ENVIRONMENTAL RISK ASSESSMENT OF VETERINARY MEDICINES USED IN ASIAN AQUACULTURE

Andreu Rico

Environmental risk assessment of veterinary medicines used in Asian aquaculture

Andreu Rico

Thesis committee

Thesis supervisor
Prof. Dr. Ir. Paul J. van den Brink
Professor of Chemical Stress Ecology
Wageningen University

Other members
Prof. Dr. Johan A. J. Verreth, Wageningen University
Prof. Dr. Jonas S. Gunnarsson, Stockholm University, Sweden
Dr. Ir. Cornelis A. M. van Gestel, VU University Amsterdam, The Netherlands
Dr. Ir. Jeroen B. Guinée, Leiden University, The Netherlands

This research was conducted under the auspices of the Graduate School for Socio-Economic and Natural Sciences of the Environment (SENSE)

Environmental risk assessment of veterinary medicines used in Asian aquaculture

Andreu Rico

Thesis
Submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr. M. J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Monday 26 May 2014
at 11.00 am in the Aula.

Andreu Rico
Environmental risk assessment of veterinary medicines used in Asian aquaculture,
216 pages

PhD Thesis, Wageningen University, Wageningen, NL (2014)
With references, with summary in English, Dutch and Spanish

ISBN 978-94-6173-956-8

-

To Neus and to my family

''The chemicals to which life is asked to make its adjustments are no longer merely the calcium and silica and copper and all the rest of minerals washed out of the rocks and carried in rivers to the sea; they are the synthetic creations of man's inventive mind, brewed in his laboratories, and having no counterparts in nature.''

(Rachel Carson, 1962)

Contents

Chapter 1. General introduction 11

Chapter 2. Use of chemicals and biological products in Asian aquaculture and their potential environmental risks: a review 19

Chapter 3. Use of veterinary medicines, feed additives and probiotics in four major internationally traded aquaculture species farmed in Asia 37

Chapter 4. Modelling environmental and human health risks of veterinary medicinal products applied in pond aquaculture 55

Chapter 5. Probabilistic risk assessment of veterinary medicines applied to four major aquaculture species produced in Asia 71

Chapter 6. Use, fate and ecological risks of antibiotics applied in tilapia cage farming in Thailand 89

Chapter 7. Ecological risk assessment of the antibiotic enrofloxacin applied to *Pangasius* catfish farms in the Mekong delta, Vietnam 109

Chapter 8. Effects of the antibiotic enrofloxacin on the ecology of tropical eutrophic freshwater microcosms 125

Chapter 9. Predicting antibiotic resistance in aquaculture production systems and surrounding environments 145

Chapter 10. General discussion and conclusions 165

References. 179

Summary 195

Samenvatting (Dutch summary) 199

Resumen (Spanish summary) 203

Acknowledgements 207

About the author 211

Chapter 1

General introduction

1. Background

Aquaculture - the cultivation of fish, crustaceans, molluscs and water plants - has dramatically evolved during the last 30 years, becoming the fastest-growing animal food-producing sector in the world (FAO, 2012a). Nowadays, nearly 90% of the global aquaculture production is produced in Asia, with China being by far the largest producer (Fig. 1). Recent technological advances such as (1) the use of fertilizers and industrial feeds, (2) the use of electricity for water exchange management and aeration, (3) the distribution of genetically improved and pathogen free seed, and (4) the use of therapeutants and other chemicals for controlling water quality and disease outbreaks, have been introduced along the main aquaculture producing regions of Asia (Bostock et al., 2010; De Silva and Davy, 2010). These technological advances have facilitated the expansion and intensification of Asian aquaculture, leading to the so-called 'blue revolution' (Costa-Pierce, 2002), a process analogous to the Green Revolution experienced by the agriculture sector during the second half of the last century, which requires assessing a range of environmental issues, including water pollution and degradation of ecosystems (Bush et al., 2013).

