmol L^{-1} (Figure 4.4).

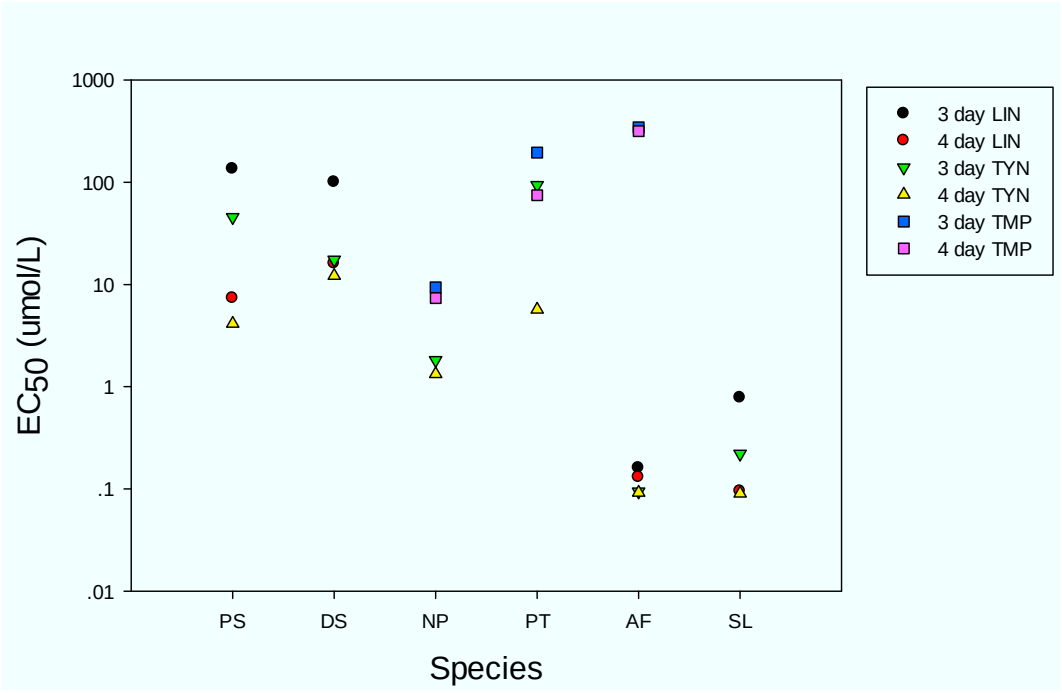


Figure 4.4: Toxicity comparison (EC_{50} µmol L^{-1}) of three antibiotics to selected algal species based on 3 day and 4 day measurement. PS, *P. subcapitata*; DS, *D. subspicatus*; NP, *N. pelliculosa*; PT, *P. tricornutum*; AF, *A. flos-aquae*; SL, *S. leopoliensis*. LIN, lincomycin; TYN, tylosin; TMP, trimethoprim.

Hypothesis-based no effect concentration (NOEC) and low effect concentration (LOEC) are common statistical approaches used to summarize ecotoxicological effects. However, the use of NOEC data has been criticized as experiments conducted using poor laboratory practice would report larger variability (Warne SJ and Van Dam, 2008). Therefore, the difference

between the control and treatments would have to be larger in order to be significant different. Instead of using NOEC, a range of studies have called for using regression-based effect concentration (ECx) value as an alternative (e,g. EC10) (Iwasaki et al., 2015). In this study therefore, in addition to determining the NOEC and LOEC values, we also have derived the EC10 value for each algal test (Table 4.1). Most of the NOEC and EC10 data are within an order of magnitude of each other.

Table 4.2: Ecotoxicity data of tested antibiotics to algal growth in literature

Species	Test duration	EC_{50} (µmol L^{-1})	Reference
Lincomycin			
P. subcapitata	4 d	3.71	(Andreozzi et al., 2006)
Cyclotella meneghiniana	4 d	4	(Andreozzi et al., 2006)
S. leopoliensis	4 d	0.49	(Andreozzi et al., 2006)
Tylosin			
P. subcapitata	3 d	0.0083	(van der Grinten et al., 2010)
P. subcapitata	3 d	1.51	(Halling-Sorensen, 2000)
P. subcapitata	3 d	0.38	(Eguchi et al., 2004)
Microcystis aeruginosa	3 d	0.037	(Halling-Sorensen, 2000)
Trimethoprim			
P. subcapitata	3 d	>31	(van der Grinten et al., 2010)

P. subcapitata	3 d	276.59	(Eguchi et al., 2004)
P. subcapitata	3 d	444.34	(Kolar et al., 2014)
A. flos-aquae	3 d	871.45	(Kolar et al., 2014)

4.3.3 Species sensitivity comparisons towards antibiotics at EC_{50} level

Sensitivities of the seven algal species exposed to the three antibiotics at EC_{50} level were assessed. For the three chlorophytes, *P. subcapitata* was slightly more sensitive to tylosin and lincomycin exposure than *D. subspicatus*, while *C. vulgaris* was not sensitive at the highest concentrations tested (Table 4.1). For the cyanobacteria, while *A. flos-aquae* was slightly more sensitive to trimethoprim exposure than *S. leopoliensis,* sensitivities of the two cyanobacteria to tylosin and lincomycin exposures based on EC_{50} values were of the same order of magnitude (Table 4.1). The two diatom species were not affected by lincomycin at the highest concentration tested. But based on data for tylosin and trimethoprim, *N. pelliculosa* was more sensitive than *P. tricornutum* (Table 4.1).

In general, cyanobacteria were more sensitive than chlorophytes to lincomycin with the EC_{50} ranging from 0.095 to 0.13 μmol L^{-1}. No effects of lincomycin were seen on diatoms (Table 4.1). The result of sensitivity across algal classes agreed with the literature. For example, Andreozzi et al. (Andreozzi et al., 2006) found the 4d EC_{50} value of lincomycin on the growth of cyanobacteria *S. leopoliensis* were around eight times lower than that for *P. subcapitata*.

Cyanobacteria were also found to be most sensitive algae tested to tylosin with EC_{50} values ranging from 0.09 to 0.092 μmol L^{-1} which was more than 5 times lower than EC_{50} values for chlorophytes and diatoms (Table 4.1). The sensitivities of chlorophytes and diatoms towards

tylosin were similar (Table 4.1). These results are consistent with the findings of Halling-Sorensen (Halling-Sorensen, 2000), who observed that the cyanobacteria *M. aeruginosa* was ten times more sensitive to tylosin than the chlorophyte *P. subcapitata*.

For trimethoprim, no effects were seen on the growth of chlorophyte and cyanobacteria species at the maximum test concentration (344.45 µmol L^{-1}) whereas the diatom species were found to be much more sensitive to trimethoprim exposure with EC_{50} values ranging from 7.36 to 74.61 µmol L^{-1}.

The differences in the sensitivities within and across algal classes to the antibiotics tested might be attributed to a number of explanations, including: differences in antibiotic uptake; differences in the binding pockets in the primary targets; differences in antibiotic elimination; and differences in active efflux pumps. These are discussed below.

In this study, the tests were performed in different media with different pH values. It has long been recognised that the pH of a system can affect the toxicity of ionisable compounds such as the study antibiotics. The initial pH values of culture media for chlorophyte, cyanobacteria and diatom species were different: 6.82 (Kuhl medium for chlorophyte), 7.8 (JM medium for cyanobacteria) and 8.2 (ESAW+f/2 medium for diatoms), respectively. For acidic antibiotics such as tylosin and lincomycin, which have pKa values ranging from 7 to 8 (Table 1.1), media with higher pH values would promote ionisation of the antibiotics which would reduce uptake into the cells (Halling-Sorensen, 2000). Species tested in lower pH media might therefore be expected to accumulate more antibiotic than higher pH media and hence toxicity, expressed based on the concentration of the antibiotic in water, would be increased. In instances where the pH of the test system changes significantly over time, this will also affect uptake. Based on

the pH of the test media, uptake of tylosin and lincomycin by chlorophyte would be expected to be greater than by cyanobacteria and diatoms based on the proportion of substance present in the neutral form (Table 4.1). As the chlorophyte were never the most sensitive group to lincomycin and tylosin, it seems that the observed differences in toxicity are not explained by differences in uptake alone. For the weak base trimethoprim, a higher pH would increase the percentage of neutral compound. The neutral percentage of trimethoprim increased from 32.37% in Kuhl medium to 82.72% in JM medium, and reached 92.32% in ESAW+f/2 medium. The higher neutral percentage of trimethoprim in ESAW+f/2 medium may therefore contribute to a higher toxicity observed for the diatom species (Table 4.1).

The toxicity of antibiotics in the non-target organisms is most frequently due to interactions with the specific drug target (Gunnarsson et al., 2008). While orthologous drug targets (protein) are evolutionarily conserved in different species, they are likely to bind to the same exogenous chemicals by binding the same or similar endogenous ligands (McRobb et al., 2014). Well-conserved targets in a given species might, therefore, increase the risk of pharmacological effects in aquatic organisms after exposure to pharmaceuticals (Gunnarsson et al., 2008). Though currently no studies have reported the conservation of pharmaceutical ligand-binding sites in the algal species, the pockets of endocrine disrupting chemicals (EDCs) have already been found to be highly conserved in aquatic toxicity testing organisms such as amphibians and fish (McRobb et al., 2014).

The sensitivity of algal species to antibiotics may also be attributed to differences in antibiotic elimination (enzymatic inactivation) by direct degradation or modification of compounds

(Wright, 2005). Some organisms (e.g. bacteria) could produce enzymes that degrade the antibiotics and further inactivate them. A wide range of antibiotics have hydrolytically susceptible chemical bonds (e.g. esters and amides), the integrity of which are important for biochemicalactivity. However, for some compounds such as beta-lactam antibiotics (e.g. penicillin), the beta-lactam ring could be cleaved by beta-lactamases. Macrolide esterase hydrolyses the macrolide antibiotic (e.g. erythromycin) by opening the ring (Wright, 2005). Other antibiotic resistant enzymes are the group transferases, which impair target binding by structural alteration. A wide range of enzymes such as chloramphenicol acetyltransferases and streptogramin acetyltransferases inactivate antibiotics by this pathway (Wright, 2005). While the above antibiotic elimination has been only reported in bacteria, the potential occurrence in the algal species may result in different sensitivities towards antibiotics.

The different sensitivity of algal species towards antibiotics may be due to differences in active efflux pumps. Efflux pumps are transport proteins used to extrude intracellular toxic substrates including antibiotics to the extracellular environment (Webber and Piddock, 2003). Several efflux pumps covering a variety of substrates were found in prokaryotic bacteria, and they are believed to lead to acquired bacterial antibiotic resistance due to the broad variety of substrates they recongnise (Webber and Piddock, 2003). In eukaryotic cells, some efflux pumps were found to modulate the accumulation of antibiotics in phagocytic cells (Van Bambeke et al., 2000). As efflux pumps are specific for one substrate or multiple classes of antibiotics, differences in efflux pumps included in each organism might explain their sensitivities towards antibiotic exposures (Webber and Piddock, 2003). Though no antibiotic

efflux studies have been reported in the algae, the potential appearance of different efflux pumps in the algal species may determine their sensitivities to antibiotic exposure.

The observations of differences in species sensitivity seen in this study are probably due to a combination of these factors. We would therefore advocate that more work be done to assess the toxicokinetics and toxicodynamics of antibiotics in different algal species, and other pharmaceuticals, in order to provide a better understanding of the key drivers of species sensitivity.

4.3.4 Implication for environment risk assessment

As can be seen from Table 4.2, previously reported toxicity data for antibiotics for algal species have been predominately available for chlorophytes and cyanobacteria. The observed sensitivity of cyanobacteria to antibiotics has resulted in these organisms being recommended for use in assessing the environmental risks of antibiotics as part of the Market Authorisation process for new antibiotics (EMEA, 2006). This conclusion is partly supported by our present toxicity results for lincomycin and tylosin. However, trimethoprim appears to be significantly more toxic to diatoms than the chlorophytes and cyanobacteria (Table 4.1) so the assumption that cyanobacteria are the most sensitive species does not seem to hold true for all antibiotics. The current EMEA regulation (EMEA, 2006) on the risk assessment of antibiotics by only considering chlorophyte and cyanobacteria as indicators might, therefore, underestimate the influence on diatoms. For the purpose of risk assessment of antibiotics on the algal species in the aquatic environment and based on the OECD 201 guideline, we recommend that the inhibition effects of antibiotics on the growh of at least three species, one from each algal class,

be investigated. It would make sense that these tests are done on the species from each class that appear to be consistently most sensitive to antibiotic exposure i.e. *P. subcapitata*, *A. flos-aquae* and *N. pelliculosa*. It is also important to recognise that we have only worked with a selection of indicator species from three classes. Further work on other antibiotic classes and other species is warranted to better inform the development of risk assessment approaches.

4.4 Conclusions

This study explored the effects of lincomycin, tylosin and trimethoprim on a battery of algal species using a standard test procedure. The results showed that algal sensitivity to antibiotics varied with EC_{50} values ranging from < 1 µmol L^{-1} level to > 344.45 µmol L^{-1} for three antibiotics. For lincomycin, cyanobacteria were found to be the most sensitive group followed by chlorophytes and then diatoms. For tylosin, cyanobacteria were found to be the most sensitive group, but diatoms were more sensitive than chlorophytes. Chlorophytes and cyanobacteria were not sensitive to trimethoprim at the top concentration tested (344.45 µmol L^{-1}) but diatoms were found to be sensitive with EC_{50} values ranging from 7.36 to 74.61 µmol L^{-1}. It is concluded that the ecotoxicological information of antibiotics on model algal species (e.g. *P. subcapitata* and *D. subspicatus*) may not generalize to other algal groups in light of variations in species sensitivity. We would, therefore, recommend that future risk assessment of antibiotics in the aquatic compartment should include at least three species from different algal classes.

There are several mechanisms that might be responsible for the inhibition of antibiotics on the growth of algae. Reduction in growth could be due to the interference of antibiotics on the algal

photosynthetic pigment synthesis such as the light-harvesting pigment chlorophyll a, b and carotenoid. As photosynthesis is the primary process for algae to produce biomass, a study investigating the effects of antibiotics on the algal photosynthesis would be valuable for understanding the toxic mechanism. In the next Chapter, the impacts of the study antibiotics on photosynthetic related endpoints are therefore explored.

Chapter 5

Effects of veterinary antibiotics on the growth and physiology of chlorophytes, cyanobacteria and a diatom species

5.1 Introduction

While it was evident that algae especially cyanobacteria were particularly sensitive to some antibiotic exposure (Guo et al., 2015), limited information is currently available concerning the underlying toxic mechanism of antibiotics to algae. For eukaryotes such as chlorophytes, available evidence indicates that antibiotics are thought to inhibit photosynthetic pathways and associated protein synthesis (Liu et al., 2011) which then results in disruption of cell growth. For example, Sandmann and Peter Boger (1981) reported that amphotericin B inhibited the photosynthetic electron transport of chlorophyte *Dunaliella parva* (Sandmann and Boger, 1981) and Liu et al. (2011) found that erythromycin, ciprofloxacin and sulfamethoxazole could significantly inhibit the physiological progress including primary photochemistry, electron transport, photophosphorylation and carbon assimilation(Sandmann and Boger, 1981, Liu et al., 2011). Effects of antibiotics on prokaryote cyanobacteria are thought to be primarily due to interference of protein synthesis (e.g. as seen for chloramphenicol) and DNA replication (e.g. as seen for quinolones) (Sandmann and Boger, 1981), although a range of studies have reported that antibiotics could also interfere with the photosynthesis process in cyanobacteria. For example, Pan et al. (2009) found that the antibiotic levofloxacin inhibited electron transport and decreased the density of the active photosynthetic reaction centre in the cyanobacteria

Synechocystis sp (Pan et al., 2009).

At present little is known about the direct effects of antibiotics on light-harvesting pigment synthesis and light utilization efficiency, though they are the prerequisites for proceeding photosynthesis metabolism in algae and cyanobacteria. The energy of sunlight is captured by the light-harvesting pigments such as chlorophyll and carotenoids in the wavelength range of 700 nm to 400 nm. While light utilization efficiency involves a variety of complex processes, it could be readily investigated by exploring the relationship between the irradiance and photosynthetic rate (Bahrs et al., 2013).

While photosynthetic endpoints such as short-term oxygen evolution rate and pigment synthesis (i.e. chlorophyll a content) have been used in a range of studies investigating the effects of external stressors on algal photosynthetic process, researchers have primarily focused on the impacts of stressors such as herbicides. For example, Xia et al. (2005) compared the endpoint sensitivity of chlorophyll a, growth and oxygen evolution rate of the cyanobacterium *Nostoc sphaeroides* after 12 d exposure to 38.79 µmol L^{-1} of the herbicide thiobencarb (Xia, 2005). Significant inhibition effects were only found on the endpoints of oxygen evolution rate and growth, where oxygen evolution rate exhibited higher sensitivity (42.1% inhibition) than growth (33.3% inhibition). Wong (2000) investigated the effects of the herbicides glyohosate and paraquat on the growth, photosynthesis and pigment synthesis in the chlorophyte *Scenedesmus quadricauda*. At a concentrations of 11.83 µmol L^{-1} of glyphosate, growth was found to be the most sensitive endpoint (64.3% inhibition), followed by photosynthetic rate (48.3% inhibition) and chlorophyll a (33.3% inhibition) (Wong, 2000). While growth was still the most sensitive endpoint (85.7% inhibition) after exposure to 0.78 µmol L^{-1}

paraquat, chlorophyll a showed higher sensitivity (66.7% inhibition) than the photosynthetic rate (38.6% inhibition). Only a handful of publications has explored the inhibition effects of antibiotics including erythromycin and chloramphenicol on the photosynthesis of chlorophytes *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardis*. Both substances were proved to signifcantly inhibit algal oxygen evolution rate and the degree of inhibition was enhanced at the higher exposures (Liu et al., 2011, Hudock et al., 1964). However, no attempts were made to compare the sensitivity of photosynthesis related endpoints and growth (i.e. cell counts). For the effect assessment of antibiotics on algal species, an understanding of the endpoint sensitivity for a battery of species from the chlorophyte, cyanobacteria and diatom groups would be valuable in understanding the potential impacts of antibiotics on ecosystems and also to provide information to help understand the mechanisms of action of antibiotics in different algal species.

The objectives of the work described in this Chapter were, therefore: 1) to compare the sensitivity of photosynthesis related endpoints (i.e. oxygen evolution rate) and growth (i.e. cell counts) following 4d exposure to antibiotics; 2) to obtain information on the underlying toxic mechanisms of the antibiotics regarding light-harvesting pigment synthesis and utilization efficiency. The work focused on the three antibiotics studied in the work described in the previous Chapter and the four species *Pseudokirchneriella subcapitata*, *Desmodesmus subspicatus*, *Anabaena flos-aquae* and *Navicula pelliculosa*, which were shown to be the sensitive organisms of the seven species studies in Chapter 4.

5.2 Method

5.2.1 Chemicals

The sources and purities of the test chemicals are described in Chapter 4 (Section 4.2.1).

5.2.2 Algae culture

Algal species *P. subcapitata* (CCAP 278/4), *D. subspicatus* (CCAP 258/137), *A. flos-aquae* (CCAP 1403/13A) and *N. pelliculosa* (CCAP 1050/9) were supplied by the Institute of Freshwater Ecology (Culture Collection of Algae and Protozoa, UK). For detailed culture methods refer to Section 4.2.2.

5.2.3 Effects on growth

The effects of the study antibiotics on algal growth were explored over 4 d using OECD Guideline 201. Initial range-finding studies were used to estimate the EC_{50} range based on the growth (cell density) inhibition tests. Triplicates of six concentrations (maximum 93.79, 225.73 and 344.45 µmol L^{-1} for tylosin, lincomycin and trimethoprim, respectively) around the estimated EC_{50} in geometric series were then selected for use in the definitive study.

All glassware and stoppers used in the tests were autoclaved at 121 °C for 30 min prior to use. Each concentration of antibiotic was prepared in the corresponding culture medium. After addition of the antibiotic solution, samples were filtered to a 25 ml vial using a 0.2 µm sterilized syringe filter. The algal solution grown in the logarithmic phase was then inoculated into the vial to obtain 15 ml solution with an initial density 5 X 10^5 cells mL^{-1}. Following the inoculation, these vials were caped with air-permeable stoppers made of cotton and muslin. All the

operations were undertaken in a sterilized chamber, and the vials were then incubated for 4 d under the same conditions as the cultures.

Cell density in each sample was measured at 24 h intervals using UV-Visible spectrophotometry. Cell density was calculated from a calibration curve of known cell density counted by a haemocytometer against adsorption measured by an ultraviolet and visible (UV-Vis) spectrophotometry (R^2>0.999) for each test species. Measurement of turbidity (adsorption) using a spectrophotometer set at a selected wavelength is a reliable method to determine cell density (ABO, 2013). Each algal culture was diluted and scanned over the 600-800 nm range. The wavelengths with the highest absorbance were selected for experiments. *P. subcapitata* was detected at a wavelength of 750 nm and *D. subspicatus*, *A. flos-aquae, N. pelliculosa* at a wavelength 682 nm.

The prepared concentration of tested samples before the test was confirmed by chemical analysis. Samples with the highest and lowest concentrations were analysed again after the test to determine the antibiotic stability. In several algal toxicity tests, the recoveries of antibiotics in the highest and lowest test concentrations were less than 80% after 4d test. In these cases, the first-order degradation reaction (Equation 5.1) was used to estimate a dissipation rate constant (k). The k was then applied in Equation 5.2 to estimate the time-weighted average concentration (TWAC) over t days (where t=1, 2, 3, 4). By comparing the TWAC with the nominal concentration, a correction factor was then obtained for use in the concentration response analyses. Observations from the low concentration recovery tests were used for correcting the three lowest concentrations used in the ecotoxicity study while concentrations for the high concentration recovery were used for correction of the three

highest concentrations.

$C_t = C_0 \text{ X } e^{-kt}$ Equation 5.1

$C_{avet} = C_0 \text{ X } (1- e^{-kt}) / kt$ Equation 5.2

Where C_0: initial concentration (µmol L^{-1}); Ct: concentration at the t day (µmol L^{-1}); C_{avet}: average concentration over t days (µmol L^{-1}); k: rate constant (day^{-1}); t: time (day) (Boesten et al., 1997).

5.2.4 Photosynthetic oxygen evolution

After 4 d exposure to the antibiotics, algae from the growth studies were taken and the oxygen evolution rate was determined using a DW2 Oxygen Electrode Chamber (Hansatech Instruments Limited, UK). The measurement was firstly performed for 10 min under dark conditions at 20 °C to give the respiration rate (R). A 15 min measurement under illumination of 76 µmol $m^{-2} s^{-1}$ actinic light intensity was then performed to give the photosynthesis rate (P_n). The gross photosynthesis rate (P_g) was the sum of these two processes.

5.2.5 Photosynthetic pigment content

After 4 d exposure in the growth studies, 5 ml of each treated sample was firstly filtered using a 25 mm fibre filter (Pall Corporation, UK). Afterwards, the filter was put into a vial with 5 ml methanol, and kept for 24h in a spark-free fridge to extract photosynthetic pigment content. Chlorophyll a and b were estimated using the Wellburn coefficient equation (Equation 5.3 & 5.4) (Wellburn, 1994) and the total carotenoid (carotene and xanthophyll) were estimated using the Lichtenthaler equation (Equation 5.5) (Henriques et al., 2007). Absorbance was measured by UV - Vis spectrophotometry at 470 nm, 653 nm and 666 nm. For each

experimental measurement, the result was corrected for cell density.