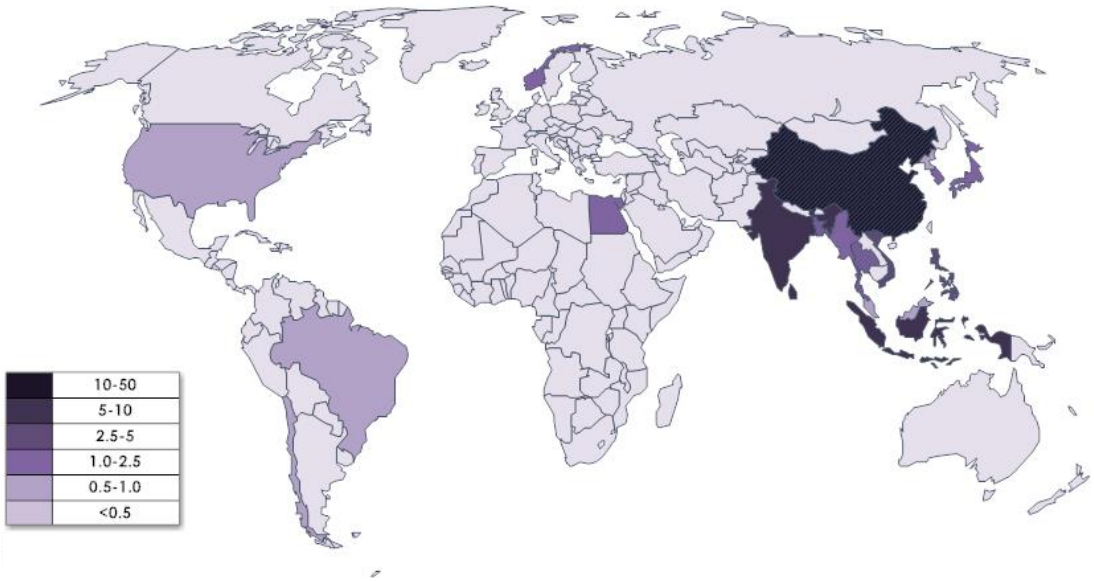


Figure 1. Aquaculture production quantities in 2010 (Mt). Data source: FAO (2012b).

The majority of the Asian aquaculture is produced in inland systems such as excavated ponds and net pens or cages suspended in rivers and reservoirs, and discharge their effluents into surrounding freshwater and brackish water ecosystems (Bostock et al., 2010; Fig. 2). Effluent discharges from intensive and semi-intensive aquaculture facilities may contain high loads of nutrients and potentially toxic chemicals such as heavy metals and veterinary medicines. The increasing pressure of aquaculture production on surrounding aquatic ecosystems has been largely criticised because of their potential negative impacts on biodiversity (Primavera, 2006; De Silva, 2012; Diana, 2012), and important ecosystem services such as nutrient cycling and the provision of water and food (Beveridge et al., 1997; Soto et al., 2008). The contamination of streams and rivers may also result in negative impacts for aquaculture farmers since these

constitute their main source of input water for their production activities, and excessive water quality deterioration may be responsible for higher disease outbreaks and unaffordable economic losses (Bondad-Reantaso et al., 2005). The sustainable development of the Asian aquaculture industry demands an increased awareness of its environmental impacts as well as a greater commitment of science to develop the knowledge and tools required to quantify and minimize those impacts.

Figure 2. Main aquaculture production systems in Asia include earthen ponds (left) and floating cages in freshwater rivers and reservoirs (right), which are hydrologically connected with the surrounding aquatic environment.

2. Veterinary medicines used in Asian aquaculture

The proliferation of disease outbreaks in Asian aquaculture and the need to increase biosecurity and prevent unaffordable mortality losses has led to the introduction of a wide range of veterinary medicines (Bondad-Reantaso et al., 2005). Veterinary medicines are substances used for treating or preventing animal diseases, or for modifying physiological functions in animals[1]. Veterinary medicines reported to be used in Asian aquaculture include antibiotics, antifungals, anaesthetics, anthelmintics, parasiticides, hormones and growth promoters (Boyd and Massaut, 1999; Gräslund and Bengtsson, 2001; Le and Munekage, 2004; Sapkota et al., 2008; Bosma et al., 2009). They constitute a very diverse group of compounds with varied origin (e.g. synthetized by microorganisms, produced by plants, naturally occurring chemicals, synthetized by humans) and with specific modes of action. With the exception of some injectable anaesthetics, the majority of the veterinary medicines used in Asian aquaculture are administered either mixed with feed or directly to water. Aquaculture medicines may enter the environment by a number of different routes. For example, antibiotics applied mixed with feed to fish cages can enter the environment via fish faeces and urine, by the dissolution from feed, or by the sinking of uneaten feeds. Veterinary medicines applied to ponds may enter the environment via effluent discharges, by the environmental or agricultural deposition of sludge, and to a lesser extent by volatilization, percolation or leaching from aquaculture facilities (Boxall et al., 2004; Kümerer, 2009). Some studies have demonstrated that veterinary medicines applied to confined Asian aquaculture facilities constitute an important source of environmental pollution (Le and Munekage, 2004; Zou et al., 2011; Barbosa et al., 2013). For example, residues of 17α-methyl-testosterone, a hormone used to induce sex-reversal in tilapia hatcheries, have been detected in water samples collected in the surroundings of aquaculture facilities in Thailand (Barbosa et al., 2013), and antibiotics used in shrimp and fish ponds of Vietnam and China have been measured at relatively high concentrations in water and sediments of down-stream aquatic ecosystems (Le and Munekage,