Chlorophyll a (mg L^{-1}) = 15.65 A_{666} - 7.34 A_{653} Equation 5.3

Chlorophyll b (mg L^{-1}) = 27.05 A_{653} - 11.21 A_{666} Equation 5.4

Total carotenoids (mg L^{-1}) = (1000 A_{470} - 44.76 A_{666}) / 221 Equation 5.5

5.2.6 Irradiance-Photosynthesis (I- P) relationship measurement

To investigate the potential effects of antibiotics on the algal light utilization efficiency, algae were exposed, in triplicate, to the EC_{50} of each antibiotic based on photosynthesis endpoint for 4 d after which photosynthesis of the samples was measured under 5 different light intensities: 76, 150, 300, 450 and 600 µmol m^{-2} s^{-2}. Bar charts of gross photosynthesis rate (P_g) versus light intensity were plotted to analyse the effects of antibiotics on the algal light utilisation efficiency.

5.2.7 Chemical analysis procedures

Concentrations of the study antibiotics in the test solutions were confirmed analytically using the methodologies described in Section 4.2.4.

5.2.8 Statistical methods

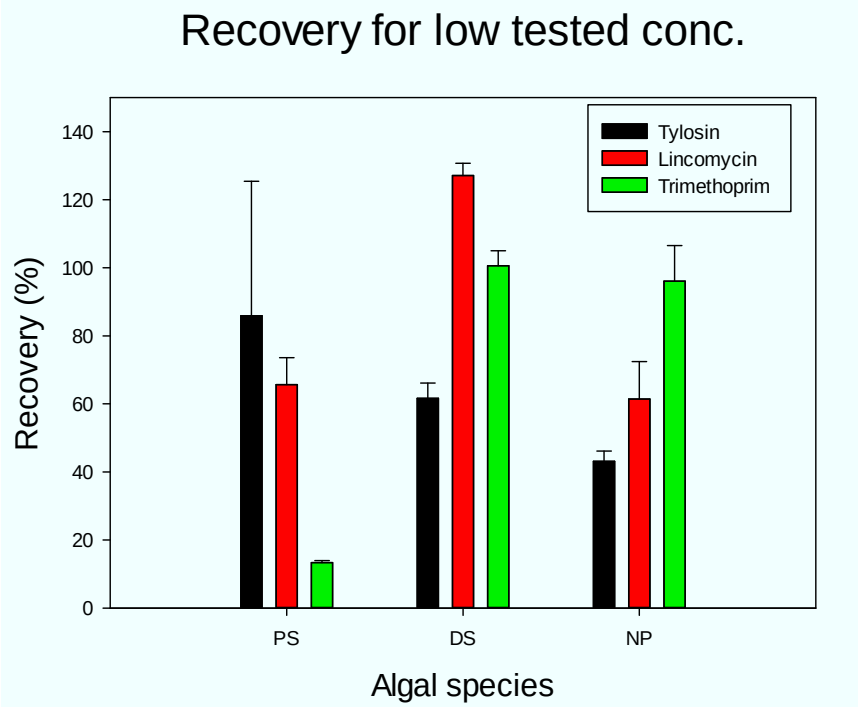
The inhibition data were analysed using Sigma-plot software. Dose-response curves were fitted using sigmoid curve regression analysis. Significant differences between treatments and controls were identified using the One way ANOVA Dunnett test with a p value <0.05. A Two way ANOVA Tukey test was used in Irradiance-photosynthesis relationship study, where all data passed the test for normality.

5.3 Results and discussion

5.3.1 Analysis of chemical stability, pH variation and reference substance

While SPE was performed to concentrate the exposure solutions for the tests on *A. flos-aquae* prior to the chemical analysis, the volume of solution in the test vial was less than 15 ml so it was not possible to conduct SPE again. While no stability data of the antibiotics for studies with *A. flos-aquae* during the 4d period are available, stability data of lincomycin and tylosin obtained in Chapter 4 were applied to extrapolate to the intermediate concentration. The mean recovery of tylosin, lincomycin and trimethoprim before the test on *A. flos-aquae* were 120.13%, 120.28% and 84.7%, respectively.

Data on the stability of the study compounds during the tests on the two chlorophytes and the diatom are presented in Figure 5.1 and Appendix 4. Stability varied depending on test concentration and species. For tylosin, concentrations at the end of the study ranged from 40.96% (*N. pelliculosa* exposed to a concentration of 7.25 µmol L^{-1}) to 129% (*P. subcapitata* exposed to 0.4 µmol L^{-1}) of the starting concentration. For lincomycin, the range of was 33% (*N. pelliculosa* exposed to a concentration of 225.73 µmol L^{-1}) to 131.1% (*D. subspicatus* exposed to 18.87 µmol L^{-1}). For trimethoprim the range was 12.75% (*P. subcapitata* exposed to 30.69 µmol L^{-1}) to 105.08% (*N. pelliculosa* exposed to 146.32 µmol L^{-1}). The disappearance of antibiotics might be due to photolysis and metabolism to algae. The potential occurrences and effects of two factors on algal tests have been thoroughly discussed in Chapter 4 and will therefore not be repeated here.



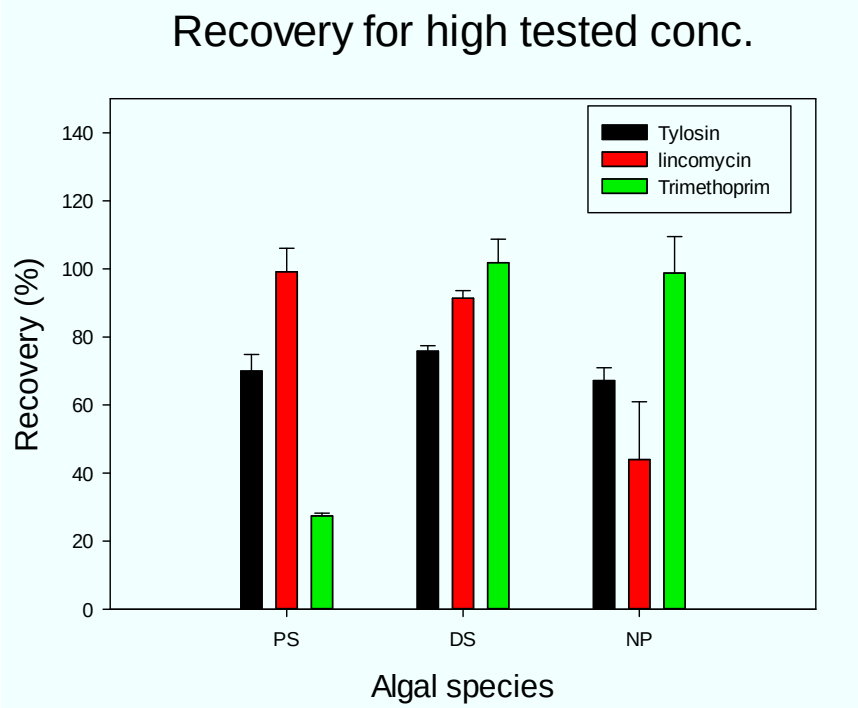


Figure 5.1: The amount (expressed as a % of the starting concentration) of the three study antibiotics remaining in the exposure media used in the growth samples (data are shown for the lowest and highest test concentration for each study). Data represent mean ± standard deviation (n=3). DS, *D. subspicatus*; PS, *P. subcapitata*; NP, *N. pelliculosa*.

For most studies, there was no significant difference between the pH of the medium at the start and the end of the study (Figure 5.2). The exceptions were tests with trimethoprim on *P. subcapitata*, *N. pelliculosa* and *A. flos-aquae*, lincomycin on *N. pelliculosa* and tylosin on *P. subcapitata* where a maximum increase of 0.8 units was observed - this value is within the variation considered acceptable by the OECD 201 guideline (less than 1.5 units). An explanation is that CO_2 mass transfer from the surrounding air could not fulfil the growth of algae due to the carbon demand of algal growth. CO_2 was then derived from biocarbonate in the medium resulting in an increase in pH (Luetzhoft et al., 1999). This result agreed with published work e.g. in previous tests of trimethoprim on the chlorophyte *P. subcapitata* and cyanobacteria *A. flos-aquae*, the pH values were within the range of 7.1-8.3 (Kolar et al., 2014). The pH values of the different algal media (6.8—8.2) would promote the ionisation of the tested antibiotics in solutions, which resulted in the neutral fractions ranging from 20.08% to 92.32% (Table 5.1). Effects of antibiotic ionisation on algal toxicity and sensitivity have been discussed in Chapter 4 and therefore will not be repeated here.

EC_{50} values for the reference toxicant, potassium dichromate on two chlorophytes *D. subspicatus* and *P. subcapitata* were 4.59 and 5.23 µmol L^{-1}, respectively. These results are consistent with previously reported data where the EC_{50} for the substance was found to range from 1.33 to 4.86 µmol L^{-1} for *D. subspicatus* and 1.29-8.89 µmol L^{-1} for *P. subcapitata* (Pattard, 2009). The EC_{50} found for diatom *N. pelliculosa* and *A. flos-aquae* were > 33.99 and 15.94µmol L^{-1}, respectively. However, no information on the toxicity of potassium dichromate to these two species is available in the literature for comparison purposes.

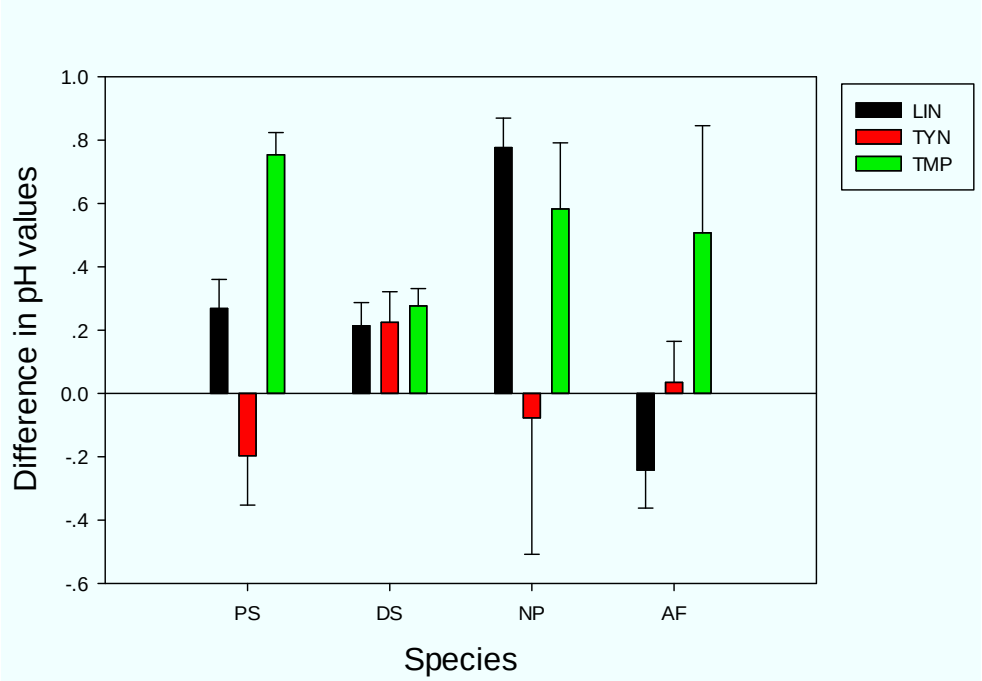


Figure 5.2: Changes in pH during 4 days of exposure to antibiotics. Data represent mean ± standard deviation (n=21). PS, *P. subcapitata*; DS, *D. subspicatus*; NP, *N. pelliculosa*; AF, *A. flos-aquae*; LIN, lincomycin; TYN, tylosin; TMP, trimethoprim.

5.3.2 Endpoint sensitivity comparison

All the exposure concentrations used for plotting concentration-response curves have been revised using modified chemical recoveries (Table 5.2). While this study characterised the inhibition effects of antibiotics on the pigment synthesis, the results of pigment content (chlorophyll a, b and carotenoid) after 4d exposure could not be fitted to dose-response curves. Therefore, it was only possible to derive dose-response curves based on effects on growth and oxygen evolution rate to derive EC_{50} values. These data are described in the next section along with a discussion of the sensitivity of the different endpoints.

5.3.2.1 Toxicity test analysis based on growth

Studies into the effects of the three study antibiotics on the growth of a selection of algal

species have been reported previously. In our study the 96 h EC_{50} for tylosin for growth inhibition of *P. subcapitata* was 4.8 µmol L^{-1} (Table 5.1), which agrees with the previous studies where 72h EC_{50} values have been reported to range from from 0.0083 to 1.51 µmol L^{-1} (Halling-Sorensen, 2000, van der Grinten et al., 2010, Eguchi et al., 2004). For *A. flos-aquae,* we obtained a 96h EC_{50} of 0.06 µmol L^{-1}, which is at the order of magnitude of a published EC_{50} of 0.037 µmol L^{-1} which was reported for another cyanobacterial species, *Microcystis aeruginosa,* after 72h exposure to tylosin (Halling-Sorensen, 2000). The 96 h EC_{50} for lincomycin for *A. flos-aquae* growth inhibition was 1.2 µmol L^{-1}, this is not dissimilar to the 96h EC_{50} value reported for the cyanobacteria *Synechococcus leopoliensis* of 0.49 µmol L^{-1} (Andreozzi et al., 2006). The 96 h EC_{50} for lincomycin to the chlorophyte *P. subcapitata* was 24.14 µmol L^{-1} (Table 5.1), which is higher than previously reported values for the same species 0.16 µmol L^{-1} [72 h EC_{50}] (Isidori et al., 2005) and 3.71 µmol L^{-1} [96 h EC_{50}] (Andreozzi et al., 2006). There are numerous explanations for variations between our data and previous studies including: differences test conditions (e.g. in initial inoculation cell number) or differences in the sensitivities of individual species within an algal class. As suggested by OECD 201 guideline (OECD, 2011), low cell numbers ranging from 5 X 10^3 to 5 X 10^4 cells mL^{-1} were usually used for pure toxicity tests (van der Grinten et al., 2010, Andreozzi et al., 2006, Isidori et al., 2005). In this study, the inoculated cell number was set at 5 X 10^5 cells mL^{-1} to allow the pigments to be extracted and analysed after the 4 day exposure. The pigment extraction could be favoured by a higher initial biomass to obtain measurable pigment content. A higher initial cell density could lead to less toxicant content bonding to the cells (both intercellular and extracellular), and further lead to less toxicant uptake and lowering of toxicity

(Franklin et al., 2002). This trend has been reported in tests with copper on the chlorophyte *P. subcapitata*, where significantly more extra- and intracellular copper was accumulated at algal initial cell density at 10^3 cells ml^{-1} compared to 10^4 and 10^5 cells ml^{-1} for the medium with the same copper concentration. The toxicity at 72h EC_{50} level in terms of growth rate significantly decreased from 97.56 to 118.02 and 267.51 µmol L^{-1} as cell density increased (Franklin et al., 2002). Despite previous studies showing lincomycin to affect the diatom *Cyclotella meneghiniana* with a reported at 96 h EC_{50} of 4 µmol L^{-1} (Andreozzi et al., 2006), in the current study, no effect was found for the diatom *N. pelliculosa* at the top test concentration of 153.91 µmol L^{-1}. It was inferred to the difference in species sensitivity. Potential effects of trimethoprim were recorded for the chlorophyte *P. subcapitata* (72h EC_{50} 276.59 – 444.34 µmol L^{-1}) (Kolar et al., 2014, Eguchi et al., 2004) and cyanobacteria *A. flos-aquae* (72h EC_{50} 871.45 µmol L^{-1}) (Kolar et al., 2014), which agreed with the results of this study (> 307 µmol L^{-1}). The 96h EC_{50} for trimethoprim for the diatom *N. pelliculosa* was 70.48 µmol L^{-1}, this compound does not appear to have been tested previously on diatoms.

Table 5.1 Summary of EC50 (μmol L^{-1}) data based on two endpoints (growth and gross photosynthesis) for three antibiotics on four algal species over 4 d exposures (Numbers in parenthese indicate 95% confidence limits).

	Tylosin				Trimethoprim				Lincomycin			
	Growth	Photosynthesis	pH range	Neutral fraction (%)	Growth	Photosynthesis	pH range	Neutral fraction (%)	Growth	Photosynthesis	pH range	Neutral fraction (%)
DS	38.27 (30.23-47.08)	17.6 (10.13-13.39)	6.65-7.76	89.49	>272.7	>272.7	5.99-6.31	32.37	>188.71	79.41 (60.27-103.3)	7.38-7.8	86.32
PS	4.8 (4.26-5.47)	2.1 (n.a.)	6.69-6.86	89.49	>307	>307	6.77-7.03	32.37	24.14 (21.84-27.6)	12 (n.a.-20.68)	5.92-6.06	86.32
AF	0.06 (n.a.-0.068)	0.33 (0.21-0.52)	6.99-8.04	45.98	>341.69	>341.69	7.21-7.85	82.72	1.2 (1.04-1.51)	4.75 (0.49-n.a.)	7.28-7.78	38.69
NP	4.4 (3.66-5.05)	7.35 (0.44-17.49)	7.75-8.36	25,31	70.48 (57.79-96.03)	136.36 (95.34-n.a.)	8.54-9.1	92.32	>153.91	>153.91	8.81-9.07	20.08

n.a. not available;

5.3.2.2 Toxicity test analysis based on photosynthesis and endpoint sensitivity comparison

For the two chlorophytes, photosynthesis was found to be a more sensitive endpoint than growth. After 4d exposure to tylosin, the EC_{50} values for the two chlorophytes *D. subspicatus* and *P. Subcapitata,* based on photosynthesis as an endpoint, were 17.6 and 2.1 µmol L^{-1}, respectively. Similar results were also observed for two chlorophytes exposed to lincomycin (Table 5.1). However, for cyanobacteria *A. flos-aquae* and diatom *N. pelliculosa*, the situation was reversed and growth appeared to be a more sensitive endpoint than photosynthesis (Table 5.1). For example, after 4d exposure of *A. flos-aquae* to lincomycin, the EC_{50} derived based on growth was 1.2 µmol L^{-1} (Table 5.1), which was nearly one third of that derived based on photosynthesis. While no explanation for the sensitivity behaviour of both endpoints was available, the results of this study indicate that when testing antibiotics on chlorophytes for the environmental risk assessment purpose, oxygen evolution rate measurements might be an additional endpoint that could be included which, in some cases, may be more sensitive as well a being ecologically relevant as photosynthesis is such an important process for ecosystem functioning.

5.3.3 Analysis of the toxic effects on the algal physiology

5.3.3.1 Toxic effects on the oxygen evolution rate

All three antibiotics significantly inhibited the oxygen evolution rate of gross photosynthesis (Table 5.2). The inhibition effects were strengthened with the increasing concentrations of antibiotics. However, not all tested antibiotics (e.g. trimethoprim on *N. pelliculosa* and lincomycin on *A. flos-aquae*) affected pigment synthesis (e.g. chlorophyll a). The effects of

these antibiotics on algal photosynthetic reaction (i.e. oxygen evolution rate) may be due to the damage on structural development of the chloroplast, where it was not correlated with a factor directly related to chlorophyll synthesis/ content (Hudock et al., 1964). Similar results were reported in the study of Hudock et al. (1964) who investigated the effects of the antibiotic chloramphenicol on a strain of the chlorophyte *Chlamydomonas reinhardi*. Algal cultures were kept in the dark for 96h where this strain could neither form a normal chloroplast nor synthesize chlorophyll. The cells were then returned to the light to ensure that the chlorophyll and chloroplasts were newly synthesised and formed (called regreening; Hudock et al., 1964). It was found that algal cultures treated with 61.89 µmol L^{-1} chloramphenicol during the first three hours of regreening would inhibit the oxygen evolution rate but had no effect on chlorophyll synthesis rate/content.

5.3.3.2 Toxic effects on the pigment synthesis

The pigment contents of algal cells were not significantly affected by the three antibiotics for *D. subspicatus*. However, in some cases, pigment contents were significantly increased for *P. subcapitata*, *N. pelliculosa* and *A. flos-aquae* at the concentrations affecting growth (Table 5.2). For example, after 4d exposure to tylosin at 18.23 µmol L^{-1}, the chlorophyll a and carotenoid contents per cell of *P. subcapitata* increased by 45% and 165% compared to that in control. Similar stimulation effects have been reported by study testing other toxicant (polyamidoamine (PAMAM) 1,4-diaminobutane core, G2), where total chlorophyll content increased by 121% compared to the control at a concentration of 0.76 µmol L^{-1} (Petit et al., 2010). However, not all the antibiotics could promote the synthesis of chlorophyll and carotenoids. Liu et al. (2011) reported that erythromycin at the concentration of 0.41 µmol L^{-1} could lead to a reduction in the

chlorophyll content of *P. subcapitata* to 0.4 mg g^{-1} fresh weight compared to 0.95 mg g^{-1} in the control. A few of studies only present the measured pigment contents in the unit of mg L^{-1}, without correction for cell density or weight. For example, the carotenoid content of the prokaryote *Sarcina lutea* was reduced from 63 mg L^{-1} in the control to 38 mg L^{-1} over 24 h exposure to 14.24 µmol L^{-1} chloramphenicol (Portoles et al., 1970), In this case, the reduction in pigment might be attributed to less algae exisitng in the solution due to reduced growth.

Table 5.2 Values of net photosynthesis, respiration, gross photosynthesis rate, chlorophyll a, b and total carotenoid content per cell of *Desmodesmus subspicatus* (D.S.), *Pseudokirchneriella subcapitata*, (P.S.) *Navicula pelliculosa* (N.P.) and *Anabaena flos-aquae* (A.F.) over 4 d antibiotic exposures for three antibiotics: tylosin (TLN), trimethoprim (TMP) and lincomycin (LIN) (n.a not available as chlorophyll b only occurred in chlorophyte; Data are presented as Mean values ± standard deviation (n=3); asterisks indicate significant difference; Data are shown for the control, the lowest and highest test concentration for each study).

Algae	Pharma	4d TWAC (µmol L^{-1})	net photosynthesis/cells (umol O2 h^{-1} $cell^{-1}10^6$)	respiration/cells (umol O2 h^{-1} $cell^{-1}10^6$)	gross photosynthesis/cells (umol O2 h^{-1} $cell^{-1}$ 10^6)	chloro a/cell (10^9 mg L^{-1} $cell^{-1}$)	chloro b/cell (10^9 mg L^{-1} $cell^{-1}$)	total chloro/cell (10^9 mg L^{-1} $cell^{-1}$)	total carotenoid/ cells (10^9 mg L^{-1} $cell^{-1}$)
D.S.	TLN	control	0.233±0.108	-0.27±0.077	0.507±0.045	0.98±0.11	1.41±0.19	2.4±0.31	0.59±0.073
		6.49	0.282±0.067	-0.16±0.083	0.44±0.027	0.91±0.3	1.42±0.65	2.32±0.95	0.56±0.204
		12.99	0.339±0.028	-0.11±0.011*	0.45±0.018	0.97±0.104	1.41±0.18	2.38±0.29	0.60±0.066
		25.97	0.092±0.022	-0.0058±0.018*	0.097±0.016*	1.12±0.37	1.77±0.64	2.88±1.01	0.72±0.202
		42.94	0.074±0.037*	-0.051±0.033*	0.125±0.039*	0.72±0.071	1.1±0.096	1.82±0.17	0.49±0.042
		57.26	0.093±0.091*	-0.093±0.077*	0.185±0.12*	0.65±0.14	1.02±0.31	1.67±0.45	0.45±0.107
		71.56	0.076±0.0085*	-0.045±0.039*	0.12±0.048*	0.86±0.089	1.51±0.18	2.37±0.27	0.61±0.07
	LIN	Control	0.38±0.031	-0.076±0.024	0.46±0.055	1.24±0.14	1.76±0.3	3±0.44	0.71±0.1
		18.87	0.25±0.031*	-0.092±0.0068	0.34±0.035*	1.17±0.07	1.78±0.21	2.95±0.25	0.72±0.063
		37.74	0.19±0.047*	-0.112±0.016	0.304±0.034*	1.44±0.18	2.12±0.31	3.56±0.49	0.88±0.12
		75.49	0.11±0.054*	-0.11±0.0072	0.22±0.053*	1.03±0.16	1.42±0.35	2.45±0.52	0.63±0.12
		113.23	0.07±0.05*	-0.13±0.014*	0.2±0.041*	1.12±0.058	1.6±0.13	2.72±0.19	0.69±0.037

		150.97	0.027±0.015*	-0.14±0.035*	0.17±0.023*	1.2±0.098	1.85±0.15	3.05±0.24	0.78±0.07
		188.71	0.02±0.018*	-0.11±0.015	0.13±0.0046*	1±0.1	1.5±0.12	2.5±0.22	0.64±0.067
	TMP	control	0.23±0.11	-0.19±0.01	0.43±0.1	1.25±0.052	1.77±0.12	3.02±0.17	0.72±0.039
		27.25	0.2±0.16	-0.14±0.0063	0.34±0.16	0.99±0.13	1.31±0.19	2.3±0.32	0.57±0.069
		54.53	0.25±0.13	-0.18±0.034	0.43±0.16	1.05±0.1	1.36±0.13	2.41±0.24	0.59±0.045
		109.09	0.24±0.13	-0.2±0.019	0.44±0.14	1.14±0.15	1.51±0.31	2.65±0.46	0.65±0.089
		163.61	0.31±0.11	-0.18±0.033	0.49±0.13	0.99±0.34	1.65±0.44	2.64±0.63	0.66±0.15
		218.14	0.3±0.088	-0.15±0.053	0.45±0.13	1.22±0.21	1.66±0.33	2.88±0.54	0.703±0.12
		272.7	0.36±0.033	-0.15±0.033	0.51±0.066	0.98±0.057	1.27±0.13	2.25±0.18	0.56±0.051
P.S.	TLN	Control	0.086±0.055	-0.11±0.023	0.2±0.046	0.49±0.068	0.25±0.12	0.745±0.18	0.2±0.042
		0.4	0.098±0.045	-0.095±0.013	0.19±0.052	0.5±0.07	0.246±0.083	0.746±0.15	0.21±0.03
		1.2	0.1±0.038	-0.098±0.012	0.2±0.045	0.52±0.054	0.23±0.031	0.749±0.081	0.205±0.024
		3.61	-0.08±0.006*	-0.13±0.027	0.052±0.03*	0.53±0.015	0.29±0.061	0.82±0.063	0.23±0.026
		9.12	-0.2±0.045*	-0.22±0.04	0.023±0.006*	0.6±0.097	0.63±0.072*	1.24±0.16	0.307±0.041
		18.23	-0.3±0.095*	-0.32±0.092*	0.012±0.006*	0.89±0.044*	1.22±0.089*	2.12±0.13*	0.53±0.036*
		27.35	-0.32±0.083*	-0.33±0.083*	0.008±0.006*	0.43±0.038	0.38±0.14	0.81±0.17*	0.19±0.068*
	LIN	Control	0.073±0.036	-0.063±0.0078	0.136±0.039	0.29±0.036	0.15±0.015	0.44±0.049	0.14±0.014
		17	-0.029±0.022*	-0.082±0.023	0.053±0.007	0.31±0.078	0.203±0.14	0.51±0.204	0.18±0.069
		34	-0.055±0.01*	-0.096±0.037	0.041±0.044	0.28±0.083	0.3±0.14	0.58±0.22	0.19±0.056
		68	-0.078±0.014*	-0.089±0.014	0.0107±0.002	0.33±0.092	0.45±0.204	0.78±0.3	0.23±0.069
		125	-0.104±0.032*	-0.11±0.032	0.0073±0.002*	0.29±0.1	0.405±0.18	0.7±0.28	0.214±0.071
		166.61	-0.124±0.039*	-0.13±0.035*	0.0069±0.005*	0.17±0.074	0.19±0.14	0.36±0.21	0.12±0.05
		208.28	-0.131±0.014*	-0.14±0.016*	0.0052±0.002*	0.34±0.31	0.52±0.55	0.85±0.86	0.23±0.18
	TMP	Control	0.044±0.022	-0.073±0.0205	0.117±0.034	0.52±0.035	0.35±0.095	0.88±0.13	0.26±0.03
		13.2	0.058±0.038	-0.059±0.014	0.017±0.04	0.45±0.018	0.26±0.037	0.707±0.054	0.208±0.013
		26.42	0.059±0.036	-0.063±0.023	0.12±0.046	0.54±0.054	0.37±0.098	0.908±0.15	0.26±0.038

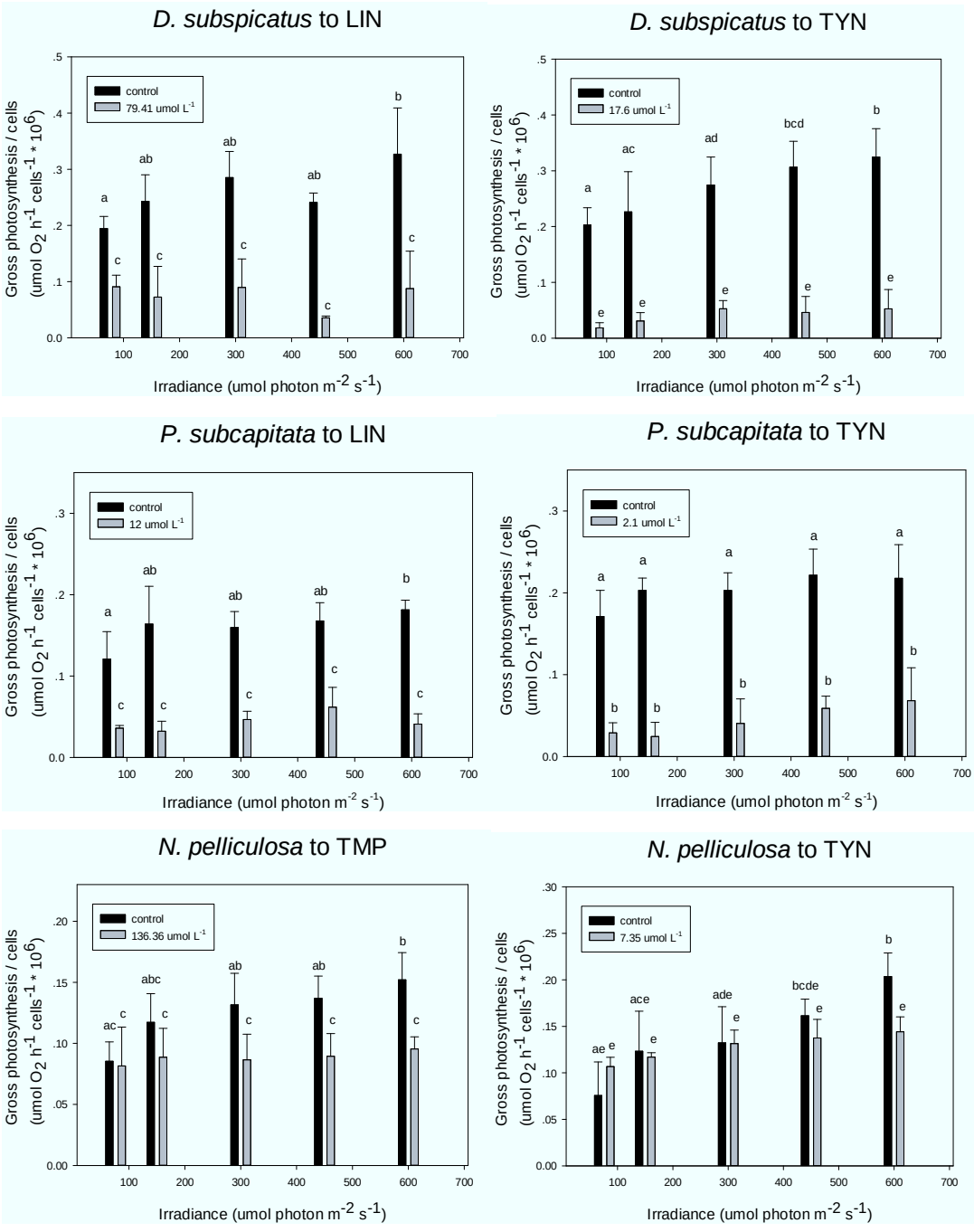
		52.83	0.058±0.014	-0.073±0.015	0.13±0.024	0.56±0.048	0.41±0.082	0.97±0.13	0.28±0.025
		103.29	0.05±0.015	-0.07±0.018	0.12±0.023	0.5±0.0104	0.32±0.03	0.818±0.039	0.24±0.002
		137.73	0.043±0.01	-0.068±0.022	0.11±0.025	0.52±0.018	0.408±0.081	0.928±0.086	0.27±0.013
		172.15	0.033±0.006	-0.072±0.019	0.1±0.022	0.45±0.044	0.35±0.0503	0.801±0.093	0.23±0.023
N.P.	TLN	control	0.071±0.016	-0.081±0.013	0.15±0.014	0.74±0.053	n.a.	n.a.	0.502±0.041
		4.88	-0.01±0.012*	-0.1±0.061	0.086±0.051*	0.86±0.1	n.a.	n.a.	0.64±0.067
		9.77	-0.04±0.009*	-0.11±0.013	0.07±0.012*	1.05±0.12	n.a.	n.a.	0.8±0.089
		19.53	-0.06±0.019*	-0.11±0.011	0.051±0.017*	1.1±0.2	n.a.	n.a.	0.85±0.19
		41.72	-0.06±0.007*	-0.096±0.02	0.032±0.021*	1.05±0.34	n.a.	n.a.	0.8±0.28
		59.6	-0.06±0.02*	-0.099±0.04	0.036±0.03*	1.24±0.4	n.a.	n.a.	0.95±0.32
		77.4	-0.06±0.014*	-0.12±0.022	0.054±0.023*	1.34±0.17	n.a.	n.a.	1.06±0.13*
	LIN	Control	0.02±0.023	-0.12±0.025	0.14±0.037	0.76±0.18	n.a.	n.a.	0.56±0.14
		21.44	0.031±0.022	-0.089±0.004	0.12±0.021	0.56±0.024	n.a.	n.a.	0.42±0.023
		42.88	0.035±0.023	-0.09±0.031	0.13±0.051	0.73±0.24	n.a.	n.a.	0.5±0.13
		64.33	0.026±0.014	-0.1±0.027	0.13±0.041	0.77±0.19	n.a.	n.a.	0.58±0.14
		82.03	0.048±0.007	-0.1±0.031	0.15±0.038	0.64±0.1	n.a.	n.a.	0.38±0.1
		102.61	0.05±0.009	-0.095±0.022	0.15±0.031	1.03±0.34	n.a.	n.a.	0.77±0.24
		153.91	0.053±0.027	-0.093±0.007	0.15±0.033	0.78±0.2	n.a.	n.a.	0.58±0.17
	TMP	control	0.026±0.016	-0.17±0.047	0.19±0.032	0.57±0.096	n.a.	n.a.	0.43±0.077
		10.85	0.035±0.009	-0.16±0.02	0.19±0.013	0.66±0.038	n.a.	n.a.	0.49±0.031
		16.26	0.026±0.004	-0.16±0.03	0.19±0.034	0.6±0.053	n.a.	n.a.	0.45±0.031
		32.52	0.059±0.012	-0.16±0.04	0.22±0.042	0.7±0.04	n.a.	n.a.	0.51±0.039
		48.77	-0.01±0.016	-0.18±0.085	0.17±0.075	0.63±0.048	n.a.	n.a.	0.49±0.033
		97.55	-0.15±0.061*	-0.29±0.101	0.14±0.041	1.68±0.6*	n.a.	n.a.	1.4±0.48*
		146.32	-0.19±0.068*	-0.27±0.051	0.086±0.023*	1.14±0.2*	n.a.	n.a.	0.97±0.17*
A.F.	TLN	control	0.058±0.041	-0.1±0.0093	0.16±0.05	0.26±0.032	n.a.	n.a.	0.194±0.034

	0.032	0.07±0.039	-0.097±0.17	0.17±0.051	0.24±0.062	n.a.	n.a.	0.166±0.052
	0.064	0.03±0.014	-0.074±0.033	0.1±0.019	0.27±0.033	n.a.	n.a.	0.215±0.024
	0.19	-0.1±0.026*	-0.19±0.031	0.092±0.034	0.24±0.002	n.a.	n.a.	0.191±0.007
	0.5	-0.18±0.084*	-0.21±0.065	0.034±0.054*	0.4±0.064	n.a.	n.a.	0.332±0.045
	1.06	-0.18±0.091*	-0.18±0.051	-0.0032±0.042*	0.47±0.178*	n.a.	n.a.	0.366±0.137*
	2.11	-0.2±0.073*	-0.2±0.095	-0.0071±0.022*	0.49±0.048*	n.a.	n.a.	0.384±0.038*
LIN	control	0.028±0.025	-0.13±0.023	0.16±0.026	0.35±0.137	n.a.	n.a.	0.25±0.095
	0.12	-0.01±0.034	-0.14±0.013	0.13±0.034	0.56±0.267	n.a.	n.a.	0.363±0.14
	0.23	-0.04±0.007	-0.144±0.016	0.104±0.021	0.31±0.112	n.a.	n.a.	0.227±0.074
	1.38	-0.11±0.042*	-0.21±0.078	0.101±0.054	0.47±0.146	n.a.	n.a.	0.35±0.091
	2.93	-0.17±0.054*	-0.25±0.087	0.079±0.034*	0.83±0.176*	n.a.	n.a.	0.57±0.113*
	5.87	-0.17±0.065*	-0.25±0.08	0.08±0.02*	0.6±0.05	n.a.	n.a.	0.43±0.035
TMP	control	0.091±0.019	-0.066±0.034	0.16±0.035	0.27±0.046	n.a.	n.a.	0.18±0.032
	23.21	0.094±0.055	-0.064±0.027	0.16±0.03	0.22±0.016	n.a.	n.a.	0.14±0.008
	46.42	0.056±0.056	-0.078±0.0098	0.13±0.065	0.22±0.015	n.a.	n.a.	0.13±0.008*
	92.83	0.085±0.034	-0.067±0.023	0.15±0.052	0.22±0.041	n.a.	n.a.	0.12±0.029*
	205.02	0.084±0.057	-0.067±0.021	0.15±0.073	0.27±0.029	n.a.	n.a.	0.17±0.02
	273.35	0.101±0.025	-0.067±0.018	0.168±0.041	0.23±0.025	n.a.	n.a.	0.14±0.017
	341.69	0.069±0.019	-0.064±0.017	0.13±0.035	0.24±0.041	n.a.	n.a.	0.15±0.013

5.3.3.3 Toxic effects on the Irradiance - Photosynthesis relationship

The gross oxygen evolution rate in the control cultures of *D. subspicatus*, *P. subcapitata and N. pelliculosa* measured at increasing irradiance levels increased and the trend followed a typical irradiance – photosynthesis (I-P) curve (Figure 5.3),where significant differences between controls and treated samples were observed for these species. While the oxygen evolution rate in the treated samples exhibited a similar increasing trend, each evolution rate was still lower than that of the control (except for *A. flos-aquae*). The gap of gross oxygen evolution rate between control and treated samples was enlarged with higher irradiance. However, in the cyanobacteria *A. flos-aquae*, no significant differences between controls and treated samples were observed, though EC50s of lincomycin and tylosin based on photosynthesis were applied. The reason might be due to that the EC50 derived was not significantly different. For example, after 4d exposure to tylosin, EC50 derived from concentration-response curve (gross oxygen evolution rate) was 0.33 µmol L^{-1}, which was lower than the lowest-observed-effect-concentration (LOEC, 0.5 µmol L^{-1}; Table 5.2). No increasing trend of oxygen evolution rate was shown with increasing irradiance as light has already achieved saturation or higher (Figure 5.3). These findings agreed with other published work e.g. Bahrs et al. (2013) found significant differences in P – I relationship could be observed for the chlorophyte *Desmodesmus armatus* and the cyanobacteria *Synechocystis sp*. between the control and samples treated with polyphenol p - benzoquinone at the EC_{90} level based on growth. In particular, the maximum gross oxygen production of *Synechocystis sp*. in treated sample was five times lower than that in the control. However, no significant effects of p - benzoquinone were found on the P - I relation of cyanobacteria *Microcystis aeruginosa*. The light - saturation

photosynthesis rate was determined by the light acclimation state and the nutrient conditions (Blache et al., 2011). As the nutrients in control and treated samples in this study were the same, a reduction in light - saturation photosynthesis rate might be due to antibiotic interacting with algal acclimation state via the disruption of the photosynthesis process.



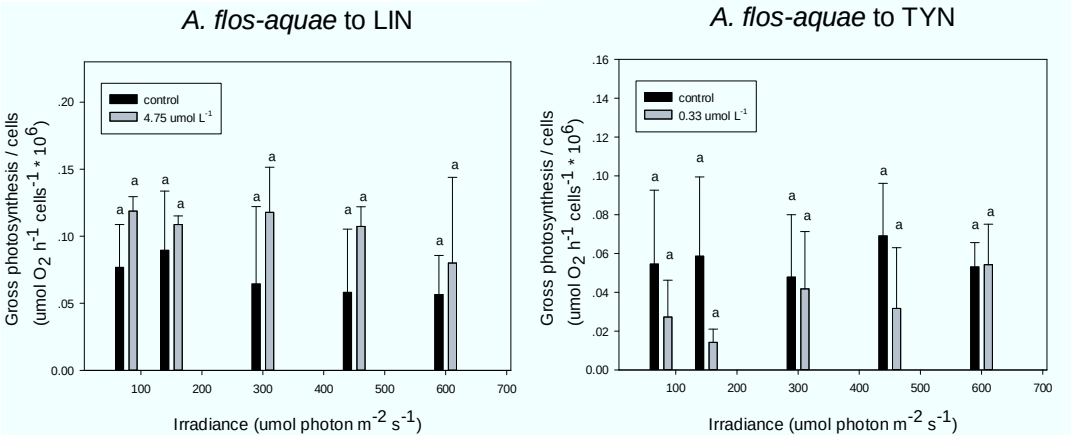


Figure 5.3 Responses of the gross photosynthetic rate on irradiance for algal species with evident photosynthesis inhibition effect from antibiotics. Data represent mean ± standard deviation (n=3). Bars sharing the same letter code are not significantly different; LIN, lincomycin; TYN, tylosin; TMP, trimethoprim.

5.3.3.4 Toxic mechanism analysis

While antibiotics are designed to interact with receptors in pathogenic bacteria, the fact that similar receptors and/or pathways might also be conserved in algal species means that the exposure of antibiotics in the culture medium could pose potential threat to the growth of algae. For tylosin and lincomycin, the mode of action is by interference with bacterial protein synthesis by binding to the 50s ribosomal subunit (Drugbank, 2013, Sigma-Aldrich, 2015). 50s is the larger subunit of the 70s ribosome of prokaryotes (PDB, 2010). In the eukaryote 60s is the larger subunit of the 80s ribosome (Wilson and Cate, 2012). An antibiotic (e.g. erythromycin) with a similar mode of action is entirely selective for 70s ribosomes and does not affect 80s ribosomes (Scholar and Pratt, 2000). This evidence could explain why a high sensitivity to tylosin and lincomycin was observed for the cyanobacteria. However, tylosin and lincomycin could inhibit the growth of eukaryotic species by interfering with the protein and enzyme synthesis involved in the photosynthesis process (Liu et al., 2011). For example,

approximately 30 proteins of cytochrome bf complex, which are the important component for the membrane in the thylakoid of algae, are involved in photosynthesis Ⅰ and Ⅱ. The macrolide erythromycin has been found to reduce the membrane content by interfering with the electron transport from PS Ⅱ to PS Ⅰ and reducing the size of the receptor- side of PS Ⅱ (Liu et al., 2011). Ribulose bisphosphate carboxylase (Rubisco) is an essential enzyme to catalyse the addition of CO_2 to ribulose-1,5-bisphosphate (RuBPCase) during the Calvin Cycle in the algal photosynthesis (Cooper, 2000). Macrolides could adversely influence the activity of rubisco and further inhibit the synthesis and activity of the RuBPCase in algae i.e. erythromycin could inhibit the synthesis of RuBPCase in *P. subcapitata*, and the inhibitory degree enhanced with higher exposure concentration (Liu et al., 2011).

For trimethoprim, the mode of action is to interact with dihydrofolate reductase (FolA) (Drugbank, 2013). However, for prokaryotic species such as *Nostoc sp.* and *Synechocystis sp.*, FolA is not included as these enzymes seem not to be essential in their folate metabolism (Myllykallio et al., 2003). This may indicate why trimethoprim did not affect the chlorophyll synthesis in cyanobacteria. For eukaryotes, information on the toxic mechanism of trimethoprim is still lacking.

5.4 Conclusions

This study indicated that after 4d exposure to antibiotics tylosin, lincomycin and trimethoprim, the photosynthesis related endpoint (oxygen evolution rate) exhibited higher sensitivity than the growth endpoint in the test with chlorophytes. The situation was reversed when testing antibiotics on cyanobacteria and diatoms. It is recommended that more species from each

class should be involved in testing antibiotics to confirm this conclusion. Once the verdict has been confirmed, the endpoint of oxygen evolution rate might be an endpoint that could be used in regulatory ecotoxicity studies in the future in addition to growth.

This study revealed that antibiotics might promote the pigment synthesis in some algal species (*P. subcapitata, N. pelliculosa* and *A. flos-aquae*). Despite the light utilization efficiency of eukaryote chlorophytes and diatom being reduced after exposure to the antibiotics that affecting growth, no significant inhibition effect on prokaryote cyanobacteria was observed.

While Chapters 2 - 5 have attempted to understand and characterise the potential risk and effects of antibiotics on a wide range of algal species in surface waters, results from these studies were obtained based on single compound tests. However, surface waters are more likely to be exposed to antibiotic mixtures than single substances so the combination effects and potential risks of antibiotic mixtures warrant further consideration. The next Chapter therefore explores the risks arising from combined exposure to the three study compounds.

Chapter 6

Risks of mixtures of major-use veterinary antibiotics to blue-green algae

6.1 Introduction

The work described in the previous Chapters (Chapter 4 & 5) investigated the effects of the three study antibiotics on the growth and physiology of algal species. The studies worked with single compounds. The results indicated that algal species, especially cyanobacteria, are more likely to be affected by antibiotics than other aquatic organisms. However, agricultural surface waters are unlikely to be exposed to single antibiotics (Backhaus et al., 2011). The reason being that some antibiotic products contain mixtures of antibacterially active substances (e.g. sulfonamides and trimethoprim are often used in combination) (Kienzler et al., 2014) and a number of different antibiotics are likely to be in use in a catchment at any one time (Kienzler et al., 2014). When assessing the risks of antibiotics, it is therefore important to consider the potential combined effects of antibiotics. A number of studies have explored the effects of pharmaceutical mixtures on aquatic organisms. For examples, Cleuver (2003) assessed the joint toxicity of clofibric acid and carbamazepine on the green algae *Desmodesmus subspicatus*, and showed that the mixture toxicity could be predicted by the concept of independent action (IA). Christensen et al. (2007) investigated the effects of binary mixtures of citalopram, fluoxetine, fluvoxamine, paroxetine and sertraline on algae and daphnids, and also showed that the combined toxicity of the compounds could be predicted by

concentration addition (CA).

Methods for assessing the risks of mixtures of chemicals to the natural environment have been proposed (Kienzler et al., 2014). For example REACH (Regulation No 1907/2006) presents a tiered approach for assessment of industrial chemical mixtures (EC, 2006). At tier 1, a conservative approach based on concentration addition (CA) is applied. Risk quotients (RQ) for individual mixture components are determined from PECs and PNECs and then summed to determine the RQ of the mixture (RQ_{mix}) (Kienzler et al., 2014). A similar approach could be used to evaluate the risks of antibiotic mixture.

This Chapter describes a study to assess the risks of the three study antibiotics to the cyanobacterial species *Anabaena flos-aquae*. *A. flos-aquae* was selected for use in the mixture studies as the studies described in Chapter 4 demonstrated that this species was the most sensitive to tylosin and lincomycin with the 4d EC_{50} being 0.13 µmol L^{-1} and 0.14 µmol L^{-1}, respectively, while the 4d EC_{50} for trimethoprim was 285.75 µmol L^{-1}.