[1] When referring to veterinary medicines within this thesis, terms like chemotherapeutants, drugs, chemicals, compounds and substances are used interchangeably.

2004; Zou et al., 2011). Some of the chemotherapeutants used in Asian aquaculture have been classified as highly hazardous for the environment and show high toxicity potential for non-target aquatic organisms (Boyd and Massaut, 1999; Gräslund and Bengtsson, 2001). To date, the number of available studies reporting the environmental contamination and the environmental impacts of aquaculture medicines in Asia is very limited, and has been restricted to qualitative descriptions (e.g. Gräslund and Bengtsson, 2001). The expansion and intensification of Asian aquaculture suggests that the environmental pollution with veterinary medicines and their contribution to biodiversity loss and water quality deterioration are likely to become more evident in the near future, and therefore research and guidance for assessing and managing their environmental risks is urgently required.

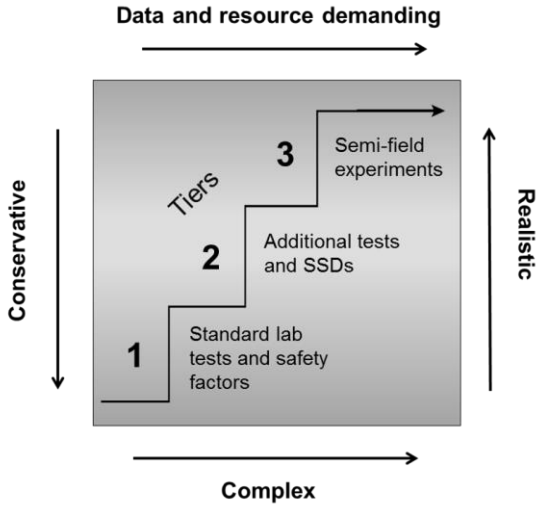


Figure 3. Conceptual framework showing the tiered approach used in the environmental effect assessment of chemicals. Lower tiers require less data and are more conservative than higher tiers. Higher tiers rely on more costly and complex experiments, and show a higher level of ecological realism. Adapted with permission from Solomon et al. (2008).

3. Environmental risk assessment of aquaculture medicines

Environmental Risk Assessment (ERA) can be defined as the estimation of the likelihood and extent of an adverse effect to occur in ecosystems as a result of an exposure to a chemical or a mixture of chemicals. The ERA usually entails three different phases: the exposure assessment, the effect assessment, and the risk characterization phase (Van Leeuwen and Vermeire, 2007). The exposure assessment uses chemical-related data and empirical environmental data, in combination with established environmental fate models or analytical measurements, to describe chemical exposure patters in different environmental matrices such as water or sediments (Beltman et al., 1996; Koelmans et al., 2001). The effect assessment aims at the estimation of the relationship between the dose or level of chemical exposure and the incidence of a particular physiological or ecological effect in response to this exposure. Because it is impossible to assess the effects of chemicals on all species and all processes occurring in ecosystems, a tiered approach is normally used in prospective ERAs (Fig. 3). Such tiered approach uses different methods to extrapolate chemical effects from low (individual level) to high levels of biological organization (populations, communities, ecosystem). Normally, first-tier evaluations are based on the assessment of the chemical effects on a limited number of standard test species and on the use of assessment factors to derive safe environmental concentrations (Brock et al., 2006). In intermediate tiers, the effects of the chemical are assessed on a larger number of species and probabilistic methods, such as the Species Sensitivity Distribution (SSD) approach, are used to estimate safe environmental concentrations (Posthuma et al., 2002). Higher-tiers normally predict safe environmental concentrations at the population and community level by evaluating their sensitivity at more realistic exposure patterns in model ecosystem experiments (micro- and

mesocosms), which can be complemented by ecological model computer simulations (Van den Brink et al., 2002; Van den Brink et al., 2005; Galic et al., 2010). Finally the risk characterization phase combines the output of the exposure and effect assessments in order to provide an estimation of the environmental risk, which is usually expressed as a risk quotient or as a risk probability (Solomon et al., 2000; Brock et al., 2006; Van Leeuwen and Vermeire, 2007).