Initially, an experimental investigation into the effects of a mixture of the study antibibiotics was performed and the results used to evaluate the performance of two commonly used mixture effect models, the concentration addition (CA) and independent action (IA) models. One of the validated models was then used alongside surface water exposure models, proposed by the Forum for Pesticide Fate Models and their Use (FOCUS) (FOCUS, 2011), to assess the combined risks of the use of the three compounds, resulting from use as veterinary medicines, to European surface waters.

6.2 Methods

6.2.1 Chemicals

The sources and purities of the test antibiotics are described in Section 4.2.1.

6.2.2 Algae culture

Algal culturing procedures for *Anabaena flos-aquae* are described in Section 4.2.2.

6.2.3 Ecotoxicity studies

Exposure models described in the CVMP Guidance Document (EMEA, 2008) were used to define relative concentrations, on a molar basis, of the three study compounds for use in the experimental mixture toxicity study. Based on this modelling (see Equation 6.1, Appendix 5), a ratio of 1 part tylosin to 4.31 parts trimethoprim and 4.18 parts lincomycin was selected for mixture toxicity testing. The 96 h EC_{50} values for the single study compounds were determined in the work described in Chapter 4. Therefore, the EC_{50} determination for the mixture was conducted without the range-finding step. Thirteen concentrations of the mixture in a geometric series around the lowest EC_{50} of the study compounds (i.e. tylosin) were used in the definitive EC_{50} test. The dose-response curve based on growth (cell density) was then generated based on the definitive data.

Prior to use in the ecotoxicity studies, all glassware and stoppers used in the tests were autoclaved at 121°C for 30 min. The antibiotics in the media were prepared and filtered into a 25 ml vial using a 0.2 µm sterilized syringe filter. The precultured algal inocula, taken from logarithmic growing precultures, were diluted into 15 ml of the prepared antibiotic solutions in the vials. The initial cell density was set at 2×10^4 cells ml^{-1}. The test vials were capped with air-permeable stoppers made of cotton and muslin. All the operations were performed on a sterilized bench. Afterwards, the prepared vials were put in the culturing incubator with the same shaking and physical conditions.

Bioassays lasted for 96 h, and the cell numbers were measured every 24 h using UV-Vis spectrophotometry. Cell numbers were estimated using a calibration curve of absorption determined by an ultraviolet and visible (UV-Vis) spectrophotometry and cell density. The wavelength for absorption measurement was 682 nm for *A. flos-aquae*. The pH values of thirteen tested exposure solutions were measured at the start and the end of the exposures. Analytical confirmation of exposure concentrations was also performed for the thirteen exposure solutions.

6.2.4 Concentration - response curve analysis

The data were analysed using Sigma-plot software. The experimental concentration - response curve was obtained by fitting experimental result to a sigmoidal regression, where x-axe was the molar sum of each component (μmol L^{-1}) and y-axe was growth inhibition (%). Two computational models concentration addition (CA) and independent action (IA) were introduced to predict the concentration – response curves. Concentration addition is defined as toxicants acting on the same biological site by the same mode of action (Equation 6.2):

$$\sum_{i=1}^{n} \frac{Ci}{\overline{ECxi}} = 1 \qquad \text{Equation 6.2}$$

Where C_i is the individual concentration of the *i*th substance present in a mixture with a total effect of x%, and EC_{xi} is the concentration of *i*th substance that causes the same x% effect by single exposure (Backhaus et al., 2000, Cleuvers, 2003). With the known mixture ratio (tylosin: trimethoprim: lincomycin, 1: 4.31: 4.18), the total concentration can be expressed as a function of the concentration of each component. To calculate the effect concentrations predicted by the concentration addition (CA), Equation 6.2 can be rewritten as Equation 6.3:

$$EC_{xmix} = \left(\sum_{i=1}^{n} \frac{Pi}{\overline{ECxi}}\right)^{-1} \qquad \text{Equation 6.3}$$

Where EC_{xmix} is the total concentration of the mixture causing x% effect and p_i is the molar fraction of components in the mixture (Backhaus et al., 2000). By using all the available effect, the total concentration was calculated (Equation 6.3) and a predicted concentration-response curve was plotted by fitting total concentration/ effect pairs to sigmoid regression. The dose – response curve of *A. flos-aquae* exposed to each single antibiotic was obtained from pervious study (Chapter 4).

The alternative concept is independent action defined by the effects of toxicants on different modes of toxic action (Equation 6.4):

$$E(c_{mix}) = 1 - \prod_i^n (1 - E(ci)) \quad \text{Equation 6.4}$$

Where $E(c_i)$ is the effect of an individual single component and $E(c_{mix})$ is the total effect of the mixture of total concentration. Equation 6.4 was used to estimate the mixture effects based on independent action (IA), and the total concentration is the sum of each component (Equation 6.5). The total concentration/ effect pairs were plotted and fitted to sigmoid regression to get the concentration-response relationship (Backhaus et al., 2000).

$$C_{mix} = \sum_{i=1}^n Ci \quad \text{Equation 6.5}$$

6.2.5 Mixture model evaluation

The 5% effective concentration values (EC_{05}) and the median effective concentration values (EC_{50}) with approximate 95% confidence intervals were calculated for the experimental mixture concentration – response curves as well as the CA and IA predictive curves. EC_{50}/EC_{05} ratio provides a measure of slope. These parameters were used to evaluate the predictive capability of the two models.

6.2.6 Antibiotic analysis

Concentrations of the antibiotics in the exposure solutions were confirmed using analytical methodologies described in Section 4.2.4.

6.2.7 Estimation of PECs based on FOCUS model

Concentrations of the study antibiotics in representative surface waters in agricultural areas in Europe were estimated using models and scenarios recommended by the Forum for Pesticide Fate Models and their Use (FOCUS) (FOCUS, 2011). The application rate, which is a required input for the models, was estimated based on recommended dosages and treatment frequencies and durations for each antibiotic, obtained from Compendium of Data Sheets for Animal Medicines 2012 (NOAH, 2011), using the approach recommended by the European Medicines Agency (EMEA, 2008). For each antibiotic, the maximum application rate (A_{max}), the average application rate (A_{ave}) and the minimum application rate (A_{ave}) of all products and indications were used for the FOCUS modeling. The medical products used to derive the maximum application rates were Synutrim (trimethoprim) and Pharmasin (tylosin) used for the treatment of broilers, and Lino-spectin 100 (lincomycin) for treating pigs (NOAH, 2011). The medical products used to derive the minimum application rates were Trimacare injection (trimethoprim) and TYLAN 200 (tylosin) used for the treatment of cattle, and Lincocin Premix (lincomycin) for pig treatment.

Modeling of the eight scenarios (five covering systems with soil drainage: D1, D2, D4, D5, D6; and three systems that are vulnerable to runoff: R1, R3, R4) covering drainage and runoff inputs to different watercourses (ditch, pond and stream) was performed using the winter cereal scenario. The ground incorporation method of application was selected and inputs from spray drift were set at zero. No uptake by plants was assumed (EMEA, 2008).

Physico-chemical properties of the antibiotics, needed for the modeling, were derived from a variety of sources and are given in Table 6.1. Estimation procedures can be found in the FOCUS model manual. The 4 d time-weighted averaged exposure concentrations (TWAEC) in the water layer for each scenario and antibiotic were obtained and used in the risk characterization work, as the PNEC in this study was based on 4 d EC_{50} derived from the effects of antibiotics on a cyanobacteria *A. flos-aquae*.

Table 6.1: Input parameters for the three antibiotics used in the FOCUS modelling

	Trimethoprim	Tylosin	Lincomycin
Molecular weight (g mol^{-1})	290.32	916.12	406.53
Log Kow	0.91[1]	1.63[2]	0.56[1]
$DT_{50water}$ (d)	29.1[3]	9.5[4]	37.5[5]
DT_{50soil} (d)	110[6]	54[7]	4.5[8]
$DT_{50sediment}$ (d)	542[5]	1000[5]	337.5[5]
VP (pa)	1.32 x 10^{-6} [9]	2.65 x 10^{-32} [5]	1.79 x 10^{-15} [5]
Water solubility (mg L^{-1})	400[10]	5[11]	29300[10]
Enthalpy of vaporization (J mol^{-1})[12]	95000	95000	95000
Molar enthalpy of dissolution (J mol^{-1}) [12]	27000	27000	27000
A_{max} (kg ha^{-1})	6.652	8.448	0.456
A_{ave} (kg ha^{-1})	0.711	1.194	0.257
A_{min} (kg ha^{-1})	0.0273	0.0656	0.0652
Koc	1680[9]	553[13]	59[6]

1 (Drugbank, 2013); 2 (Loke et al., 2002); 3 (Giang et al., 2015); 4 (Brain et al., 2005); 5 Data predicted by EPI Suite (EPA, 2013); 6 (Boxall et al., 2005); 7 (Boxall et al., 2006); 8 (Kummerer, 2004); 9 (straub, 2013); 10 (Drugbank, 2013); 11 (EPA, 2013); 12 (EMEA, 2008); 13 (Rabolle and Spliid, 2000)

6.2.8 Mixture risk assessment for the three antibiotics

Concentration addition was used as the basis for the risk characterization for the mixtures of the three antibiotics for each of the FOCUS scenarios. Initially, a risk quotient (PEC/PNEC) for each veterinary antibiotic was calculated based on the concentration estimated for the

antibiotic in each scenario. The risk quotient for the mixture (RQ_{mix}) of antibiotics for a scenario was then obtained by summing up the PEC/PNEC ratios for the individual antibiotics (Kienzler et al., 2014). If the RQ_{mix} was lower than one then the risk of the mixture to algae was deemed to be acceptable.

6.3 Results and discussion

6.3.1 Chemical analysis and pH variation

With an increase in the exposure concentration, the pH in the antibiotic mixture studies decreased gradually from 7.99 to 6.96. While a pH variation (1 unit) was observed, it was within the scope of OECD 201 guideline (less than 1.5 units). A drift in pH can be caused by CO_2 mass transfer from the surrounding air to the test solution (OECD, 2011). The variation in pH was consistent with a previous study (Chapter 4). The chemical recoveries for tylosin, lincomycin and trimethoprim were 122±16% (mean± standard deviation), 191±37% and 80±24%, respectively. As the chemical recovery of lincomycin was far above 100%, this measured concentration of lincomycin was used to modify the mixture ratio (tylosin: trimethoprim: lincomycin, 1: 4.31: 6.65)

6.3.2 Mixture toxicity analysis and model evaluation

Dose-response curves based on the experiments with cyanobacteria *A. flos-aquae* as well as CA and IA predictions are shown in Figure 6.1. The mixture had an observed 4d EC_{50} of 0.248 µmol L^{-1} (trimethoprim 0.089 tylosin 0.021 and lincomycin 0.138 µmol L^{-1}) (Table 6.2). While both the CA and IA concepts provided good estimations of the combined effects of the different mixtures of tylosin, lincomycin and trimethoprim (Table 6.2; Figure 6.1), a more accurate

predictability by CA of the joint toxicity of the mixture was observed. The IA predicted an EC_{50} of 0.34 µmol L^{-1} which was 37.1% higher than the observed EC_{50}. While CA predicted a slightly higher toxicity (EC_{50} 0.21 µmol L^{-1}) which was only 15.3 % lower than the observed EC_{50}. This finding was consistent with other publications investigating combination effects of pharmaceuticals. For examples, Cleuvers (2003) reported that the toxic effect of a binary mixture of pharmaceuticals ibuprofen and diclofenac, both belonging to the nonsteroidal anti-inflammatory drugs (NSAID), on chlorophyte *D. subspicatus* could be predicted well using the CA concept yet the IA predicted a lower combination effect. The binary mixture toxicity of three selective serotonin reuptake inhibitors (SSRIs) citalopram, fluoxetine and sertraline to freshwater algae *P. subcapitata* was predictable by CA model (Christensen et al., 2007).

The fact that the CA model works well is probably explained by the modes of action of the three antibiotics as well as the relative concentrations. Trimethoprim acts by inhibiting dihydrofolate reductase (DHFR) (Drugbank, 2013), while tylosin and lincomycin act by inhibiting bacterial protein synthesis by binding to 50s ribosome (Drugbank, 2013, Sigma-Aldrich, 2015). The relative concentrations mean that tylosin and lincomycin, which act by the same mode of action, are the two components within the mixture that dominant toxicity in this mixture (both EC_{50}s are 1000 times lower than that of trimethoprim; Table 6.2).

Table 6.2 Concentration - response models, EC05, EC50 and EC50/EC05 ratio of the tested antibiotics and the mixture

Substance	Model	EC05 (µmol L^{-1})	EC50 (µmol L^{-1})	EC50/EC05
Trimethoprim	Three-parameter sigmoid	<1.56	285.95 (246.88- n.a.)	183
Tylosin	Three-parameter hill	0.025	0.13 (0.09-0.18)	5.2
Lincomycin	Three-parameter hill	0.036	0.14 (0.11-0.15)	3.89
Mixture	Three-parameter hill	0.05	0.248 (0.22-0.29)	5
CA	Calculated	<0.061	0.21	3.44
IA	Calculated	<0.12	0.34	2.83

Steepness is important in determining the predictability of CA and IA models for a mixture at EC50 level. While no universal measure for slope of a concentration-response curve exists, it can be defined as a ratio between two EC values (e.g. the EC50/EC05 ratio), which has been reported in a range of literatures [47]. Brosche and Backhaus (2010) reported that with an EC50/EC05 ratio of 13.5, CA and IA models will predict quantitatively identical toxicity despite their mutually exclusive conceptual ideas. CA will predict a lower EC50 (higher toxicity) for the mixture than IA if the ratio for the concentration – response curve of the mixture is lower. In this study the EC50/EC05 ratio (5) indicated a high steepness of the observed concentration – response curve for the mixture (Table 6.2; Figure 6.1) (Brosche and Backhaus, 2010). The steepness of the mixture curve was within the range of slope for each single component, e.g. the ratio of EC50/EC05 ranged from 3.89 for tylosin up to 183 for trimethoprim. The CA model predicted a more accurate steepness with a factor of 0.31 lower than the experimental value

(Table 6.2), despite only a lower factor of 0.43 was observed in the steepness of IA predicted curve. Smit et al. (2001) found average value for the EC50/EC05 ratio of 7.2 for typical bioassay with algae, which is in line with finding for our single and mixture studies (Table 6.2). Typical EC50/EC05 ratios for algal test were substantially smaller than the critical threshold of 13.5 (Smit et al., 2001). The application of CA to a mixture tested on algae would therefore result in a slight overestimation of the mixture toxicity and IA predicted higher toxicity value, which agreed with the current study (Table 6.2). In conclusion, CA predicted more accurately the combined effect of three antibiotics on *A. flos-aquae* than IA. Therefore, risk assessment for the antibiotic mixture was performed based on the CA model.

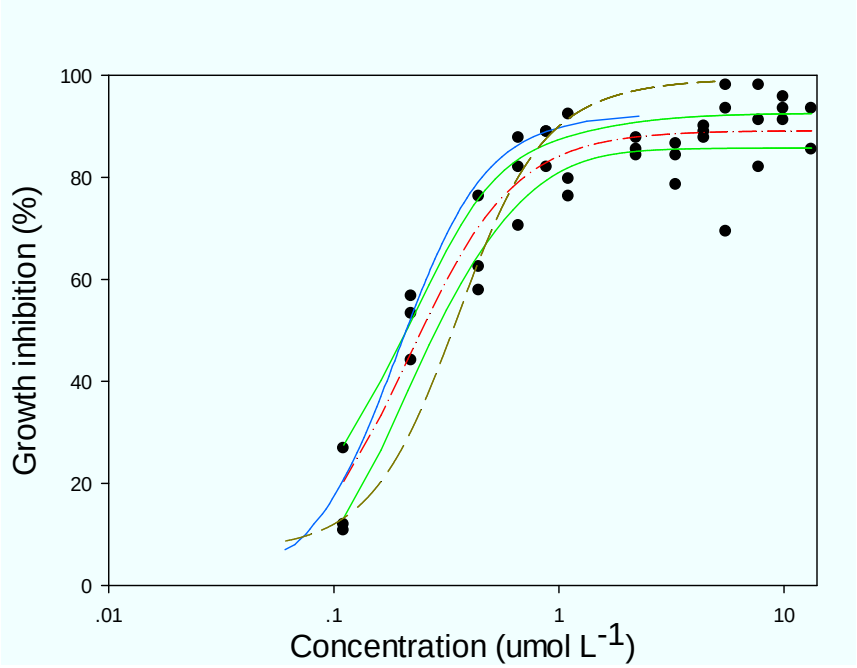


Figure 6.1: Predicted and observed mixture toxicity. Solid line (blue) = prediction according to concentration addition (CA); dashed line (dark yellow) = prediction according to independent action (IA); dashed-dotted line (red) = fit to the experimental mixture data; Solid line (green) = 95% confidence band; solid symbols= treated samples. X axis (C_{mix}) is the sum concentrations of three antibiotics. Molar rate of tylosin: trimethoprim: lincomycin = 1: 4.31: 6.65.

6.3.3 Estimation of exposure concentrations

The maximum occurrence of three substances were found in scenario R3 for stream systems (Table 6.3). R3 is a southern Europen scenario considering the superficial loading from run-off to surface water, where run-off is determined by annual rainfall and slope. The R3 stream scenario had a higher annual rainfall (800-1000 mm) than R1 & 4 scenarios (600-800 mm), and a slope of 4-10% in comparison with the intermediate case 2-4% of R1 (FOCUS, 2011).

The occurrence of three antibiotics has been reported worldwide. The concentration of trimethoprim ranged from less than 3.4×10^{-5} µmol L^{-1} in UK surfacewaters (Ashton et al., 2004) to 0.0061 µmol L^{-1} (US) (Kolpin et al., 2002) in US. These reported concentrations of trimethorpim were within the range of the predicted concentrations obtained using the FOCUS models. While very limited information on the occurrence of tylosin and lincomycin in surface waters was available, the presensce of lincomycin in surfacewater has been recorded from less than 2.46×10^{-6} to 0.0018 µmol L^{-1} (US) (Monteiro and Boxall, 2010). The maximum occurrence of tylosin was found at 5.46×10^{-5} µmol L^{-1} in the downstream of agricultural land in US (Boxall et al., 2011). All these reported concentrations for both antibiotic bases were within the range of the predicted concentrations in this study (Table 6.3) which gives some confidence in the model predictions. It should be noted that only 4 d TWAEC was extracted here (Table 6.3), whereas in reality the concentration of each antibiotic can be further reduced by other factors such as degradation and dilution over time.

Table 6.3 Estimation of exposure concentrations and single-substance risk quotients for three antibiotics. 4 d TWAEC: 4 d time weighted average exposure concentration. The value range indicates the lowest and highest TWAECs and risk quotients obtained for target waterbody

type. Values in parentheses are predicted based on medium application rate. D: drainage scenario; R: runoff scenario.

Chemical	Waterbody type	Highest scenario	4 d TWAEC (µmol L^{-1})	Risk quotient
Trimethoprim	Pond	R1	$4.72x10^{-6}$ - 0.016 (0.0017)	$1.65x10^{-6}$ - 0.0054 (0.00058)
	Ditch	D1	$1.22\ x10^{-5}$ - 0.026 (0.0028)	$4.26x10^{-6}$ - 0.0092 (0.00098)
	Stream	R3	$4.82\ x10^{-6}$ - 0.084 (0.009)	$1.69\ x10^{-6}$ - 0.029 (0.0031)
Tylosin	Pond	R1	$9\ x10^{-6}$ - 0.0061 (0.00086)	0.0069 – 4.61 (0.65)
	Ditch	D2	$1.98\ x10^{-5}$ - 0.011 (0.0016)	0.015– 8.45 (1.19)
	Stream	R3	$8.27\ x10^{-6}$ - 0.073 (0.01)	0.0063 – 55.83 (7.89)
Lincomycin	Pond	R1	1.25×10^{-5} - 0.0004 (0.00022)	0.0089 - 0.28 (0.16)
	Ditch	D2	1.01×10^{-5} - 0.00075 (0.00042)	0.0072 - 0.53 (0.3)
	Stream	R3	6.74×10^{-6} - 0.011 (0.0063)	0.0048 – 8.01 (4.51)

6.3.4 Risk assessment for single antibiotics and antibiotic mixtures

In terms of single substances, it can be concluded that a potential environment risk for trimethoprim was unlikely even using maximum application rate for the exposure estimation, as the maximum risk quotient (RQ) reached 0.029 in the R3 stream scenario (Table 6.3). With the estimation of medium exposures, RQs of tylosin and lincomycin exceeded a value of one for streams (Table 6.3). The maximum RQ values for both substances were 55.83 and 8.01, respectively (Table 6.3). These data indicate a high potential risk for tylosin and lincomycin in the European aquatic environment. These risk characterisation reuslts for single antibiotics agreed with other risk assessments or risk based prioritisation studies. For example, the max RQ of trimethoprim was 0.15 in Norway (Grung et al., 2008). Both tylosin and lincomycin were classified as high priority based on the potential risk in the UK environment (Boxall et al., 2003). Risk quotients for mixtures, estimated based on maximum application rates, exceeded one for most exposure scenarios i.e. D1, D2, D5, D6, R1, R3 and R4. The RQ values of the antibiotic mixture estimated based on the three application rate scenarios ranged from 0.016 to 63.86 (Figure 6.2). While these values indicated a high potential risk of this antibiotic mixture to the aquatic environment, the risk was dominated by tylosin.

In this risk assessment exercise cyanobacteria *A. flos-aquae* was targeted for hazard assessment. A range of studies have demonstrated that cyanobacteria exhibit higher sensitivity towards anitbiotics than fish, *daphnia* and other algal species, despite there were some exceptions i.e. diatom *Navicula pelliculosa* was found to be more sensitive to trimthoprim (4 d EC_{50} 21.01 µmol L^{-1}) than *A. flos-aquae* by a factor of 10 (Guo et al., submitted). However, *N. pelliculosa* was not sensitive to lincomycin with 4 d EC_{50} > 225.7 µmol

L^{-1}, which was more than 1000 times higher than that of *A. flos-aquae* (4 d EC_{50} 0.13 µmol L^{-1}). The 4 d EC_{50} of tylosin to *N. pelliculosa* (1.33 µmol L^{-1}) was also ten times higher than that of *A. flos-aquae* (0.13 µmol L^{-1}; Chapter 4). Therefore, risk characterisation on cyanobacteria *A. flos-aquae* will likely protect the broader environment from exposure to an antibiotic mixture of trimethoprim, tylosin and lincomycin.

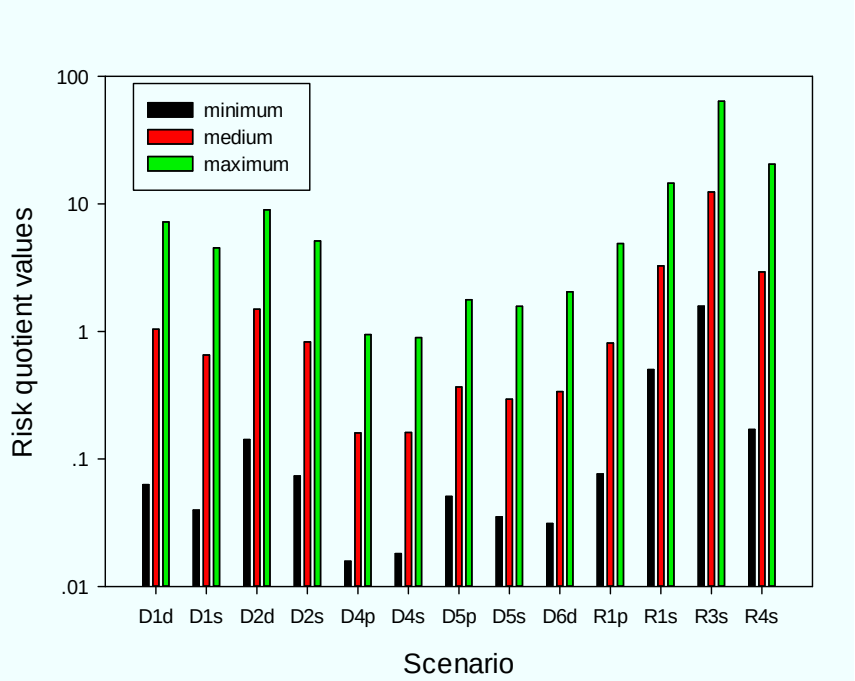


Figure 6.2: Risk quotients (PEC/PNEC) for a mixture of three antibiotics calculated based on maximum, medium and minimum application rate. d, ditch; s, stream and p, pond.