The ERA of aquaculture medicines in countries of Europe, USA, Japan, Australia and New Zealand is performed under the framework generated by the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Products (VICH). This framework establishes physico-chemical and ecotoxicological data requirements for the evaluated compounds, and comprises a tiered approach supported by several guidance documents for the calculation of environmental exposure and ecological effect thresholds (VICH, 2000; VICH, 2004; EMEA, 2008). Such an approach guarantees that veterinary medicines authorized for their use in aquaculture have been subject to a standard and conservative ERA that ensures no or acceptable negative effects on the environment when used according to recommendations. This situation substantially differs from the way this issue has been approached in the majority of the aquaculture producing countries in Asia, where lists of approved or banned aquaculture chemicals are often based on international food safety hazards and are not subjected to any kind of formal environmental scrutiny.

4. Towards the environmental risk assessment of aquaculture medicines in Asia

The ERA for veterinary medicines used in Asian aquaculture firstly requires the procurement of precise and up-to-date information (i.e., dosages and modes of application) on the active ingredients that are being used in different countries and in different phases of the aquaculture production cycle (e.g. hatcheries, nurseries, grow-out). This information is essential for performing first-tier, preliminary risk assessments and for the prioritization of aquaculture scenarios and compounds that require further (semi-)field evaluations.

Secondly, the risk assessment of aquaculture medicines requires the evaluation of chemical exposure patterns in ecosystems potentially impacted by aquaculture pollution. Several studies have monitored antibiotics and parasiticide residues in water and sediments surrounding cages and net pens anchored along the coasts of major salmon producing countries such as Ireland, Scotland, Norway and Chile (e.g. Samuelsen et al., 1992; Coyne et al., 1994; Telfer et al., 2006; Buschmann et al., 2012), and fewer studies are available reporting concentrations of antibiotics in streams impacted by freshwater aquaculture (e.g. Thurman et al., 2002; Lalumera et al., 2004). Differences in aquaculture species, production systems, compounds, and environmental characteristics between the published literature and the Asian aquaculture scenarios make it imperative to develop Asian specific exposure assessments. The development of chemical fate models will help in this task and will overcome the technical and economic limitations of some developing countries of Asia to perform extensive field monitoring campaigns.

Thirdly, veterinary medicines applied in Asian aquaculture comprise a set of compounds with varied toxicological mode of action on non-target aquatic organisms, some of which are still relatively unknown. During the last decades, the great bulk of the ecotoxicological research into the effects of chemicals on aquatic ecosystems has focused on the assessment of agricultural pesticides - some of them also used in aquaculture - through laboratory and semi-field experiments (see reviews by Van Wijngaarden et al., 2005; Van den Brink et al., 2006; Maltby et al., 2009). Likewise, the aquatic toxicity of the most widespread anthelmintic used in aquaculture, ivermectin - which is also used in livestock production - has been thoroughly investigated (Sanderson et al., 2007; Brinke et al., 2010; Boonstra et al., 2011). The amount of data available for these chemical classes (i.e., ecto- and endo-parasiticides) allows a trustworthy ecological

effect evaluation, with well understood consequences for aquatic ecosystems. Less advanced, however, is the scientific knowledge that underpins the ERA for antimicrobials used in aquaculture. For these compounds there are still large knowledge gaps regarding, for example: (1) the variation in sensitivity between standard and non-standard test organisms, (2) the variation in sensitivity between temperate species and those inhabiting (sub-)tropical aquatic ecosystems of Asia, (3) the adequacy on the use of ecological thresholds derived with single-species toxicity data to protect aquatic communities, and (4) the use of biomarkers to effectively assess sub-lethal biological effects under field conditions (Brooks et al., 2009).