6.4 Conclusions

This study explored the combined effect of mixtures of three major use veterinary antibiotics to cyanobacteria *A. flos-aquae*, followed by an evaluation on the predictability of concentration addition (CA) and independent action (IA) models. With the estimation of antibiotic exposures, concentration addition was used as the basis for the risk characterization for the mixture. The results showed that while CA slightly overestimated the combined toxicity, this model

performed better than the IA model. When the CA model was used alongside exposure assessment models, an unacceptable risk was observed for the mixture of the three antibiotics for surface water scenarios covering different regions of Europe, primarily due to the effects from tylosin and lincomycin. We advocate that target monitoring of these antibiotics in the European surface water should be performed to gather data for a more realistic risk assessment and that biological monitoring be performed to see whether effects on algae are occurring in reality.

Chapter 7

General discussion and recommendations

In the last decades much research has focused on the fate of APIs in the environment and the potential effects on a wide range of organisms. Several studies have investigated the ecotoxicological effects of APIs on algal species with the EC_{50} ranging from ug L^{-1} to mg L^{-1} levels (Chapter 2). While differences in the sensitivity of algae to antibiotics have been recognised, the available data is limited to very few species and groups. Fewer studies have investigated the sensitivity of algal species to antibiotics. As the current environment risk assessment (ERA) regulations (e.g. EMEA 2006 & 2008) on antibiotics heavily rely on the algal test results to perform hazard assessment, studies exploring the sensitivity of algal species to antibiotic exposures and the underlying toxic mechanisms are justified.

Studying the response of algal species towards APIs in the aquatic environment is important because algae, sitting at the base of the food web, act as an essential element in the nutrient - cycling processes in the environment, so impacts of APIs on algae might threaten entire ecosystems (Chapter 2). In contrast to *Daphnia* and fish, algae are known to be particularly sensitive to APIs with antimicrobial properties.

The research in this thesis initially prioritised the APIs for experimental investigation, based on the potential risk in the UK environment (Chapter 3). An approach was developed for prioritising pharmaceuticals in the environment in terms of risks to aquatic and soil organisms,

avian and mammalian wildlife and humans. Compared to other prioritisation studies that have tended to focus on single use categories (e.g. prescription or hospital use), the developed approach is more complete as it includes assessment of parent compounds with high primary and secondary care usage, associated metabolites, over the counter (OTC) drugs, APIs suggested by environmental experts and substance detected in a recent chemical monitoring program (Gardner, 2013).

A summary of the highly ranked APIs, identified based on the ecotoxicological data for algae in Chapters 2 & 3 are presented in Table 7.1. The chemicals with RQ > 1 (i.e. those that are likely to occur at concentrations above effects concentrations) are all antibiotics. The antibiotics identified as a priority in the two Chapters were also slightly different (Table 7.1). The differences in priorities between the two Chapters are explained by differences in the exposure assessment methodologies used. In Chapter 2, a total residue method that only relied on the usage data in England was used, whereas the usage data considered in Chapter 3 included England, Scotland and Wales and the exposure assessment considered metabolism and removal in Waste Water Treatment Plants (WWTP). In this case the compound that is extensively metabolised after oral administration and easily to be adsorbed to sludge would be removed from the priority list (Chapter 3), but the primary metabolite would be added i.e. After oral administration, less than 5% of the administered dose of erythromycin can be recovered in the active form in the urine (Drugbank, 2013). This fact resulted in the low ranking of erythromycin. However, its main metabolite norerythromycin appeared 11th in the final list with a risk score in the range 0.1-1 (Chapter 3). In contrast with the research in Chapter 2, the priority compounds identified in Chapter 3 are more likely to occur in the environment due to

the realistic exposure assessment approach (Metcalfe et al., 2008) and a wide range of toxicological data characterised in the hazard assessment. Dissimilar ranking results for veterinary and human APIs in Chapter 2 are due to different exposure estimation approaches. Unlike exposure for human APIs that rely on total residue, the $PEC_{surfacewater}$ for veterinary APIs is dependent on the daily dose and number of days of treatment (EMEA, 2008). Furthermore, only a small proportion of veterinary APIs have available toxicological data to algae (Chapter 2). The priority substances for veterinary APIs are therefore not comparable to that for human APIs.

While this research (Chapter 2 & 3) only focuses on the UK environment, several studies (Roos et al., 2012, Kim et al., 2008) indicated that a variety of highly prioritised compounds identified in this study also showed high usage in other nations. The prioritisation approach for APIs also be transferred to other countries for the purpose of designing monitoring program, setting priorities and developing environmental risk management plans (Boxall et al., 2003). For example, risk scores (RQ) obtained for three veterinary antibiotics tylosin, lincomycin and trimethoprim identified for laboratory study (Table 7.1) were comparable to prioritisation studies based on RQ in other countries i.e. In a prioritisation exercise of veterinary APIs in China, tylosin and trimethoprim were classified as high priority, and lincomycin was classified as medium priority (Wang et al., 2014).

Table 7.1 Summary of priority compounds derived in Chapter 2 & 3, based on the ecotoxicological data to algal species

Risk score	Human APIs Chapter 2	Veterinary APIs Chapter 2	Human APIs Chapter 3
>10	clarithromycin amoxicillin	tylosin erythromycin lincomycin tiamulin amoxicillin	Amoxicillin
1-10	erythromycin	trimethoprim	clarithromycin azithromycin ciprofloxacin
0.1-1	oxytetracycline mycophenolate mofetil fluoxetine propranolol	florfenicol oxytetracycline tetracycline	mycophenolic acid oxytetracycline

The research then systematically evaluated the sensitivity of different algal species from chlorophytes, cyanobacteria and diatoms to three antibiotics by deriving a dose-response relationship for each species/antibiotic combination (Chapter 4). This was followed by evaluating the sensitivity between cell density and photosynthetic related endpoints that directly link to viable cells such as the oxygen evolution rate in the standard algal bioassays (Chapter 5). A summary of the ecotoxicological data generated from the experiments detailed in this thesis can be found in Table 7.2. In the tests with *P. subcapitata*, *D. subspicatus*, *A. flos-aquae* and *N. pelliculosa*, it is important to recognise that the EC_{50}s based on the growth endpoint in Chapter 4 are always lower than that in Chapter 5. For example, in the test of

lincomycin on *P. subcapitata*, the EC_{50} ranged from 7.36 µmol L^{-1} with initial cell density $5X10^3$ cells L^{-1} to 24.14 µmol L^{-1} with $5X10^5$ cells L^{-1}. The discrepancy in the EC_{50}s is likely due to the difference in the initial cell density, $5X10^3$ - $2X10^4$ cells mL^{-1} recorded in Chapter 4 and $5X10^5$ cells mL^{-1} in Chapter 5, respectively. A higher initial cell density could lead to less toxicant content bonding to the cells (both intercellular and extracellular), and further lead to less toxicant uptake and the lowering of toxicity (Franklin et al., 2002). A report studying the effects of copper on the chlorophyte *P. subcapitata*, where the initial cell density ranging from 10^3 to 10^5 showed that increasing cell number decreased the toxicity from 97.56 to 267.51 µmol L^{-1} based on EC_{50} values (Franklin et al., 2002).

The research presented in this thesis demonstrates that oxygen evolution rate is a more senstivie endpoint than growth for chlorophytes *P. subcapitata* and *D. subspicatus*. While the toxic mechanisms determining the effects of antibiotics on algal growth are still uncertain, this research indicates that antibiotics could affect biomass synthesis by interfering with the algal photosynthesis process such as light-harvesting pigment synthesis and light utilisation efficiency (Chapter 5).

The research generated knowledge on the effect and risk assessment of major use antibiotic mixtures based on the toxicological data to the cyanobacterial species *A. flos-aquae*, where the CA model was found to perform best in describing the combination effects of the antibiotic mixture (Chapter 6). While the FOCUS models have been proposed for the exposure assessment of veterinary medicines in the EMEA (2008) guideline, the research documented in Chapter 6 was the first attempt to estimate exposures of major use veterinary antibiotics in European surface water and use these data to assess mixture risks. The results indicated that

a potential risk of the antibiotic mixtures to the environment was likely in the regions of Europe, primarily due to the effects from tylosin and lincomycin.

While algal toxicity testing is required for environmental hazard evaluation as recommended by the Organization for Economic Cooperation and Development (OECD), these studies are often conducted as a screening toxicity tests rather than for predicting environmental impact (Rand, 2003). The ecological relevance of results from algal toxicity tests is still unknown. For example, the results from the standard 4 day tests using single-species may not be predictive of effects on natural algal communities exposed to the same compound (Calow, 1998). Available data indicate that, in general, laboratory-based algal studies are more sensitive to chemical exposure than natural algal communities (Calow, 1998). Potential reasons for this include that there is considerable interspecific variation in response of algae to a chemical, that unrealistic experimental conditions are used in laboratory tests, and that natural communities are able to adapt to tolerate exposure to a pollutant (Ogilvie and Grant, 2008), The ability of algal toxicity tests using single species to predict ecosystem effects therefore is still unclear (Rand, 2003).

Table 7.2 Summary of the EC50 values for tested antibiotics obtained in ecotoxicological studies. All toxicity values are in µmol L-1 (values in brackets are the range of 95% confidence limits). Initial cell densities (cell no.) are in cells mL^{-1}. Seven algal species are *P. subcapitata* (PS), *D. subspicatus* (DS), *C. vulgaris* (CV), *N. pelliculosa* (NP), *P. tricornutum* (PT), *A. flos-aquae* (AF) and *S. leopoliensis* (SL). Three antibiotics are lincomycin (LIN), tylosin (TYN) and trimethoprim (TMP). n.a. not available.

	LIN					TYN					TMP				
	Cell no.	EC_{50} growth	Cell no.	EC_{50} growth	EC_{50} photosynthesis	Cell no.	EC_{50} growth	Cell no.	EC_{50} growth	EC_{50} photosynthesis	Cell no..	EC_{50} growth	Cell no..	EC_{50} growth	EC_{50} photosynthesis
P.S.	$5X10^3$	7.36 (4.88-11.98)	$5X10^5$	24.14 (21.84-27.6)	12 (n.a.-20.68)	$5X10^3$	4.14 (3.4-5.06)	$5X10^5$	4.8 (4.26-5.47)	2.1 (n.a.)	$5X10^3$	>218.28	$5X10^5$	>306.9	>306.9
D.S.	$5X10^3$	16.07 (11.2-23.72)	$5X10^5$	>188.71	79.41 (60.27-103.3)	$5X10^3$	12.19 (10.57-15.42)	$5X10^5$	38.27 (30.23-47.08)	17.6 (10.13-13.39)	$5X10^3$	>344.45	$5X10^5$	>272.7	>272.7
C.V.	2×10^4	>225.7	n.a.	n.a	n.a	2×10^4	>81.2	n.a	n.a	n.a	2×10^4	>344.45	n.a	n.a	n.a
N.P.	1×10^4	>225.7	$5X10^5$	>153.91	>153.91	1×10^4	1.33 (1.14-1.76)	$5X10^5$	4.4 (3.66-5.05)	7.35 (0.44-17.49)	1×10^4	7.36 (6.74-8.28)	$5X10^5$	70.48 (57.79-96.03)	136.36 (95.34-n.a.)
P.T.	1×10^4	>225.7	n.a	n.a	n.a	1×10^4	5.7 (3.67-9.6)	n.a	n.a	n.a	1×10^4	74.61 (55.47-105.23)	n.a	n.a	n.a

A.F.	2× 10^4	0.13 (0.11-0.15)	5X10^5	1.2 (1.04-1.51)	4.75 (0.49-n.a.)	2× 10^4	0.092 (0.073-0.12)	5X10^5	0.06 (n.a-0.068)	0.33 (0.21-0.52)	2× 10^4	315.78 (285.16-n.a.)	5X 10^5	>341.69	>341.69
S.L	5× 10^5	0.095 (0.076 - 0.13)	n.a	n.a	n.a	5× 10^5	0.09 (0.068-0.13)	n.a	n.a	n.a	5× 10^5	>344.45	n.a	n.a	n.a

7.1 Implications for Environmental Risk Assessment

7.1.1 Multi - species involved in the APl risk assessment

Cyanobacteria have previously been shown to demonstrate high sensitivity to a wide range of antibiotics and have therefore been recommended for use in assessing the environment risks of antibiotics as part of the Market Authorisation process for new antibiotics (EMEA, 2008). While our toxicity study results for tylosin and lincomycin partly support this approach, the study found that trimethoprim only influences the growth of the diatom species rather than chlorophytes and cyanobacteria. This evidence indicates the assumption that cyanobacteria are the most sensitive species does not hold for all antibiotics. Therefore, to avoid the underestimation of environmental hazards to algae, this research suggests that the future risk assessment should consider inhibitiory effects of antibiotics on the growth of at least three species, one from each algal class. It would make sense that these tests are done on the species from each class that appear to be consistently most sensitive to antibiotic exposure i.e. *P. subcapitata*, *A. flos-aquae* and *N. pelliculosa*. It is also important to recognise that this research has only used a selection of indicator species from three classes. Further work on other antibiotic classes and other species is warranted to better inform the development of risk assessment approaches.

7.1.2 Mixtures in pharmaceutical risk assessment

As a broad range of substances are used as human and veterinary pharmaceuticals, the occurrence of APIs in the aquatic environment is more likely to be a multi-component mixture instead of a pure substance. Concerns about the mixture effects have been raised due to the

facts that: 1. the ecotoxicity of a mixture is almost always higher than that of single substance; and 2. a mixture could cause considerable ecotoxicological effects even when all the components are below the low observed effect concentrations (LOEC) (Backhaus et al., 2008). In view of these facts, current environmental risk assessment of antibiotics regulations (e.g. EMEA 2006 & 2008) only using individual substances may therefore underestimate the potential risk. This study demonstrates that environmental risk assessment of chemical mixtures based on the CA concept could be applied in the assessment for antibiotic mixture. In this approach PNECs of the individual substances are calculated first, and then extrapolation from a single substance to a mixture is undertaken by adding the PEC/PNEC ratios (risk characterisation ratios for the individual compounds). This approach makes optimum use of the existing individual compound assessments and it could be applied as a cautious step for mixture assessment (Backhaus and Faust, 2012).

7.1.3 The use of algal photosynthesis as an additional endpoint

Standardised algal ecotoxicological tests (e.g. OECD 201 guideline) do not consider important physiological endpoints such as algal photosynthesis, in which alteration could affect the ecological balance (Petit et al., 2010). Effects of external chemical stressors on algal photosynthetic activity are widely studied by the direct measurements of short-term oxygen evolution rate (Petit et al., 2010, Liu et al., 2011). In this study the sensitivity between two endpoints, oxygen evolution rate and growth, was compared. The results demonstrated that photosynthesis was a more vulnerable endpoint than growth for two chlorophytes, whereas higher sensitivities on the growth of cyanobacteria and the diatom species were observed. For

example, the effect of tylosin on the photosynthesis of *D. subspicatus* at EC_{50} level was 21.67 µmol L^{-1}, which was nearly half of that calculated based on growth (43.24 µmol L^{-1}); after 4d exposure to lincomycin, the photosynthesis based EC_{50} value of *P. subcapitata* was 8.8 µmol L^{-1}, which was approximately three times lower than that calculated based on growth (32.62 µmol L^{-1}). While the reason for this particular observation is still unclear, the endpoint of oxygen evolution rate might be an endpoint that could be used in the future in addition to growth. It is important to recognise that this comparison work only focused on a selection of indicator species, more antibiotics and algal species from three classes need to be involved in further research to confirm this finding.

7.2 Conclusion

This research has prioritised the APIs based on their risk in the UK environment, followed by systematically evaluating the sensitivity of a battery of species from chlorophytes, cyanobacteria and diatom to antibiotic exposures. The research also investigated the effects of target antibiotics on the growth and physiology of a selection of algal species. A risk assessment approach for antibiotic mixtures was developed and performed. The main conclusions of this thesis are the following:

1. The environmental occurrence and effects of APIs in the aquatic environment are issues that are increasingly important to the public and researchers. Algal species are interesting model organisms as they are sensitive to APIs and their short generation time allows the observation of negative effects from APIs.
2. The use of a prioritization approach is practical to identify the substances of most concern.

Apart from the effects on the aquatic and terrestrial organisms at low trophic levels considered in previous prioritization exercises, the approach developed in this research firstly considered secondary poisoning on avian and mammalian wildlife (i.e. fish and earthworm-eating birds and mammals) via the food chain, but no potential risk was found through these pathways.

3. Algal sensitivity towards antibiotics has been systematically evaluated using a battery of indicator species from chlorophytes, cyanobacteria and diatoms. One of the most significant findings is that the toxicity of trimethoprim to diatom is an order of magnitude higher than to chlorophytes and cyanobacteria. This evidence indicates that for some antibiotics chlorophytes and cyanobacterial species might not be the most appropriate test organisms.
4. Photosynthesis of chlorophytes was a more sensitive endpoint than growth, but the situation was reversed when testing cyanobacteria and diatom. The ecotoxicological effects of three antibiotics could partly be explained by the influence on physiological endpoints including oxygen evolution rate, light-harvesting pigment synthesis and light utilisation efficiency.
5. Research in this thesis demonstrated that CA-based model could well predict the combined effects of antibiotic mixtures on the cyanobacteria *A. flos-aquae*. Therefore, the concentration addition (CA) based risk assessment approach could be applied for antibiotic mixtures. The potential risk of antibiotic mixtures was likely in the regions of Europe.

7.3 Recommendations

7.3.1 Recommendations specific resulting from this research

1. Further development of the prioritisation approach – The risk based prioritisation approach that has been used, has employed available predictive models and data. There are uncertainties in the validity of some of these approaches for selected classes of pharmaceuticals. In the future, work should focus on further developing and validating the prioritisation approach to reduce these uncertainties. One important aspect would be the consideration of biodegradation during wastewater treatment. This process was not considered during the prioritisation and compounds that are susceptible to biodegradation will have had their environmental risk significantly overestimated.

2. Filling of data gaps for less well studied priority substances – For the compounds identified as a priority, based on predicted data, and which are found to occur in effluents, it is recommended that attempts are made to develop experimental data on the chronic aquatic ecotoxicity of these compounds. Some of these data may have already been developed by the pharmaceutical industry as part of the market authorisation process so contact should be made with relevant companies and trade associations (ABPI, EFPIA) to attempt to gain access to these data.

3. Consideration on sensitivity of different algal species in APIs risk assessment – Currently a number of toxicity data is available on the two freshwater algal species (*P. subcapitata and D. subspicatus*). Though the EMEA (2006) guideline has suggested using cyanobacteria species for testing some therapeutic class of APIs (e.g. antibiotics), the data on cyanobacteria is still limited. Almost no toxicity data is available on diatom species. The results for the single toxicity

test of three antibiotics on seven algal species indicate that the risk of some APIs to algae in the environment might be underestimated if hazards are only assessed by chlorophytes. It is therefore suggested that the toxicity data of APIs focusing on algal species such as cyanobacteria and diatom species should be produced.

4. Assessment of mixture interactions in the prioritisation study - This assessment has considered single pharmaceutical ingredients. However many compounds will have the same mode of action and some compounds are known to interact toxicologically in patients (i.e. they are contraindicated). A logical extension to the prioritisation exercises would be to consider the potential interactions of high priority compounds which have the same mode of action of those which are contraindicated. The results of the joint toxicity tests indicate the reliable predictive capacity of concentration addition (CA) models, and therefore, the CA-based approach could be included in risk assessment of API mixtures.

5. Investigation of APIs on the impairment process in algae – The results of the physiology study reveal that the algal physiological endpoints including oxygen evolution rate and light-harvesting pigment contents were vulnerable to a wide range of external stressors such as APIs. To completely understand the damage process and mechanisms of APIs to algal photosynthetic activity, it would be valuable to explore the effects of APIs on the algal physiology and biology such as enzymes and translation/ transcription process involved in the photosynthesis process.

6. Utilisation of environment relevant exposure concentration in algal studies – The effects of APIs on the algal growth and physiology were investigated using high exposure concentrations which could obtain dose – response curves, to enable the comparison of endpoint sensitivities

between growth and photosynthesis at EC_{50} levels. In this case the adverse influence on algal physiology might be overestimated. To investigate whether these observed effects occur in the field, environment relevant exposure concentrations should be used in the future algal studies.

7.3.2 General recommendations

1. Targeted monitoring of less well studied prioritised substances and metabolites – Due to significant data gaps, a number of compounds were identified as a priority based on data generated from predictive methods. It is recommended that a targeted monitoring study be undertaken at a few treatment works to identify whether these high priority substances do occur in wastewater effluents and sludge or not.

2. Development of data on the use and emissions of over - the - counter (OTC) medicines – In the prioritisation exercise quantitative information on the usage of OTC medicines was not obtained so it was not possible to prioritise these substances based on risk. Given the likely high use of these substances, it would be beneficial to generate data on usage patterns for these products and on the likelihood of emissions of these to the environment. It may be appropriate to monitor these substances in the future targeted monitoring study described in 1.

3. Implementation of risk assessment by using occurrence data – To undertake exposure assessment of APIs in the environment, the current risk assessment regulation such as EMEA (2006) was used. However this only calculated predicted exposure concentrations, as monitored data was not always available for most of the substances. With the development of analytical instruments which have lowered the limit of detection in the last decade, an increasing number of detected concentrations of APIs have been reported worldwide. These

data might be collated and synthesised along with the growing dataset of ecotoxicity data to perform a more realistic risk assessment of APIs in the environment.

4. Investigation of biotransformation and metabolism products of APIs – So far a substantial number of studies have focused on parent compounds. However, information on the occurrence, fate and effects of biotransformation products and metabolites in the environment is still very limited and therefore, more data should be produced to enable risk assessment on biotransformation products.

Appendix 1

Table A1.1 Toxicity and environmental risk assessment of pharmaceuticals to chlorophytes

Species	Pharmaceutical/ Products	Ingredient	Test duration	EC_{50} (mg L^{-1})	Reference	PEC (mg L^{-1})	PEC:PNEC ratio*
Chlorella pyrenoidosa	Chloramphenicol	I	72h	14	(Lai et al., 2009) [1]	6.4E-08	4.57E-07
	Florfenicol	I	72h	215		NA	NA
	Thiamphenicol	I	72h	1283		NA	NA
	Carbamazepine	I	96h	49.4	(Zhang et al., 2012) [3]	0.00085	0.0017
Chlorella vulgaris	Oxytetracycline	I	48h	6.4	(Pro et al., 2003) [3]	0.00047	0.0073
	Streptomycin	I	96h	20.08	(Qian et al., 2012) [3]	7.44E-10	3.7E-09
Desmodesmus subspicatus (Scenedesmus subspicatus)	Atenolol	I	72h	620	(Cleuvers, 2005) [2]	0.00061	9.8E-05
	Captopril	I	72h	168	(Cleuvers, 2003)[3]	9.71E-06	5.78E-06
	Carbamazepine	I	72h	74		0.00085	0.0011
	Clofibrinic acid	I	72h	115		NA	NA
	Diclofenac	I	72h	72		0.0033	0.0046
	Ibuprofen	I	72h	315		0.0057	0.0018
	Metoprolol	I	72h	7.3		5.6E-05	0.00077
	Metformin	I	72h	>320		0.018	0.0057
	Naproxen	I	72h	>320		0.0012	0.00036
	Propranolol	I	72h	5.8		0.00017	0.003
	Allegra	Fexofenadin	72h	>200	(FASS, 2012)[3]	NA	NA
	Alvedon	Paracetamol	72h	134		4.59E-07	3.43E-07
	Amaryl	Glimepirid	72h	610		1.84E-08	3.02E-09
	Aptivus	Tipranavir	72h	>40.4		3.28E-10	8.12E-10
	Bisolvon	Bromhexin	72h	0.25		NA	NA
	Bisoprolol	I	72h	11.5		2.65E-05	0.00023
	Bisostad	Bisoprolol	72h	11.5		NA	NA
	Buscopan	Butylscopolamine	72h	>80		1.43E-05	1.785E-05
	Carvedilol	I	72h	14.8		7.11E-06	4.806E-05
	Carveratio	Carvedilol	72h	14.8		NA	NA
	Ciklosporin	Cyclosporin	72h	>100		NA	NA
	Clozapine	I	72h	2.38		8.41E-07	3.53E-05
	Coramil	Diltiazem	72h	33.5		NA	NA
	Daonil	Glibenclamide	72h	735.5		4.75E-11	6.45E-12
	Diklofenak	I	72h	72		NA	NA

Emconcor	Bisoprolol	72h	11.5	NA	NA
Fexofenadin Orifarm	Fexofenadin	72h	>200	0.00017	8.67E-05
Furosemide	I	72h	322.21	0.00032	9.77E-05
Glimepirid	I	72h	610.72	1.63E-06	2.62E-07
Glucophage	Metformin	72h	>320	0.0032	0.00101
Granisetron	I	72h	22.6	1.18E-09	5.22E-09
Impugan	Furosemide	72h	322.2	NA	NA
Intelence	Etravirine	72h	>0.0049	7.87E-10	1.61E-05
Invega	Paliperidon	72h	14	2.5E-09	1.79E-08
Kredex	Carvedilol	72h	14.8	NA	NA
Kytril	Granisetron	72h	22.6	6.82E-10	3.02E-09
Lariam	Mefloquine	72h	0.16	9.55E-07	0.0006
Lasix Retard	Furosemide	72h	322.21	1.1E-09	3.426E-10
Leponex	Clozapine	72h	2.5	NA	NA
Magnevist	Gadopentetsyra	72h	>100	NA	NA
Medikinet	Methylphenidate	72h	6	1.19E-06	1.98E-05
Metformin	I	72h	320	0.018	0.0057
Micardis	Telmisartan	72h	9.88	3.03E-05	0.00031
Midazolam	I	72h	11.4	2.09E-07	1.83E-06
Minitran	Glyceryl trinitrate	72h	0.4	2.44E-07	6.09E-05
Naproxen	I	72h	39	0.001161	0.00298
Naprosyn	Naproxen	72h	39	2.16E-05	5.53E-05
Naramig	Naratriptan	72h	>100	9.61E-08	9.61E-08
Nefoxef	Fexofenadine	72h	>200	NA	NA
Nexavar	Sorafenib	72h	0.00054	3.06E-11	5.71E-6
Pamol	Paracetamol	72h	134.4	3.57E-10	2.66E-10
Paracetamol	I	72h	134	0.022	0.017
Perfalgan	Paracetamol	72h	134.4	3.73E-08	2.77E-08
Persantin Depot	Dipyridamole	72h	>2.36	0.0005	0.021
Pindolol	I	72h	11	8.11E-08	7.37E-07
Pramipexol	I	72h	240	1.7E-07	7.1E-08
Primolut- Nor	Noretisteron	72h	0.4	4.91E-08	1.23E-05
Pronaxen	Naproxen	72h	39	NA	NA
Ramipril	Ramipril	72h	>100	9.62E-05	9.62E-05
Rilutek	Riluzol	72h	4.48	1.05E-06	2.35E-05
Riluzol	I	72h	4.48	1.05E-06	2.35E-05
Sandimmun	Ciclosporin	72h	>100	1.11E-07	1.11E-07
Sandomigrin	Pizotifen	72h	0.98	NA	NA
Serevent	Salmeterol	72h	2.8	2.33E-09	8.33E-08
Sertralin	I	72h	240	0.00019	7.88E-05
Sifrol	Pramipexol	96h	240	NA	NA
Sinalfa	Terazosin	72h	160	NA	NA
Sumatriptan	I	72h	26	1.39E-05	5.34E-05
Telfast	Fexofenadine	72h	>200	6.88E-06	3.44E-06

	Telzir	I	72h	>100		1.11E-08	1.11E-08
	Terazosin	I	96h	160		6.4E-07	4E-07
	Testim	Testosterone	72h	0.5		1.26E-06	0.00025
	Testogel	Testosterone	72h	0.5		4.73E-06	0.00095
	Tradil	Dexibuprofen	72h	7.1		NA	NA
	Transiderm-Nitro	Glyceryl trinitrate	72h	0.4		1.04E-06	0.00026
	Triatec	Ramipril	72h	>100		NA	NA
	Ultravist	Iopromide	72h	10000 NOEC		NA	NA
	Undestor Testocaps	Testosterone	72h	0.5		5.06E-10	1.01E-07
	Velcade	Bortezomib	72h	0.3		NA	NA
	Visanne	Dienogest	72h	>16		NA	NA
	Visken	Pindolol	72h	11		1.07E-07	9.69E-07
	Voltaren	Diclofenac	72h	72		2.54E-09	3.53E-09
	Voxra	Bupropion	96h	0.95		NA	NA
	Xeplion	Paliperidon	72h	14		3.72E-10	2.66E-09
	Zyban	Bupropion	96h	0.95		5.8E-06	0.000604
	Mixture						
	Angemin	Drospirenone Estradiol	72h	5.5		NA	NA
	Asasantin Retard	Aspirin Dipyridamole	72h	>2.36		8.58E-05	0.0036
	Codalvonil	Codeine Paracetamol	72h	134.4		NA	NA
	Mollipect	Bromhexine Ephedrine	72h	0.25		NA	NA
	Neovletta	Etinylestradiol Levonorgestrel	72h	130		NA	NA
	Norgesic	Orphenadrine Paracetamol	72h	134.4		NA	NA
	Panocod	Codeine Paracetamol	72h	134.4		NA	NA
	Qlaira	Dienogest Estradiol	72h	>16		5.51E-08	3.44E-07
	Trionetta	Etinylestradiol Levonorgestrel	72h	>0.13		NA	NA
	Yasminelle	Drospirenone Ethinylestradiol	72h	130		NA	NA
	Yasmin	Drospirenone Ethinylestradiol	72h	130		4.98E-08	3.83E-08
	Yaz	Drospirenone Ethinylestradiol	72h	130		NA	NA
Dunaliella	Clofibric acid	I	96h	224.2	(DeLorenzo	NA	NA
tertiolecta	Diclofenac	I	96h	185.7	and Fleming,	0.0033	0.0018

	Fluoxetine	I	96h	0.17	2008) [3]	0.0001	0.06
	Simvastatin	I	96h	22.8		0.00096	0.0042
	Triclosan	I	96h	0.0036		3.55E-10	9.99E-06
Pseudokirchneriella subcapitata (Selenastrum capricornutum)	Flumequine	I	72h	16	(van der Grinten et al., 2010)[2]	NA	NA
	Oxytetracyline	I	72h	0.6		0.00047	0.078
	Streptomycin	I	72h	1.5		7.44E-10	4.96e-08
	Sulphamethoxazole	I	72h	0.52		NA	NA
	Trimethoprim	I	72h	9		0.00019	0.0021
	Tylosin	I	72h	0.0089		NA	NA
	Chlortetracycline	I	72h	3.1	(Isidori et al., 2005b)[3]	NA	NA
	Clarithromycin	I	72h	0.002		0.00025	12.33
	Erythromycin	I	72h	0.02		0.00045	2.23
	Lincomycin	I	72h	0.07		1.51E-05	0.022
	Ofloxacin	I	72h	1.44		4.89E-06	0.00034
	Olaquindox	I	72h	40		NA	NA
	Spiramycin	I	72h	2.3		NA	NA
	Tetracycline	I	72h	2.2	(Halling-Sorensen, 2000)[2]	2.36E-05	0.0011
	Tiamulin	I	72h	0.17		NA	NA
	Streptomycin	I	72h	0.13		7.44E-10	5.59E-07
	Naproxen	I	72h	31.82	(Isidori et al.,2005a)[3]	0.0012	0.0037
	Tylosin	I	72h	1.38		NA	NA
	Aciclovir	Acyclovir	72h	>99	(FASS, 2012)[3]	2.31E-06	2.33E-06
	Albyl	Aspirin	72h	15		NA	NA
	Alfuzosin	I	72h	0.7		4.68E-06	0.00067
	Alimata	Pemetrexed	72h	63		NA	NA
	Alli	Orlistat	10d	1.92		1.29E-06	6.7E-05
	Amiodaron	Amiodaron	72h	>100		7.96E-05	7.96E-05
	Amoxicillin	I	72h	630		0.0022	0.00035
	Aprovel	Irbesartan	96h	460		0.00039	8.54E-05
	Arava	Leflunomide	72h	22.4		3.48E-08	1.55E-07
	Arkolamyl	Olanzapine	14d	>141		NA	NA
	Asmanex Twisthaler	Mometasone	72h	>3.2		2.62E-08	8.19E-07
	Atriance	Nelarabine	72h	>100		5.47E-12	5.47E-12
	Bambec	Bambuterol	72h	475		6.47E-08	1.36E-08
	Bamyl	Aspirin	72h	15		NA	NA
	Baraclude	Entecavir	72h	110		9.6E-10	8.73E-10
	Bondronat	Ibandronate	72h	1.4		3.43E-07	2.44E-05
	Bonviva	Ibandronate	72h	1.4		1.22E-06	8.73E-05
	Brevoxyl	Benzoyl peroxide	72h	0.07		0.000011	0.015
	Bricanyl	Terbutaline	72h	>500		1.05E-07	2.1E-08

Budenofalk	Budesonide	72h	>8.6	1.02E-07	1.2E-06
Budesonide	I	72h	>8.6	5.02E-06	5.84E-05
Candesartan	Candesartan	72h	>56	4.24E-05	7.58E-05
Candexetil	Candesartan	72h	>56	NA	NA
Ceftriaxon	Ceftriaxone	72h	100 NOEC	1.42E-07	1.42E-07
Cefuroxime	I	72h	>76	1.81E-06	2.38E-06
Cellcept	Mycophenolate--mofetil	72h	0.068	4.76E-05	0.07
Cialis	Tadalafil	72h	>1.2	1.86E-06	0.00015
Citanest	Prilocaine	72h	154	1.58E-11	1.03E-11
Copegus	Ribavirin	72h	100 NOEC	2.28E-07	2.28E-07
Cordarone	Amiodarone	72h	>100	3.97E-07	3.97E-07
Cymbalta	Duloxetine	72h	0.2	2.56E-05	0.013
Danafusin	Alfuzosin	72h	52.7	NA	NA
Demoson	Mometasone	72h	3.2	NA	NA
Dermovat	Clobetasol	72h	4.2	2.23E-05	0.00053
Desonix	Budesonide	72h	8.6	NA	NA
Dimor	Loperamide	72h	76	NA	NA
Durogesic	Fentanyl	72h	15.1	1.8E-09	1.2E-08
Elocon	Mometasone	72h	3.2	1.43E-05	0.00045
EMEND	Aprepitant	72h	0.18	2.18E-09	1.19E-06
Epivir	Lamivudine	72h	96.9	4.05E-08	4.18E-08
Ergenyl	Valproic acid	72h	>100	NA	NA
Ery-Max	Erytromycin	72h	0.037	NA	NA
Exelon	Rivastigmine	72h	83	8.74E-07	1.37E-07
Exjade	Deferasirox	72h	0.32	5.86E-07	0.00018
Ezetrol	Ezetimibe	72h	4	1.66E-05	0.00042
Felodipine	Felodipine	72h	0.32	1.12E-05	0.0035
Fentanyl	I	72h	15.1	2.07E-08	1.37E-07
Fevarin	Fluvoxamine	72h	0.1	NA	NA
Flagyl	Metronidazole	72h	>39.1	5.09E-07	1.3E-06
Fontex	Fluoxetine	72h	0.027	NA	NA
Formatris	Formoterol	72h	94	NA	NA
Fucidin	Fusidic acid	72h	4.3	0.00049	0.011
Fucithalmic	Fusidic acid	72h	4.3	6.62E-07	1.54E-05
Fundan	Ketoconazole	72h	0.032	NA	NA
Fungoral	Ketoconazole	72h	0.032	NA	NA
Furadantin	Nitrofurantoin	72h	2.3	2.38E-08	1.03E-06
Galantamine	I	72h	>100	2.69E-06	2.69E-06
Geavir	Acyclovir	72h	>99	NA	NA
Gemcitabine	I	72h	5.4	NA	NA
Gemzar	Gemcitabine	72h	5.4	NA	NA
Glibenklamid Recip	Glibenclamide	72h	>1000	NA	NA
Glivec	Imatinib	72h	2.5	1.04E-06	4.18E-05

Glytrin	Glyceryl trinitrate	72h	0.4	2.51E-07	6.29E-05
Ibandronate	I	72h	1.4	NA	NA
Ibandronic acid	Ibandronate	72h	1.4	1.99E-06	0.00014
Imacillin	Amoxicillin	72h	630	NA	NA
Imigran	Sumatriptan	72h	26	1.82E-06	7.0E-06
Inside	Ranitidine	72h	150	NA	NA
Instanyl	Fentanyl	72h	15.1	6.52E-12	4.3E-11
Instillagel	Lidocaine	72h	780	2.65E-08	3.4E-09
Invanz	Ertapenem	72h	545	8.45E-08	1.55E-08
Iomeron	Jomeprol	72h	>1000	NA	NA
Irbesartan	I	72h	79	0.004	0.0005
Iressa	Gefitinib	72h	1.02	NA	NA
Ivemend	Fosaprepitant	72h	0.18	NA	NA
Januvia	Sitagliptin	72h	39 NOEC	8.38E-05	0.00022
Jevtana	Cabazitaxel	72h	0.013	NA	NA
Kestine	Ebastine	72h	9	NA	NA
Ketoconazole	I	72h	0.032	8.36E-07	0.0026
Klopidogrel	Clopidogrel	72h	0.85	NA	NA!
Lafunomy	Alfuzosin	72h	52.7	NA	NA
Lamictal	Lamotrigine	72h	39.7	2.06E-05	5.18E-05
Lamotrigine	I	72h	39.7	0.00017	0.00042
Leflunomide	Leflunomide	72h	22.4	1.84E-06	8.22E-06
Leptanal	Fentanyl	72h	15.1	NA	NA
Levofloxacin	I	72h	7.4	2.25E-06	3.04E-05
Lipanthyl	Fenofibrate	72h	>0.102	NA	NA
Livostin	Levocabastine	72h	>10	1.09E-13	1.09E-12
Loperamide	Loperamide	72h	>54	4.49E-06	8.3E-06
Loratadin	Loratadine	72h	0.7	1.65E-05	0.0024
Losec	Omeprazole	72h	85	3.22E-06	3.79E-06
Matrifen	Fentanyl	72h	7.6	1.37E-09	1.8E-08
Metomylan	Metoprolol	72h	22.8	NA	NA
Metoprolol	I	72h	22.8	5.6E-05	9.6E-05
Metronidazol	Metronidazole	72h	39.1	0.000025	2.45E-4
Montelukast	I	72h	100	9.4E-06	9.4E-06
Moxonidin	Moxonidine	96h	210	1.34E-07	6.39E-08
Mozoc	Metoprolol	72h	58.3	NA	NA
Mucoangin	Ambroxol	72h	25.6	NA	NA
Multaq	Dronedarone	72h	0.045	1.47E-05	0.033
Mycophenolate mofetil	I	72h	0.068	0.00017	0.26
Narop					
Nexium	Ropivacaine	72h	59	NA	NA
Nimvastid	Esomeprazole	72h	85	2.72E-05	3.19E-05
Nitroglycerin	Rivastigmine	72h	>83	NA	NA
Nitrolingual	Glyceryl trinitrate	96h	0.4	NA	NA

Novopulmon	Glyceryl trinitrate	96h	0.4	1.54E-06	0.00038
Noxafil	Budesonide	72h	>8.6	NA	NA
Olanzapine	Posaconazole	72h	0.19	4.02E-08	2.13E-05
Omecat	I	14d	> 141	8.6E-06	6.1E-06
Omeprazole	Omeprazole	72h	85	NA	NA
Omniscan	I	72h	85	0.00037	0.00043
Optinate Septimum	Gadodiamide	72h	>3200	NA	NA
Oxis Turbuhaler	Risedronic acid	72h	0.76	NA	NA
Panodil	Formoterol	72h	94	2.9E-11	3.09E-11
Pevaryl	Paracetamol	72h	134	NA	NA
Physiotens	Econazole	72h	0.17	1.28E-07	7.53E-05
Prezista	Moxonidine	96h	210	2.2E-09	1.05E-09
Primodium	Darunavir	72h	>43	1.7E-08	3.96E-08
Pulmicort	Loperamide	72h	>54	NA	NA
Ranitidine	Budesonide	72h	>8.6	2.56E-08	2.98E-07
Reminyl	I	72h	>150	0.00073	4.8E-4
Requip	Galantamine	72h	>100	2.69E-06	2.69E-06
Reyataz	Ropinirole	72h	29.3	1.01E-06	3.44E-06
Risedronat	Atazanavir	72h	>4.1	2.01E-08	4.91E-07
Risperdal	Risedronic acid	72h	0.76	3.12E-06	0.00041
Risperidone	Risperidone	72h	26	9.73E-08	3.74E-07
Rivastigmine	I	72h	26	1.73E-06	6.66E-06
Ropinirole	I	72h	>83	9.41E-07	1.13E-06
Ropivacaine	Ropinirole	72h	29.3	1.56E-06	5.33E-06
Rosazol	I	72h	59	NA	NA
Seloken	Metronidazole	72h	39.1	NA	NA
Sporanox	Metoprolol	72h	58.3	NA	NA
Stilnoct	Itraconazole	10d	>1000	1.12E-06	1.12E-07
Stioxyl	Zolpidem	72h	2.2	1.04E-07	4.73E-06
Stocrin	Benzoyl peroxide	72h	0.07	NA	NA
Stomacid	Efavirenz	72h	>0.012	NA	NA
Tamiflu	Ranitidine	72h	>150	NA	NA
Tasigna	Oseltamivir	96h	463	4.13E-07	8.92E-08
Tavanic	Nilotinib	72h	>0.016	NA	NA
Temodal	Levofloxacin	72h	7.4	1.01E-06	1.37E-05
Temomedac	Temozolomide	72h	>90	5.47E-13	6.07E-13
Temozolomide	Temozolomide	72h	>90	NA	NA
Tetracyklin	I	72h	>90	5.47E-13	6.07E-13
Teveten	Tetracycline	72h	0.31	NA	NA
Topirmate	Eprosartan	72h	>100	7.81E-05	7.81E-05
Valaciclovir	I	72h	>93	NA	NA
Valtrex	I	72h	>99	8.92E-06	9.01E-06
Viramune	Valaciclovir	72h	>99	7.54E-07	7.6E-07
Votubia	Nevirapine	72h	>43	3.26E-08	7.58E-08

Warfarin	Everolimus	72h	>16		NA	NA
Xylocard	I	72h	11		2.1E-05	0.00019
Zantac	Lidocaine	72h	>780		NA	NA
Zeffix	Ranitidine	72h	>150		2.45E-05	1.64E-05
Ziagen	Lamivudine	72h	>96.9		5.17E-07	5.34E-07
Zidoval	Abacavir	72h	49.06		2.02E-08	4.12E-08
Zinacef	Metronidazole	72h	39.1		1.59E-07	4.06E-07
Zinnat	Cefuroxime	72h	>76		2.84E-08	3.74E-08
Zolpidem	Cefuroxime	72h	>76		2.1E-07	2.77E-07
Zometa	I	72h	2.2		3.32E-06	0.00015
Zovirax	Zoledronic acid	72h	15		5.04E-12	3.36E-11
Zyprexa	Acyclovir	72h	>99		NA	NA
ZYTIGA	Olanzapine	14d	>141		6.6E-06	4.68E-06
	Abiraterone	72h	>1.0		NA	NA
Atenolol	I		257.5 LOEC	(Kuester et al., 2010) [3]	0.00061	0.00024
Diclofenac	I	96h	10 NOEC	(Ferrari et al., 2003) [1]	0.0033	0.033
Mixtures						
Asasantin Retard	Aspirin Dipyridamole	72h	>2.36	(FASS, 2012)[3]	0.000086	0.0036
Atacand Plus	Hydrochlorothiazide Candesartan	72h	>56		9.49E-09	1.69E-08
Axanum	Aspirin Esomeprazole	72h	85		NA	NA
Bactrim	Sulfamethoxazole Trimethoprim	72h	70		NA	NA
Bioclavid	Amoxicillin Clavulanic	72h	630		NA	NA
Duac	Benzoyl peroxide Clindamycin	72h	0.07		1.97E-05	0.028
Elosalic	Mometasone Salicylic acid	72h	3.2		NA	NA
Foradil	Budesonide Formoterol	72h	0.094		2.3E-10	2.44E-07
Kivexa	Abacavir Lamivudine	72h	49.06		5.63E-08	1.15E-07
Logimax	Felodipine Metoprolol	72h	>0.32		NA	NA
Riamet	Artemether Lumefantrine	72h	0.33		5.52E-10	1.67E-07
Spektramox	Amoxicillin Clavulanic	72h	630		NA	NA

	Symbicort	Budesonide Formoterol	72h	>8.6		2.19E-08	2.54-07
	Xylocain	Epinephrine Lidocaine	72h	>780		3.93E-09	5.04E-10
	Xyloproct	Hydrocortisone Lidocaine	72h	>780		4.43E-06	5.68E-07
Scenedesmus intermedius	Chloramphenicol	I	72h	0.1	(Sanchez-Fortun et al., 2009)[3]	6.4E-08	6.4E-05
Scenedesmus obliquus	Cefradine	I	72h	1.77	(Chen and Guo, 2012) [3]	2.52E-05	0.0014
	Enrofloxacin	I	72h	45.1	(Qin et al., 2012) [3]	NA	NA
	Carbamazepine	I	96h	70.1	(Zhang et al., 2012)[3]	0.00085	0.0012
Scenedesmus vacuolatus	Diuron	I	24h	0.012	(Neuwoehner and Escher, 2011) [3]	NA	NA
	Lidocaine	I	24h	134.7		0.00013	0.000093
	Norfluoxetine	I	24h	0.47		NA	NA
	Trimipramine	I	24h	15.6		5.12E-06	0.000033
Tetraselmis chuii (chlorophyta)	Chloramphenicol	I	72h	41	(Goncalves et al., 2007)[3]	6.4E-08	1.56E-07
	Florfenicol	I	96h	6.06		NA	NA
	Oxytetracyline	I	96h	11.18		0.00047	0.0042
	Florfenicol	I	72h	8	(Lai et al., 2009)[1]	NA	NA
	Thiamphenicol	I	72h	158		NA	NA

*PNEC= EC_{50}/100

1 real concentration used; 2 nominal concentration used; 3 unknown.

I represents ingredient.