Another important question regarding the ERA of aquaculture antibiotics is to know how and to what extent antibiotics can affect environmental bacteria. Standard ERA procedures for aquaculture antibiotics accept the alteration of the structure of bacterial communities as long as their ecological functions are preserved (VICH, 2004; EMEA, 2008), though these do not offer standard monitoring protocols to assess effects on ecological functions. Yet, it is rather unknown what are the implications of structural changes in bacteria communities regarding their mediated ecological functions like nitrification and organic matter mineralization, and how this could result in side-effects at higher trophic levels. Similarly, the selective pressure that antibiotic pollution may exert on natural bacterial communities and the consequent development of antibiotic resistance is not included as part of the current ERA framework (see critics by Montforts, 2005 and Tello et al., 2012). Recent studies have stressed the dimensions of this worldwide problem for the future treatment of human and animal bacterial diseases (Martinez, 2008; Miranda et al., 2013; Cabello et al., 2013), which has particularly been debated in the context of emerging aquaculture industries like the Asian (Suzuki and Hoa, 2012). Many researchers have claimed the need to find alternatives in order to reduce the use of antibiotics, as well as to derive science-based risk assessment tools aiming at quantifying and minimizing the development of antibiotic resistance in aquaculture environments (Miranda et al., 2013; Cabello et al., 2013; Pruden et al., 2013). This thesis aimed to contribute to reduce the current uncertainties in the risk assessment of aquaculture antibiotics by tackling the issues highlighted in this section.

5. Research objectives and scope

This thesis consists of a series of studies that collectively contribute to increase our knowledge on the use and environmental risks posed by the use of veterinary medicines in Asian aquaculture production, with an emphasis on the evaluation of the risks posed by aquaculture antibiotics for tropical aquatic ecosystems.

The specific research objectives of this thesis are:
1. To assess the current use of veterinary medicines in Asian aquaculture production.
2. To develop new modelling tools to support the risk assessment of aquaculture medicines.
3. To identify compounds and Asian aquaculture production scenarios that may pose high environmental risks and, therefore, require further in-depth risk evaluations.
4. To monitor the environmental fate and ecological risks posed by the use of aquaculture antibiotics for tropical aquatic ecosystems.

This thesis was carried out as part of the EU-FP7 Sustaining Ethical Aquaculture Trade project (SEAT; www.seatglobal.eu). The SEAT project was originated to assess and support the economic, social and environmental sustainability of the Asian aquaculture industry, with the aim of providing better informed choices to seafood consumers in Europe and other developed countries. The SEAT project focused on assessing aquaculture value chains of four aquaculture species groups with high export potential (i.e. Penaeid shrimp, *Macrobrachium* prawn, tilapia and *Pangasius* catfish) produced in four major aquaculture producing countries of Asia: China, Vietnam, Thailand and Bangladesh (Table 1). This thesis contributed to the assessment of the environmental performance and sustainability of the aquaculture production in the mix of

countries and aquaculture species studied within the SEAT project by studying several aspects related to the use of veterinary medicines and their potential environmental risks.

Table 1. Matrix of countries and major aquaculture species included as part of the SEAT project and studied within this thesis.

	Bangladesh	P.R. of China	Thailand	Vietnam
 Penaeid shrimp	✓	✓	✓	✓
 *Macrobrachium* prawn	✓			
 Tilapia		✓	✓	
 Pangasius catfish				✓

6. Thesis outline

This thesis starts with a profound analysis of chemical use practices in Asian aquaculture that includes not only veterinary medicines but also other compounds such as fertilizers, water quality treatments, hormones, and other biological compounds. **Chapter 2** provides a literature review on the use of chemicals and biological products in the top-seven aquaculture producing countries of Asia. This study also discusses the factors that influence the control on chemical use at the farm-level, describes the available international guidelines for the ERA of veterinary medicines, and highlights the research needs for the implementation of ERA studies in Asian countries. **Chapter 3** continues with the analysis of chemical use practices in Asian aquaculture and describes the outcomes of a survey performed to 252 grow-out farmers and 56 chemical supply shops in the countries studied within the SEAT project. Furthermore, a comparative analysis is performed between the studied aquaculture species and countries with regard to chemical use frequencies, reported dosages, applied quantities relative to production, and the factors underlying the observed chemical use patterns.