Table A1.2 Toxicity and risk assessment of pharmaceuticals to cyanobacteria

Species	Pharmaceuticals	Ingredient	Test duration	EC_{50} (mg L^{-1})	Reference	PEC (mg L^{-1})	PEC:PNEC ratio*
Anabaena flos-aquae	Ceftazidim	I	72h	0.025	(FASS, 2012)[3]	4.15E-07	0.0017
	Fortum	Ceftazidim	72h	0.025		2.14E-07	0.00086
	Multaq	Dronedarone	72h	0.25		1.47E-05	0.0059
Microcystis aeruginosa	Amoxicillin	I	7d	0.008	(Liu et al., 2012)[3]	0.0022	27.36
	Spiramycin	I	7d	0.0012		NA	NA
	BenzylpenicilliG	I	72h	0.006	(Halling-Sorensen, 2000)[2]	1.99E-07	0.0033
	chlortetracycline	I	72h	0.05		NA	NA
	Olaquindox	I	72h	5.1		NA	NA
	Spiramycin	I	72h	0.005		NA	NA
	Streptomycin	I	72h	0.007		7.44E-10	1.06E-05
	Tetracycline	I	72h	0.09		2.36E-05	0.026
	Tiamulin	I	72h	0.003		NA	NA
	Tylosin	I	72h	0.034		NA	NA
	Cefradine	I	72h	1.38	(Chen and Guo,2012)[3]	2.52E-05	0.0018
	Octylphenol	I	72h	0.068	(Baptista et al., 2009)[2]	NA	NA
	Minocycline	I	72h	0.24	(Stoichev et al., 2011)[2]	1.53E-05	0.0064
	Streptomycin	I	96h	0.28	(Qian et al., 2012)[3]	NA	NA
	Amoxicillin	I	7d	0.0037	(FASS, 2012)[3]	0.0022	59.4
	Livostin	Levocabastine	72h	>32		1.09E-13	3.42E-13
	Sporanox	Itraconazole	10d	>1000		1.12E-06	1.12E-07
	Stocrin	Efavirenz	12d	>0.76 EC_{10}		NA	NA
	Visacor	Rosuvastatin	16d	330 NOEC		NA	NA
	Mixture						
	Bactrim	Sulfamethoxa-zole Trimethoprim	7d	112		NA	NA
	Bioclavid	Amoxicillin Clavulanic	7d	0.0037		NA	NA
	Spektramox	Amoxicillin Clavulanic	7d	0.0037		NA	NA
Synechococcus leopoliensis	Amoxicillin	I	96h	0.0022		0.0022	444.84

*PNEC= EC_{50}/100

1 real concentration used; 2 nominal concentration used; 3 unknown

I represents ingredient.

Table A1.3 Toxicity and environmental risk assessment of pharmaceuticals to diatoms

Species	Pharmaceuticals	Test duration	EC_{50} (mg L^{-1})	Reference	PEC (mg L^{-1})	PEC:PNEC ratio*
Skeletonema costatum	Ibuprofen	96h	7.1	(Halling-Sorensen et al., 1998)[2]	0.0057	0.081

*PNEC= EC_{50}/100

1 real concentration used.

Table A1.4 Toxicity and environmental risk assessment of pharmaceuticals to algal communities

Pharmaceuticals	Sampling spot	Test duration	EC_{50} (mg L^{-1})	Reference	PEC (mg L^{-1})	PEC:PNEC ratio*
Clotrimazole	Bay of Kalvhagefjorden	96h	0.15	(Backhaus et al., 2011)[1]	9.1E-05	0.06
Fluoxetine		96h	0.038		0.0001	0.26
Propranolol		96h	0.084		0.00017	0.2
Triclosan		96h	0.34		3.55E-10	1.05E-07
Zinc-Pyrithione		96h	0.0023		NA	NA
Clotrimazole	Bay of Kalvhagefjorden		0.0034-0.034 mg L^{-1} lead to evident reduction in growth	(Porsbring et al., 2009)[1]		
Ciprofloxacin Tergitol NP 10 Triclosan	upstream and downstream of the wastewater treatment plant in Kansas		No significant effects on growth showed.	(Wilson et al., 2003)[3]		

*PNEC= EC_{50}/100

1 real concentration used; 2 nominal concentration used; 3 unknown

Table A1.5 Measured environment concentrations (MEC) and MEC versus algal EC50 ratios of 19 herbicides and 17 pharmaceuticals in surface water

Pharmaceuticals	MEC (ug L^{-1})	Type	Country	Species	72h EC_{50} (mg L^{-1})	MEC:EC_{50}
amoxicillin	0.04	pharma.	Spain[1]	*Synechococcus leopoliensis*	0.0022	0.018
atenolol	0.3	pharma.	Spain[2]	*Pseudokirchneriella subcapitata*	257.5 (LOEC)	1.2E-6
bentazone	0.042	herbicide	UK[3]	*Anabaena flos-aquae*	10.1 (120h)	4.2E-6
bromoxynil	0.047	herbicide	UK[3]	*Navicula pelliculosa*	0.12	4E-4
carbamazepine	2.37	pharma.	Ireland[4]	*Chlorella pyrenoidosa*	49.4	4.8E-5
clarithromycin	2.4	pharma.	Spain[2]	*Pseudokirchneriella subcapitata*	0.002	1.2
chloridazon	0.34	herbicide	Swiss[5]	*Pseudokirchneriella subcapitata*	3	1.1E-4
clopyralid	0.055	herbicide	UK[3]	*Raphidocelis subcapitata*	30.5	1.8E-6
clozapine	8.18	pharma.	China[6]	*Desmodesmus subspicatus*	2.38	0.0034
dicamba	0.76	herbicide	Swiss[5]	*Skeletonema costatum*	1.8	4.2E-4
dichlorprop-p	0.047	herbicide	UK[3]	*Raphidocelis subcapitata*	67	7E-7
diclofenac	0.35	pharma.	Spain[7]	*Pseudokirchneriella subcapitata*	10 (96h NOEC)	3.5E-5
diquat	1.54	herbicide	UK[3]	*Psuedokirchneriella subcapitata*	0.011	0.14
enrofloxacin	0.17	pharma.	Spain[7]	*Scenedesmus obliquus*	45.1	3.7E-6
erythromycin	0.065	pharma.	Spain[2]	*Pseudokirchneriella subcapitata*	0.02	0.0033
fluoxetine	0.01	pharma.	China[6]	*Dunaliella tertiolecta*	0.17	5.9E-5
fluroxypyr	0.045	herbicide	UK[3]	*Pseudokirchneriella subcapitata*	49.8	9E-7
furosemide	0.4	pharma.	Spain[8]	*Desmodesmus subspicatus*	322.21	1.2E-6
glyphosate	0.1	herbicide	UK[3]	*Scenedesmus quadricauda*	4.4	2.3E-5
ibuprofen	0.14	pharma.	Spain[1]	*Skeletonema costatum*	7.1	1.9E-5
irbesartan	0.69	pharma.	Spain[1]	*Pseudokirchneriella subcapitata*	79	8.7E-6
linuron	0.14	herbicide	Swiss[5]	*Raphidocelis*	0.016	0.0088

				subcapitata		
ioxynil	200	herbicide	UK[3]	*n.a.*	24	0.0083
mcpa	0.14	herbicide	Swiss[5]	*Pseudokirchneriella subcapitata*	79.8	1.7E-6
mcpb	0.15	herbicide	Swiss[5]	*Psuedokirchneriella subcapitata*	41	3.6E-6
mecoprop-p	0.24	herbicide	Swiss[5]	*n.a*	16.2	1.5E-5
metazachlor	1.5	herbicide	UK[3]	*Psuedokirchneriella subcapitata*	0.016	0.093
metoprolol	0.006	pharma.	Spain[2]	*Desmodesmus subspicatus*	7.3	8.1E-7
napropamide	0.043	herbicide	Swiss[5]	*Pseudokirchneriella subcapitata*	3.4	1.3E-5
naproxen	0.53	pharma.	Spain[2]	*Pseudokirchneriella subcapitata*	31.82	1.6E-5
propyzamide	0.034	herbicide	UK[3]	*Raphidocelis subcapitata*	2.8	1.2E-5
tralkoxydim	0.057	herbicide	UK[3]	*Pseudokirchneriella subcapitata*	5.1	1.1E-5
triclopyr	0.046	herbicide	UK[3]	*Raphidocelis subcapitata*	75.8	6.1E-7
triclosan	0.046	pharma.	Spain[8]	*Dunaliella tertiolecta*	0.0036	0.013
thiamphenicol	0.011	pharma.	Spain[8]	*Tetraselmis chuii*	158	7E-8
trimethoprim	1.19	pharma.	Ireland[4]	*Pseudokirchneriella subcapitata*	9	1.3E-4

1 (Ortiz de Garcia et al., 2013); 2 (Moreno-Gonzalez et al., 2014); 3 (EA, 2013); 4 (McEneff et al., 2014); 5 (Moschet et al., 2015); 6 (Yuan et al., 2013); 7 (Collado et al., 2014); 8 (Carmona et al., 2014). All EC_{50} values for herbicides are extracted from Pesticide properties database (PPDB, 2014).

Pharma.: pharmaceutical

Appendix 2

$Sub_{inhab} = \frac{AP\ x\ 10^6}{UKpop\ x\ 365}$ (TGD, 2003) Equation 3.1

Where:

Sub_{inhab}: Substance consumed per inhabitant per day for the UK population [mg inh d^{-1}]

AP: Annual pharmaceutical usage [kg $year^{-1}$];

UK_{POP}: UK population: 63.7 million (Statistics, 2012).

$PEC_{TR} = \frac{Subinhab}{WasteWinhabxDilution}$ (TGD, 2003) Equation3.2

Where:

PEC_{TR}: Predicted environmental concentration for surface water assuming a total residue approach [mg L^{-1}];

Dilution: Dilution factor, default value 10 (from TGD (TGD, 2003)); and

$W_{aste}W_{inhab}$: Amount of wastewater per inhabitant per day, 200 [L inh d^{-1}]

$PEC_{MET} = \frac{Subinhab\ x\ Fexc}{WasteWinhab\ x\ Dilution}$ (TGD, 2003) Equation 3.3

Where:

PEC_{MET}: Predicted environmental concentration for surface water assuming removal through patient metabolism (mg L^{-1}); and

F_{exc}: Fraction of pharmaceutical excreted unchanged.

$$PEC_{WW} = \frac{Subinhab}{WasteWinhab\ x\ Dilution} \text{ x } (1 - \frac{Sludgeinhab\ x\ Koc\ x\ focsludge}{WasteWinhab + (Sludgeinhab\ x\ Koc\ x\ focsludge)}) \text{ (TGD, 2003)}$$

Equation 3.4

Where:

PEC_{WW}: Predicted environmental concentration for surface water assuming removal through wastewater treatment (adsorption only) [mg L^{-1}];

$Sludge_{inhab}$: Mass of waste sludge per inhabitant per day, 0.074, [kg inh d^{-1}];

K_{OC}: Soil organic carbon-water partitioning coefficient [g mL^{-1}]; and

foc_{sludge}: Fraction of sludge organic carbon, 0.326, calculated from Struijs et al. (Struijs et al., 1991).

$$PEC_{SW} = \frac{Subinhab\ x\ Fexc}{WasteWinhab\ x\ Dilution} \text{ x } (1 - \frac{Sludgeinhab\ x\ Koc\ x\ focsludge}{WasteWinhab + (Sludgeinhab\ x\ Koc\ x\ focsludge)}) \text{(TGD, 2003)}$$

Equation 3.5

Where:

PEC_{SW}: Predicted environmental concentration for surface water assuming removal through patient metabolism and wastewater treatment (adsorption only) [mg L^{-1}].

$$PEC_{sludge} = Koc \times foc_{sludge} \times \frac{Subinhab}{WasteWinhab}$$ (TGD, 2003) Equation 3.6

Where:

PEC_{sludge}: Predicted environmental concentration for sludge [mg kg^{-1}].

$$PEC_{SOIL} = \frac{PECsludge \times Asludge}{Dsoil \times RHOsoil}$$ (TGD, 2003) Equation 3.7

Where:

PEC_{SOIL}:Predicted environmental concentration for soil [mg kg^{-1}];

A_{sludge}: Sludge application rate to land, 0.5, [kg m^{-2} yr^{-1}];

D_{SOIL}: Soil mixing depth, 0.2, [m]; and

RHO_{SOIL}: Bulk density of soil, 1700, [kg m^{-3}]

$$\Phi_n = \frac{1}{1 + 10^{a(pH - pKa)}}$$ (Franco and Trapp, 2008) Equation 3.8

$$\Phi_{ion} = 1 - \Phi_n$$ (Franco and Trapp, 2008) Equation 3.9

Where a =1, pH = 5.8 for acids and a = -1, pH=4.5 for bases. pK_a is the negative logarithm (log_{10}) of the dissociation constant.

$$\text{Log } K_{oc} = \log (\Phi_n X 10^{0.54 logPn + 1.11} + \Phi_{ion} 10^{0.11 logPn + 1.54}) \quad \text{for acids}$$

(Franco and Trapp, 2008) Equation 3.10

$$\text{Log } K_{oc} = \log (\Phi_n X 10^{0.37 logPn + 1.70} + \Phi_{ion} 10^{pKa^{0.65}} Xf^{0.14}) \quad \text{for bases}$$

(Franco and Trapp, 2008) Equation 3.11

Where log P_n is the log K_{ow} of the neutral molecule; $f = \frac{Kow}{Kow + 1}$ (Franco and Trapp, 2008)

When Log K_{OW} <3, Log $P_{blood : water}$ = 0.73 x log K_{OW} – 0.88 (Fick et al., 2010) Equation 3.12

When Log K_{OW} >3, Log $P_{blood : water} = \log[(10^{0.73 \text{ x log KOW}} * 0.16) + 0.84]$

(Fitzsimmons et al., 2001) Equation 3.13

Where:

$P_{blood:water}$: Aqueous phase and fish arterial blood partition coefficient

K_{OW}: Octanol/water partition coefficient

$F_{SS}PC = P_{blood : water} * PEC$ Equation 3.14

Where:

$F_{SS}PC$: Fish steady state plasma concentration [mg L^{-1}]; and

PEC_{SW}: Predicted environmental concentration for surface water [mg L^{-1}].

$RCR = F_{SS}PC/H_TPC$ Equation 3.15

Where:

RCR: Risk characterisation ratio (this was converted from a toxicity exposure ratio in the original work)

$f_n = \frac{1}{1 + 10^{i(pKa - pH)}}$ (Fu et al., 2009) Equation 3.16

$f_d = 1 - f_n$ (Fu et al., 2009) Equation 3.17

where i is 1 for bases and -1 for acids, pK_a is the negative logarithm (log_{10}) of the dissociation constant; pH is 7.

$\log BCF = \log [f_n X\ 10^{(0.64 logKow - 0.12)} + f_d X\ 10^{(0.37 logKow + 0.06 pKa - 0.51)}]$ for acids

(Fu et al., 2009) Equation 3.18

$\log BCF = \log [f_n X 10^{(0.62 logKow - 0.15)} + f_d X 10^{(0.28 logKow - 0.07 pKa + 0.84)}]$ for bases

(Fu et al., 2009) Equation 3.19

$PEC_{FISH} = PEC_{SW} \times BCF_{fish} \times BMF$ (TGD, 2003) Equation 3.20

Where:

PEC_{FISH}: Predicted environmental concentration in fish as food [mg kg^{-1}];

BMF: Biomagnification factor obtained from the technical guidance document (TGD, 2003).

$$C_{EARTHWORM} = \frac{BCFearthworm\ x\ Cporewater + Csoil\ x\ Fgut\ x\ CONVsoil}{1 + Fgut\ x\ CONV\ soil}$$ (TGD, 2003) Equation 3.21

Where:

$C_{EARTHWORM}$ ($PEC_{EARTHWORM}$): Concentration in earthworm on wet weight basis [mg kg^{-1}];

$C_{porewater}$: Concentration in porewater [mg L^{-1}];

C_{SOIL}: Concentration in soil [mg kg^{-1}]

F_{gut}: Fraction of gut loading in worm, 0.1;

$CONV_{SOIL}$: Conversion factor for soil concentration wet to dry weight soil, 1.133, calculated from TGD (TGD, 2003).

$C_{porewater} = PEC_{soil} / (foc_{SOIL} \times K_{oc})$ (TGD, 2003) Equation 3.22

Where

$foc_{SOIL:}$ Fraction of soil organic carbon, 0.02.

$BCF_{EARTHWORM}$ = (0.84 x 0.012 x Log Kow)/ $RHO_{earthworm}$ (TGD, 2003) Equation 3.23

Where:

$BCF_{EARTHWORM:}$ Bioconcentration factor for earthworms [L kg^{-1}]; and

$RHO_{EARTHWORM}$: Density of earthworms (default of 1) [kg L^{-1}].

$PNEC = \frac{EcoTox}{AF}$ (CHMP, 2006) Equation 3.24

Where:

PNEC: Predicted No-effect concentration [mg L^{-1} or mg kg^{-1}];

EcoTox: The most sensitive ecotoxicological data for the aquatic or terrestrial compartment [mg L^{-1} or mg kg^{-1}]; and

AF: Safety factor (acute QSAR data 1000, acute experimental data 100, chronic QSAR data 100, and chronic experimental data, 10 (CHMP, 2006)).

Log $LC_{50\ earthworm}$ = 1.405 – 0.308 Log K_{OW} (TGD, 2003) Equation 3.25

Where:

$LC_{50\ EARTHWORM}$: Acute earthworm ecotoxicity, [mM kg^{-1} dry soil]

$$PNEC_{DW} = \frac{ADI \times BW \times AT}{IngRDW \times EF \times ED}$$ (Schwab et al., 2005) Equation 3.26

Where:

$PNEC_{DW}$: Predicted no-effect concentration through consumption of drinking water [mg];

ADI: Acceptable daily intake, [mg day^{-1}];

BW: Body weight, adult 70 and 14 child, [kg];

AT: ADI averaging time, adult 10950 and child 2190, [days];

$IngR_{DW}$: Water consumption, adult 2 and child 1, [L day^{-1}];

EF: Exposure frequency, adult 350 and child 350, [days $year^{-1}$]; and

ED: Exposure duration, adult 30 and child 6, [years] (Schwab et al., 2005).

Appendix 3

Table A3.1: Extrapolation of chemical recovery (%) for each time points (d)

Tylosin	Chemical recovery (%)			
	1d	2d	3d	4d
Low				
AF	89.54	80.51	72.2	65.91
SL	80	65.15	53.98	45.45
High				
CV	96.39	92.96	89.69	86.57
AF	96.46	93.09	89.87	86.81
SL	92.49	85.72	79.61	74.09
Lincomycin				
Low				
NP	95.14	90.59	86.33	82.34
PT	94.64	89.65	85.02	80.71
Trimethoprim				
Low				
PS	91.39	83.76	76.97	70.93
AF	90.3	81.83	74.43	67.92
SL	90.12	81.51	74	67.43
High				
PS	88.54	78.8	70.49	63.37

Table A3.2: Summary of the effects of tested antibiotics in 1d ecotoxicological biotests. All toxicity values are in µmol L-1 (values in brackets are the range of 95% confidence limits). Seven algal species are *P. subcapitata* (PS), *D. subspicatus* (DS), *C. vulgaris* (CV), *N. pelliculosa* (NP), *P. tricornutum* (PT), *A. flos-aquae* (AF) and *S. leopoliensis* (SL).

Spe.	Lincomycin						Tylosin						Trimethoprim					
	EC_{50}	EC_{10}	EC_5	NOEC	LOEC	Model, r^2	EC_{50}	EC_{10}	EC_5	NOEC	LOEC	model	EC_{50}	EC_{10}	EC_5	NOEC	LOEC	model
PS	n.a	2.16	1.52	135.4	>135.4	Weibull, 0.5	>45,58	n.a	n.a	45.58	>45,58	n.a	>304.99	n.a	n.a	304.99	>304.99	n.a.
DS	>135.4	1.32	1.16	135.44	>135.44	Weibull,0.3	93.79	n.a	n.a	93.79	>93.79	n.a	>344.45	n.a	n.a	>344.45	>344.45	n.a.
CV	>225.73	n.a	n.a	225.73	>225.73	n.a.	>90.41	n.a.	n.a	90.41	>90.41	n.a.	>344.45	n.a	n.a	>344.45	>344.45	n.a.
NP	>225.73	n.a	n.a	225.73	>225.73	n.a	n.a	n.a	n.a	93.79	>93.79	n.a	>275.56	n.a	n.a	275.56	>275.56	n.a
PT	>225.73	n.a.	n.a	225.73	>225.73	n.a	n.a	n.a	n.a	93.79	>93.79	n.a	>344.45	n.a	n.a	344.45	>344.45	n.a
AF	>3.39	n.a.	n.a	3.39	>3.39	n.a	0.34	0.31	0.306	1.35	>1.35	Gompertz, 0.25	>344.45	n.a	n.a	344.45	>344.45	n.a
SL	>3.39	n.a.	n.a	3.39	>3.39	n.a	>1.3	n.a	n.a	1.3	>1.3	n.a	>344.45	n.a	n.a	344.45	>344.45	n.a

n.a. not available. Spe., species.

Table A3.3: Summary of the effects of tested antibiotics in 2d ecotoxicological biotests. All toxicity values are in µmol L-1 (values in brackets are the range of 95% confidence limits). Seven algal species are *P. subcapitata* (PS), *D. subspicatus* (DS), *C. vulgaris* (CV), *N. pelliculosa* (NP), *P. tricornutum* (PT), *A. flos-aquae* (AF) and *S. leopoliensis* (SL).

Spe.	Lincomycin						Tylosin						Trimethoprim					
	EC_{50}	EC_{10}	EC_5	NOEC	LOEC	Model, r^2	EC_{50}	EC_{10}	EC_5	NOEC	LOEC	model	EC_{50}	EC_{10}	EC_5	NOEC	LOEC	model
PS	>135.44	0.12	0.085	135.44	>135.44	Gompertz, 0.06	>45.59	4.14 (n.a-15.2)	1.6	45.59	>45.59	Weibull 0.41	>271.43	n.a	n.a	271.43	>271.43	n.a.
DS	>135.4	0.77	0.078	135.4	>135.4	Weibull0.23	>93.79	n.a	n.a	93.79	>93.79	n.a	>344.45	n.a	n.a	>344.45	>344.45	n.a.
CV	>225.73	n.a	n.a	225.73	>225.73	n.a.	>87.19	n.a.	n.a	87.19	>87.19	n.a.	>344.45	n.a	n.a	>344.45	>344.45	n.a.
NP	>225.73	24.14 (n.a-96.79)	5.7	225.73	>225.73	Weibull 0.31	>93.79	1.56 (n.a-6.43)	0.57	93.79	>93.79	Weibull 0.36	>275.56	7.1	5.4	275.56	>275.56	Gompertz 0.3
PT	>225.73	n.a.	n.a	225.73	>225.73	n.a	>93.79	n.a	n.a	93.79	>93.79	n.a	>344.45	n.a	n.a	344.45	>344.45	n.a
AF	0.16	0.12	0.11	0.14	0.27	Weibull 0.75	>1.31	n.a	n.a	1.31	>1.31	Gompertz 0.27	>344.45	n.a	n.a	344.45	>344.45	n.a
SL	>0.048	n.a	n.a	0.27	0.81	n.a	>1.21	0.29	0.24	0.29	0.56	logistic 0.56	>344.45	n.a	n.a	344.45	>344.45	n.a

n.a. not available. Spe., species.

Table A3.4: Summary of the effects of tested antibiotics in 3d ecotoxicological biotests. All toxicity values are in µmol L-1 (values in brackets are the range of 95% confidence limits). Seven algal species are *P. subcapitata* (PS), *D. subspicatus* (DS), *C. vulgaris* (CV), *N. pelliculosa* (NP), *P. tricornutum* (PT), *A. flos-aquae* (AF) and *S. leopoliensis* (SL).

Spe.	Lincomycin						Tylosin						Trimethoprim					
	EC_{50}	EC_{10}	EC_5	NOEC	LOEC	Model, r^2	EC_{50}	EC_{10}	EC_5	NOEC	LOEC	model	EC_{50}	EC_{10}	EC_5	NOEC	LOEC	model
PS	>135.44	9.4	8.45	4.06	12.19	Gompertz, 0.82	>45.58	n.a	n.a	1.69	5.06	Gompertz 0.81	>242.8	n.a	n.a	242.8	>242.8	n.a.
DS	100.2	0.19 (70.3.-n.a)	n.a	73.14	109.7	Gompertz 0.79	17.49 (2.74-n.a)	4.59	3.5	<9.38	9.38	Sigmoid 0.76	>344.45	n.a	n.a	344.45	>344.45	n.a.
CV	>225.73	n.a	n.a	225.73	>225.73	n.a.	>84.12	n.a.	n.a	84.12	>84.12	n.a.	>344.45	n.a	n.a	344.45	>344.45	n.a.
NP	>225.73	5.7 (1.09-24.14)	1.09	70.16	121.9	Weibull 0.48	1.82 (1.36-2.74)	0.9	0.78	1.13	1.88	Chapman 0.88	9.4 (7.56-12.95)	1.9	1.15	2.07	4.13	Chapman 0.93
PT	>225.73	n.a.	n.a	225.73	>225.73	n.a	>93.79	3.66	2	50.65	75.03	Chapman	195.2	9.7	4.1	344.45	>344.45	Chapman

								(0.9-12.42				0.55	(53.55-n.a)					0.51
								)										
AF	0.16	0.1	0.09	0.045	0.14	Hill	0.094	0.071	0.06	0.082	0.303	Chapman	>344.45	254	252.62	344.45	>344.45	Logistic
	(0.13-					0.94			7			0.84						0.34
	0.21)																	
SL	0.78	0.048	0.045	0.045	0.14	Weibull	0.22	0.03	0.01	0.061	0.27	Weibull	>344.45	n.a	n.a	344.45	>344.45	n.a
	(0.29-					0.8	(0.1-		4			0.81						
	n.a)						n.a)											

n.a. not available. Spe., species.

Table A3.5: Regression models used to derive concentration-response curves of algal species for each antibiotic tests. Seven algal species are *P. subcapitata* (PS), *D. subspicatus* (DS), *C. vulgaris* (CV), *N. pelliculosa* (NP), *P. tricornutum* (PT), *A. flos-aquae* (AF) and *S. leopoliensis* (SL).

Chemicals	species	3d Equation	Parameters	4d Equation	Parameter
LIN	PS	Gompertz f = a*exp(-exp(-(x-x0)/b))	a=45.6875, b=2.4874, x0=10.4211	Weibull f = if(x<=x0-b*ln(2)^(1/c), 0, a*(1-exp(-(abs(x-x0+b*ln(2)^(1/c))/b)^c)))	a=70.8708, b=11.6837, c=0.6998, x0=7.9763
	DS	Gompertz f = a*exp(-exp(-(x-x0)/b))	a=60.4505, b=42.1549, c=30.5704	Weibull f = if(x<=x0-b*ln(2)^(1/c), 0, a*(1-exp(-(abs(x-x0+b*ln(2)^(1/c))/b)^c)))	a=86.2329,b=20.9654, c=0.5501, x0=10.768
	CV	n.a	n.a	n.a	n.a
	NP	Weibull f = if(x<=x0-b*ln(2)^(1/c), 0, a*(1-exp(-(abs(x-x0+b*ln(2)^(1/c))/b)^c)))	A=47.0386 b=111.0582, c=0.4658, x0=50.5641	Gompertz f = a*exp(-exp(-(x-x0)/b))	a=21.6183, b=36.7259 X0=35.0287
	PT	n.a	n.a	n.a	n.a
	AF	Hill, f = a*x^b/(c^b+x^b)	a=88.8648, b=5.6126, c=0.1499	Weibull f = if(x<=x0-b*ln(2)^(1/c), 0, a*(1-exp(-(abs(x-x0+b*ln(2)^(1/c))/b)^c)))	A=93.7185, b=0.1588, c=1.3006, d=0.1191
	SL	Weibull f = if(x<=x0-b*ln(2)^(1/c), 0, a*(1-exp(-(abs(x-x0+b*ln(2)^(1/c))/b)^c)))	A=60.7292, b=0.1935, c=0.4137, x0=0.124	Hill f = a*x^b/(c^b+x^b)	a=75.0196, b=1.6001, c=0.0631
TYN	PS	Gompertz f = a*exp(-exp(-(x-x0)/b))	A=47.4673, b=0.0321,	Gompertz f = a*exp(-exp(-(x-x0)/b))	a=71.2543, b=1.8516, x0=2.1838

			c=2.1602		
	DS	Sigmoid f = a/(1+exp(-(x-x0)/b))	A=50.043, b=1.5414, c=6.8725	Chapman f = a*(1-exp(-b*x))^c	a=72.2032, b=0.192, c=3.1928
	CV	n.a	n.a	n.a	n.a
	NP	Chapman f = a*(1-exp(-b*x))^c	A=59.5793, b=2.568, c=16.9521	Chapman f = a*(1-exp(-b*x))^c	a=73.5982, b=3.2995, c=30.9286
	PT	Chapman f = a*(1-exp(-b*x))^c	a=46.7361, b=0.1217, c=1.4524	Hill f = a*x^b/(c^b+x^b)	a=70.4456, b=0.8089, c=1.8967
	AF	Chapman f = a*(1-exp(-b*x))^c	A=81.5495 b=64.4489, c=204.26	Hill f = a*x^b/(c^b+x^b)	A=95.3012, b=1.4859 c=0.085
	SL	Weibull f = if(x<=x0-b*ln(2)^(1/c), 0, a*(1-exp(-(abs(x-x0+b*ln(2)^(1/c))/b)^c)))	a=54.5819, b=0.1313, c=1.5378, d=0.0881	Chapman f = a*(1-exp(-b*x))^c	a=80.9504, b=9.9407, c=0.9057
TMP	PS	n.a	n.a	n.a	n.a
	DS	n.a	n.a	n.a	n.a
	CV	n.a	n.a	n.a	n.a
	NP	Chapman f = a*(1-exp(-b*x))^c	a=71.0205, b=0.1677, c=1.5051	Chapman f = a*(1-exp(-b*x))^c	a=85.532, b=0.4897, c=18.7533
	PT	Chapman f = a*(1-exp(-b*x))^c	a=52.2532, b=0.0152,	Chapman f = a*(1-exp(-b*x))^c	a=71.9952, b=0.022, c=1.6949

		c=0.8351		
AF	Logistic f = if(x<=0, if(b<0,0,a), if(b>0, a/(1+abs(x/x0)^b), a*abs((x/x0))^(abs(b))/(1+(abs(x/x0))^(abs(b))))) r2=0.34	a=38.3014, b=-158.5402 X0=255.6558	Logistic f = if(x<=0, if(b<0,0,a), if(b>0, a/(1+abs(x/x0)^b), a*abs((x/x0))^(abs(b))/(1+(abs(x/x0))^(abs(b)))))	a=12744.8414 b=-1.0041 x0=78305.514
SL	n.a	n.a	Sigmoid f = a/(1+exp(-(x-x0)/b))	a=41.7517 b=83.6159 x0=193.8341

Appendix 4

Table A4.1: Extrapolation of chemical recovery (%) for each time points (d)

Tylosin	Chemical recovery (%)			
	1d	2d	3d	4d
Low				
DS	94.19	88.82	83.86	79.27
NP	90.18	81.63	74.15	67.61
AF	89.54	80.51	72.7	65.91
High				
PS	95.67	91.59	87.74	84.11
DS	96.62	93.4	90.32	87.37
NP	95.2	90.7	86.48	82.53
AF	96.46	93.09	89.87	86.81
Lincomycin				
Low				
PS	94.92	90.18	85.76	81.63
NP	94.15	88.75	83.77	79.16
High				
NP	90.39	82	74.64	68.18
Trimethoprim				
Low				
PS	78.57	63.03	51.59	43.03
AF	90.3	81.83	74.42	67.92
High				
PS	85.43	73.62	63.98	56.07

Table A4.2: Regression models used to derive concentration-response curves for each antibiotics.

Chemicals	species	Equation based on the endpoint of growth	Parameters	Equation based on the endpoint of photosynthesis	Parameter
LIN	PS	**Logistic** f = if(x<=0, if(b<0,0,a), if(b>0, a/(1+abs(x/x0)^b), a*abs((x/x0))^(abs(b))/(1+(abs(x/x0))^(abs(b))))) R^2=0.99	a=85.8243 b=-1.5259 c=19.5198	**Hill** f = a*x^b/(c^b+x^b) R^2=0.84	A=103.429 B=0.9965 C=12.7343
	DS	**Hill** f = a*x^b/(c^b+x^b) R^2=0.89	a=56.1954 b=1.1138 c=37.3791	**Chapman** f = a*(1-exp(-b*x))^c R^2=0.92	A=101.6327 B=0.0035 C=0.501
	AF	**Weibull** f = if(x<=x0-b*ln(2)^(1/c), 0, a*(1-exp(-(abs(x-x0+b*ln(2)^(1/c))/b)^c))) R^2=0.95	A=65.8625 B=2.1045 C=3.5157 X0=0.7774	**Hill** f = a*x^b/(c^b+x^b) R^2=0.4	A=56.5253 B=0.6066 C=0.1657
	NP	n.a	n.a	n.a	n.a
TYN	PS	**Weibull** f = if(x<=x0-b*ln(2)^(1/c), 0, a*(1-exp(-(abs(x-x0+b*ln(2)^(1/c))/b)^c))) R^2=0.99	a=82.5802 b=4.5983 c=1.1741 x0=3.855	**Weibull** f = if(x<=x0-b*ln(2)^(1/c), 0, a*(1-exp(-(abs(x-x0+b*ln(2)^(1/c))/b)^c))) R^2=0.91	a=94.2789 b=1.366 c=0.6643 d=1.9873
	DS	**Weibull** f = if(x<=x0-b*ln(2)^(1/c), 0, a*(1-exp(-(abs(x-x0+b*ln(2)^(1/c))/b)^c))) R^2=0.92	a=96.6334 b=55.9902 c=0.833 x0=35.9337	**Sigmoid** f = a/(1+exp(-(x-x0)/b)) R^2=0.74	A=67.5547 B=2.6127 X0=14.8269
	AF	**Chapman** f = a*(1-exp(-b*x))^c R^2=0.95	a=75.3458 b=72.5115 c=29.2359	**Gompertz** f = a*exp(-exp(-(x-x0)/b)) R^2=0.76	A=103.8338 B=0.2401 C=0.2535

					D=0.2352
	NP	**Logistic** f = if(x<=0, if(b<0,0,a), if(b>0, a/(1+abs(x/x0)^b), a*abs((x/x0))^(abs(b))/(1+(abs(x/x0))^(abs(b))))) R^2=0.99	a=73.3678 b=-1.0844 c=2.1734	**Chapman** f = a*(1-exp(-b*x))^c R^2=0.73	A=73.3619 B=0.1006 C=0.5764
TMP	PS	n.a	n.a	n.a	n.a
	DS	n.a	n.a	n.a	n.a
	AF	n.a	n.a	n.a	n.a
	NP	**Chapman** f = a*(1-exp(-b*x))^c R^2= 0.94	a=70.4873 b=0.0276 c=2.4677	**Chapman** f = a*(1-exp(-b*x))^c R^2= 0.54	A=98.6262 B=0.0152 C=5.0824

Appendix 5

Predicted environmental concentration (PEC) (μmol L^{-1}) was calculated to determine the ratios of antibiotics in the mixture study (Equation 6.1)(EMEA, 2008)

$$PEC_{surface}=\frac{380.46 \times SOL \times D \times AD \times BW \times P \times Fh}{Ny \times H \times MW \times (VP \times MW + 2369.49 \times SOL + 355.42Koc)} \quad \text{Equation 6.1}$$

Where D = Daily dose of the active ingredient [$mg.kg_{bw}^{-1}.day^{-1}$]; Ad = Number of days of treatment [d]; BW = Animal body weight [kg_{bw}], calves 140kg, cattle 450kg and pig 12.5kg; P = Animal turnover rate per place per year [$place^{-1}.year^{-1}$], calves 1.8, cattle 1 and pig 6.9; Fh = Fraction of herd treated, 1 for antibiotics (feed and water medication) and 0.5 for antibiotics (injectable); Ny= Nitrogen produced in one year per place [$kg.N.place^{-1}.year^{-1}$], calves 10, cattle 35 and pig 2.25; H = housing factor, calves 1, cattle 0.5 and pig 1; VP = Vapour pressure [Pa]; MW = Molar mass [$g.mol^{-1}$]; SOL = Water solubility [$mg.L^{-1}$]; Koc = water-organic carbon distribution coefficient [1.kg]. Information on the daily dose of the active ingredient and number of days of treatment was obtained from the Compendium of Data Sheet for Animal Medicines (NOAH, 2011). Vapour pressure, water solubility and Koc were estimated by using the Environment Protection Agency EPI Suite (4.1 version) (EPA, 2013).

Equation 6.1 derivation:

The predicted environmental concentration in soil-initial, PECsoil-initial [$\mu g.kg^{-1}$] was calculated according to EMEA veterinary medicines and inspections guideline (2008) by Equation 6.6:

$$PEC_{soil\text{-}initial} = \left(\frac{D \times Ad \times BW \times P \times 170 \times Fh}{1500 \times 10000 \times 0.05 \times Ny \times H}\right) \times 1000 \quad \text{Equation 6.6}$$

Where

D = Daily dose of the active ingredient [mg.kgbw^{-1}.d^{-1}]

AD = Number of days of treatment [d]

BW = Animal body weight [kgbw]; Calves 140kg, cattle 450kg and pig 12.5kg.

P = Animal turnover rate per place per year [place^{-1}.y^{-1}]; Calves 1.8, cattle 1 and pig 6.9.

Fh = Fraction of herd treated; 1 for antibiotics (feed and water medication) and 0.5 for antibiotics (injectable).

Ny = Nitrogen produced in one year per place [kg.N.place^{-1}.y^{-1}]; Calves 10, cattle 35 and pig 2.25.

H = housing factor; Calves 1, cattle 0.5 and pig 1.

The concentration in pore$_{water}$, PEC$_{porewater}$ (equals PEC$_{groundwater}$ [µg.L^{-1}]) was calculated by Equation 6.7:

$$PEC_{porewater} = PEC_{groundwater} = \frac{PECsoil \times RHOsoil}{Ksoil-water \times 1000} \quad \text{Equation 6.7}$$

Where $K_{soil\text{-}water}$ was calculated by Equation 6.8

$$K_{soil\text{-}water} = (F_{airsoil} \times K_{air\text{-}water}) + F_{watersoil} + (F_{solidsoil} \times \frac{Kpsoil}{1000} \times RHO_{solid}) \quad \text{Equation 6.8}$$

Where $K_{air\text{-}water}$ and Kp_{soil} are calculated by Equations 6.9 & 6.10

$$K_{air\text{-}water} = \frac{VP \times MW}{SOL \times R \times TEMP} \quad \text{Equation 6.9}$$

$$Kp_{soil} = Foc_{soil} \times K_{oc} \quad \text{Equation 6.10}$$

Where

RHO_{soil} = Bulk density of fresh soil [1700 kg.m^{-3}]

RHO_{solid} = Density of soil solids [2500 kg.m^{-3}]

$F_{airsoil}$ = Fraction of air in soil [0.2$m^3.m^{-3}$]

$F_{watersoil}$ = Fraction of solids in soil [0.2$m^3.m^{-3}$]

$F_{solidsoil}$ = Fraction of solids in soil [0.6$m^3.m^{-3}$]

F_{ocsoil} = Weight of organic carbon in soil [0.02$kg.kg^{-1}$]

TEMP = Temperature at air-water interface [285K]

R = Gas constant [8.314 $Pa.m^3.mol^{-1}.K^{-1}$]

VP = Vapour pressure [Pa]

MW = Molar mass [$g.mol^{-1}$]

SOL = Water solubility [$mg.L^{-1}$]

$K_{soil\text{-}water}$ = Partition coefficient of solids and water in soil (v/v) [$m^3.m^{-3}$]

K_{psoil} = Partition coefficient of solids and water in soil (v/w) [$L.kg^{-1}$]

$K_{air\text{-}water}$ = Partition coefficient of air and water in soil [$m^3.m^{-3}$]

K_{oc} = Water-organic carbon distribution coefficient [L.kg]

PEC_{soil} = PEC_{soil} is the $PEC_{soil\text{-}initial}$ calculated based on a mixing depth of 20 cm in the soil, namely $PEC_{soil\text{-}initial}/4$ [$ug.kg^{-1}$]

Finally, the predicted environmental concentration in surfacewater, $PEC_{surfacewater}$ [$\mu g.L^{-1}$] was calculated by Equation 6.11:

$$PEC_{surfacewater} = \frac{PECporewater}{3} \quad \text{Equation 6.11}$$

Equation 6.12 was calculated by combining the default values derived from Equations 6.6 – 6.11.

$$PEC_{surfacewater} = \frac{380.46 \times SOL \times D \times AD \times BW \times P \times Fh}{Ny \times H \times (VP \times MW + 2369.49 \times SOL + 355.42Koc)} \quad \text{Equation 6.12}$$

Equation 6.1 converted the unit of $PEC_{surfacewater}$ from μg L^{-1} in Equation 12 to μmol L^{-1} by dividing the chemical molar mass [g.mol^{-1}]

$$PEC_{surfacewater} = \frac{380.46 \times SOL \times D \times AD \times BW \times P \times Fh}{Ny \times H \times \mathbb{MW} \times (VP \times MW + 2369.49 \times SOL + 355.42Koc)} \quad \text{Equation 6.1}$$

Table A5.1 Risk assessment of three veterinary antibiotics based on maximum application rate prediction. TWAEC is in μmol L^{-1}.

Scenario	TMP		TYN		LIN	
	TWAEC	PEC/PNEC	TWAEC	PEC/PNEC	TWAEC	PEC/PNEC
D1 ditch	0.026	0.0092	0.0094	7.18	$7.06x10^{-5}$	0.05
D1 stream	0.016	0.0057	0.0059	4.49	$4.72x10^{-5}$	0.034
D2 ditch	0.024	0.0085	0.011	8.45	0.00075	0.53
D2 stream	0.014	0.005	0.0064	4.88	0.00035	0.25
D4 pond	0.0012	0.000403	0.0012	0.88	$8.73x10^{-5}$	0.062
D4 stream	0.0015	0.00054	0.0011	0.81	0.00012	0.083
D5 pond	0.0019	0.00059	0.002	1.5	0.00038	0.27
D5 stream	0.0011	0.00041	0.0018	1.41	0.00024	0.17
D6 ditch	0.003	0.00104	0.0025	1.94	0.00016	0.11
R1 pond	0.016	0.0054	0.0061	4.61	0.0004	0.28
R1 stream	0.031	0.011	0.015	11.68	0.004	2.88
R3 stream	0.084	0.029	0.073	55.83	0.011	8
R4 stream	0.044	0.015	0.027	20.4	0.00011	0.081

PNEC was calculated by PNEC = EC_{50}/ AF. The 96 h EC_{50} of trimethoprim, tylosin and lincomycin to *A. flos-aquae* were 285.75, 0.13 and 0.14 μmol L^{-1}, respectively; AF=100.

Table A5.2 Risk assessment of three veterinary antibiotics based on medium application rate prediction. TWAEC is in µmol L^{-1}.

Scenario	TMP		TYN		LIN	
	TWAEC	PEC/PNEC	TWAEC	PEC/PNEC	TWAEC	PEC/PNEC
D1 ditch	0.0028	0.00098	0.0013	1.01	$3.98x10^{-5}$	0.028
D1 stream	0.0017	0.00061	0.00083	0.63	$2.66\ x10^{-5}$	0.019
D2 ditch	0.0026	0.0009	0.0016	1.19	0.00042	0.3
D2 stream	0.0015	0.00053	0.0009	0.69	0.0002	0.14
D4 pond	0.00012	$4.3\ x10^{-5}$	0.00016	0.12	$4.92x10^{-5}$	0.035
D4 stream	0.00016	$5.71\ x10^{-5}$	0.00015	0.11	$6.52x10^{-5}$	0.047
D5 pond	0.00018	$6.3\ x10^{-5}$	0.00028	0.21	0.00022	0.15
D5 stream	0.00013	$4.4\ x10^{-5}$	0.00026	0.2	0.00013	0.095
D6 ditch	0.00033	0.00011	0.00036	0.27	$8.88x10^{-5}$	0.063
R1 pond	0.0017	0.00058	0.00086	0.65	0.00022	0.16
R1 stream	0.0034	0.0012	0.0022	1.65	0.0023	1.62
R3 stream	0.009	0.0031	0.0104	7.89	0.0063	4.51
R4 stream	0.0047	0.0017	0.0038	2.88	$6.37x10^{-5}$	0.046

PNEC was calculated by PNEC = EC_{50}/ AF. The 96 h EC_{50} of trimethoprim, tylosin and lincomycin to *A. flos-aquae* were 285.75, 0.13 and 0.14 µmol L^{-1}, respectively; AF=100.

Table A5.3 Risk assessment of three veterinary antibiotics based on minimum application rate prediction. TWAEC is in µmol L^{-1}.

Scenario	TMP		TYN		LIN	
	TWAEC	PEC/PNEC	TWAEC	PEC/PNEC	TWAEC	PEC/PNEC
D1 ditch	0.00011	$3.77\ x10^{-5}$	$7.31x10^{-5}$	0.056	$1.01x10^{-5}$	0.0072
D1 stream	$6.72\ x10^{-5}$	$2.35\ x10^{-5}$	$4.57x10^{-5}$	0.035	$6.74x10^{-6}$	0.0048
D2 ditch	$9.92\ x10^{-5}$	$3.47\ x10^{-5}$	$8.62x10^{-5}$	0.066	0.00011	0.076
D2 stream	$5.86\ x10^{-5}$	$2.05\ x10^{-5}$	$4.98x10^{-5}$	0.038	$4.99x10^{-5}$	0.036
D4 pond	$4.72\ x10^{-6}$	$1.65\ x10^{-6}$	$9x10^{-6}$	0.0069	$1.25x10^{-5}$	0.0089
D4 stream	$6.27\ x10^{-6}$	$2.19\ x10^{-6}$	$8.27x10^{-6}$	0.0063	$1.66x10^{-5}$	0.012
D5 pond	$6.92\ x10^{-6}$	$2.42\ x10^{-6}$	$1.53x10^{-5}$	0.012	$5.49x10^{-5}$	0.039
D5 stream	$4.82\ x10^{-6}$	$1.69\ x10^{-6}$	$1.43x10^{-5}$	0.011	$3.39x10^{-5}$	0.024
D6 ditch	$1.22\ x10^{-5}$	$4.26\ x10^{-6}$	$1.98x10^{-5}$	0.015	$2.26x10^{-5}$	0.016
R1 pond	$6.37\ x10^{-5}$	$2.23\ x10^{-5}$	$4.7x10^{-5}$	0.036	$5.66x10^{-5}$	0.04
R1 stream	0.00013	$4.51\ x10^{-5}$	0.00012	0.091	0.00058	0.41
R3 stream	0.00034	0.00012	0.00057	0.43	0.0016	1.14
R4 stream	0.00018	$6.35\ x10^{-5}$	0.00021	0.16	$1.62x10^{-5}$	0.012

PNEC was calculated by PNEC = EC_{50}/ AF. The 96 h EC_{50} of trimethoprim, tylosin and lincomycin to *A. flos-aquae* were 285.75, 0.13 and 0.14 µmol L^{-1}, respectively; AF=100.

Table A5.4 Regressions used to derive concentration-response curves of A. flos-aquae for each antibiotic.

Chemicals	Equation based on the endpoint of growth	Parameters
Tylosin	**Hill** f = a*x^b/(c^b+x^b) R^2=0.93	a=90.808
		b=1.9117
		c=0.1126
Lincomycin	**Hill** f = a*x^b/(c^b+x^b) R^2=0.94	a=92.085
		b=2.2755
		c=0.1251
Trimethoprim	**Sigmoid** f = a/(1+exp(-(x-x0)/b)) R^2=0.88	a=65.4152
		b=86.5556
		X0=184.2818

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