In Chapter 4 and 5 modelling approaches are developed and used to perform risk evaluations for aquaculture medicines. **Chapter 4** describes the scientific background and potential applications of the ERA-AQUA model, a risk assessment model that was developed to perform environmental and human health risk assessments of aquaculture veterinary medicines applied in pond aquaculture. This chapter defines the input parameters, sub-models and underlying equations used by the ERA-AQUA model, and shows the outcomes of a sensitivity analysis that helps to identify the most important parameters to take into consideration when parameterizing the model. **Chapter 5** uses the ERA-AQUA model and the dataset generated in Chapter 3, together with up-to-date information on aquaculture production practices and chemical characteristics, to perform a probabilistic risk assessment aimed at the identification of chemotherapeutant treatments and aquaculture pond scenarios that pose a major environmental hazard.

Chapters 6, 7 and 8 describe the outcomes of monitoring studies that investigate the environmental fate and ecological effects of aquaculture antibiotics for tropical aquatic

ecosystems. **Chapter 6** focuses on the investigation of the use, fate and ecological risks of antibiotics applied in tilapia cage farming in Thailand. In this chapter, the environmental fate of the two most commonly used antibiotics, oxytetracycline and enrofloxacin, is investigated by monitoring concentrations of these compounds in water and sediment samples collected in two rivers with significant aquaculture production. Furthermore, potential ecological risks at these concentrations are calculated based on SSDs built with literature data and the results of a battery of tests performed with these two antibiotics for tropical freshwater invertebrates. **Chapter 7** investigates the environmental fate of enrofloxacin, and its main metabolite ciprofloxacin, in aquatic ecosystems exposed to *Pangasius* catfish pond effluents in Vietnam. In this chapter, the effects of enrofloxacin and ciprofloxacin are investigated on freshwater organisms belonging to three different trophic levels and a risk assessment is performed based on the monitored environmental concentrations and the obtained toxicity data. Finally, **Chapter 8** investigates the ecosystem-level effects of the antibiotic enrofloxacin by monitoring the effects of a realistic exposure pattern in tropical freshwater microcosms. In this study the antibiotic effects on five structural (macroinvertebrates, zooplankton, phytoplankton, periphyton and bacteria) and two functional (organic matter decomposition and nitrogen cycling) endpoints are described.

In **Chapter 9** the issue of antibiotic resistance is examined. This chapter proposes a modelling approach aimed at predicting the development of antibiotic resistant bacteria in aquaculture production systems and surrounding environments. The approach described in this chapter is grounded on the theory of probabilistic risk assessment and relies on the use of modelled exposure concentration distributions and SSDs derived with available antibiotic susceptibility data for clinically relevant bacteria. This chapter also discusses the need and the way forward to include relevant resistance endpoints in the prospective, screening-level risk assessment of aquaculture antibiotics.

In **Chapter 10** the results of this thesis are discussed and recommendations are provided (1) to improve the control on the use of aquaculture medicines in Asian countries, (2) to reduce the environmental discharge of aquaculture medicines, and (3) to improve the knowledge and tools that underpin the ERA of veterinary medicines used in Asian aquaculture.

Chapter 2

Use of chemicals and biological products in Asian aquaculture and their potential environmental risks: a review

Andreu Rico, Kriengkrai Satapornvanit, Mohammad M. Haque, Jiang Min, Phuong T. Nguyen, Trevor C. Telfer, Paul J. van den Brink

Abstract

Over the past few decades, Asian aquaculture production has intensified rapidly through the adoption of technological advances, and the use of a wide array of chemical and biological products to control sediment and water quality and to treat and prevent disease outbreaks. The use of chemicals in aquaculture farms has raised environmental concerns owing to their potential impacts on down-stream aquatic ecosystems. Currently little is known about the environmental fate and effects of the chemicals used in Asian aquaculture. Therefore, we reviewed recent information on the use of chemical and biological products in the most important Asian aquaculture producing countries and summarize their main potential environmental impacts. We provide an overview of the main factors controlling the use of these chemicals and describe the international risk assessment guidelines available for aquaculture chemicals. Finally, data gaps and research needs for their implementation in Asian countries are discussed. Our review aims to form a basis for developing environmental risk assessment studies of the chemicals used in Asian aquaculture.

This chapter has been published in *Reviews in Aquaculture* 2012, 4: 75-93.

1. Introduction

Asian aquaculture production accounts for nearly 90% of the global aquaculture production by volume and is primarily dominated by the culture of freshwater and brackish water species in tropical and sub-tropical regions (FAO, 2009). The Asian aquaculture industry is characterized by a high degree of diversification and differs substantially to the aquaculture practiced in temperate regions in terms of the cultured species, the types of production systems, and the levels of intensification. Asian freshwater aquaculture production is dominated by the culture of several species of carps, tilapias and catfishes in irrigated or flow-through ponds with varying degrees of intensification and, to a lesser extent, cultured in cage-based farming systems placed in water reservoirs, lakes and rivers (FAO, 2010). Brackish water aquaculture has more than doubled during the last decade (Rivera-Ferre, 2009) and is dominated by the production of crustaceans (mainly penaeid shrimps) in coastal earthen ponds and tanks (FAO, 2010).

Over the past few years, the Asian aquaculture production sector has expanded and intensified at a rapid pace largely due to the increase in the population and economic growth, the export potential of the aquatic products, and the intensification of aquaculture practices (i.e., expansion of monoculture on-growing systems, increase of stocking densities and use of high loads of artificial feeds) (Bostock et al*.,* 2010). As aquaculture practices have intensified, the Asian aquaculture industry has been overwhelmed by several aquatic animal health problems. The proliferation of viral, bacterial and fungal infections and parasitic pests has resulted in large economic losses, becoming one of the major constraints to the development and expansion of the aquaculture sector in Asia (Bondad-Reantaso et al*.,* 2005). Consequently, aquaculture farmers have relied on a wide variety of synthetic and natural chemical and biological treatments to prevent and treat disease outbreaks, to enhance the health status of the cultured species, and to improve the environmental conditions of the aquaculture production systems. These include antibiotics, disinfectants, pesticides, fertilizers, and water and soil treatment compounds (Gräslund and Bengtsson, 2001).

The majority of the Asian aquaculture production systems are hydrologically interconnected with the surrounding water bodies and produce continuous or intermittent waste water discharges into them. Thus, a considerable amount of the chemicals and biological products applied in these aquaculture production systems may eventually be released in the surrounding aquatic ecosystems, potentially damaging the natural structure and functioning of these ecosystems, and contributing to the development of resistant strains of parasites and bacteria (GESAMP, 1997; Boyd and Massaut, 1999; Gräslund and Bengtsson, 2001; Burridge et al., 2010).

To date, studies aimed at assessing the environmental risks posed by the use of chemicals in Asian aquaculture rarely exist. This is probably a result of the rapid expansion and intensification of the aquaculture sector that has taken place during the past couple of decades, a weakness of environmental regulatory frameworks and a lack of scientific and economic resources. The present study aims to provide a review on the potential environmental hazards of chemicals and biological products used in Asian aquaculture and to identify research needs for performing environmental risk assessment studies. First, we reviewed publications describing the use of chemicals and biological products in Asian aquaculture in order to identify the main compounds that have been recently used as well as the components of aquatic ecosystems that might be affected by each group or groups of these compounds. Second, we reviewed and discussed the main driving factors and regulations influencing the use of chemicals in Asian countries. Third, risk assessment guidelines for aquaculture environmentally hazardous compounds that have been already implemented in developed countries are described. Finally, the research and actions needed to implement available international risk assessment guidelines are presented and recommendations

are provided for scientists and governments in order to perform risk assessment studies in Asian aquatic ecosystems that are potentially impacted by aquaculture-induced chemical pollution.

Table 1. List of reviewed publications on chemicals and biological products used in aquaculture production in the top seven aquaculture-producing countries of Asia.

	Reference	Country†	Environment‡	Culture system§	(Group) Species¶	Information source ψ	Data collection	Dose/ Frequency
a	Yulin (2000)	CN	NS	NS	NS	R	-	No
b	Zheng and Xiang (2002)	CN	NS	NS	NS	R	-	No
c	Yang and Zheng (2007)	CN	NS	NS	NS	R	-	No
d	Qi *et al.* (2009)	CN	NS	NS	NS	R	-	No
e	Pathak *et al.* (2000)	IN	FW/BW	NS	NS	R	-	Yes
f	Amaraneni (2006)	IN	BW	P	PM	NS	-	No
g	Phillips 2000	VN/BD	FW/BW	P	F/S	IF	1994-1995	No
h	Le and Munekage (2004)	VN	BW	P	PM	IF/ICS	-	No
i	Nga (2004)	VN	BW	P	PM	IF/ICS	2003	Yes
j	Chinh (2005)	VN	FW	P	PH	IF/ICS	2004	No
k	Bosma *et al.* (2009)	VN	FW	P	PH	IF	2008	No
l	Phan *et al.* (2009)	VN	FW	P	PH	IF	2008	No
m	Tu (2009)	VN	BW	P	PM	IF	2004-2005	No
n	Supriyadi *et al.* (2000)	ID	FW/BW	NS	F/S	R	-	Yes
o	Tonguthai (2000)	TH	NS	NS	NS	IF/ICS	-	Yes
p	Gräslund *et al.* (2003)	TH	BW	P	PM	IF	2000	No
q	Holmström *et al.* (2003)	TH	BW	P	PM	IF	2000	No
r	MacRae *et al.* (2002)	BD	FW	C	MR/O	IF	1999	Yes
s	Faruk *et al.* (2005)	BD	FW	P	C/PH/MR	IF	2004	Yes
t	Faruk *et al.* (2008)	BD	FW	P	F/MR	IF/ICS	2007-2008	Yes
u	Cruz-Lacierda *et al.* (2000)	PH	BW	P	PM	IF	1995-1996	Yes
v	Tendencia *et al.* (2001)	PH	BW	P	PM	R	-	No
w	Cruz-Lacierda *et al.* (2008)	PH	BW	P	PM/CC	IF	2006-2007	Yes

† CN: China IN: India VN: Vietnam ID: Indonesia TH: Thailand BD: Bangladesh PH: Philippines
‡ NS: Non specific FW: freshwater BW: brackish water
§ NS: Non specific P: ponds C: concurrent rice-fish-shrimps systems
¶ NS: Non specific F: fish (species not reported) S: shrimps (species not reported)
PM: *Penaeus mondon* PH: *Pangasionodon hypophthalmus* MR: *Macrobrachium rosenbergii* O: others *(Hypophthalmichthys molitrix, Catla catla, Labeo rohita, Ctenopharyngodon idellus)* C: carps (species not reported) CC: *Chanos chanos*
ψ R: review national publications IF: interview farmers ICS: interview chemical sellers NS: not specified

2. Use of chemicals and biological products in Asian aquaculture and their potential environmental risks

In general, an overview of qualitative and quantitative data on the use (and/or sales) of chemicals applied in Asian aquaculture is currently lacking in the public domain. For this reason, country-specific information based on field surveys and national reviews published since 2000 was compiled for the top seven Asian aquaculture-producing countries (i.e., China, India, Vietnam, Indonesia, Thailand, Bangladesh, Philippines) (see FAO, 2009 for a complete country classification in terms of production volume). This literature search yielded 23 studies describing the use of chemicals and biological products in different aquaculture systems and species (Table 1). The identified compounds were classified according to their main use, but some of these compounds might be used for different purposes according to different aquaculture practices and in different dosages. Commonly used compounds were identified by assessing the number of publications and countries reporting their use. It should be noted, however, that this information might be somehow biased towards those countries that have good tracking and recording systems and research facilities to investigate the use of these chemicals. In addition, most of the field surveys

included in this review are focused on high-economic value species such as *Pangasius* catfish and Penaeid shrimps produced in (semi-)intensive pond systems (Table 1).

2.1. Water and sediment treatment compounds

Thirteen different compounds were reported to be applied to improve water and sediment quality in the production systems (Table 2). Liming compounds like calcium carbonate (agricultural lime), calcium hydroxide (hydrated lime) and calcium oxide (quicklime) are applied during pond preparation to the water or the sediment to neutralize acidity. To a lesser extent, however, some farmers have reported to use them to kill potential pests and predators. Other compounds are used to reduce bioavailability of heavy metals (e.g. EDTA) and to reduce turbidity in shrimp ponds (e.g. aluminium potassium sulphate).

Table 2. Water and sediment treatment compounds used in Asian aquaculture[†].

Water and sediment treatment compounds (*n* = 13)	CH	IN	VN	ID	TH	BD	PH	No. Countries
Aluminium potassium sulphate						*r*		1
Calcium carbonate		*e,f*	*i,j,k*		*o,p*	*r,s,t*	*u,v,w*	5
Calcium hydroxide		*e,f*			*o*	*t*	*u,w*	4
Calcium oxide	*a,b,c*	*e*	*j*		*o*	*t*		5
Calcium sulfphate dihydrate		*e*						1
Charcoal			*k*					1
Dolomite					*o,p*		*u,w*	2
EDTA	*a*	*e*	*i,j*		*o*	*t*		5
Potassium dichromate		*e*						1
Sodium bicarbonate					*p*			1
Sodium chloride	*a*	*e*	*k,l*			*r,s,t*		4
Sodium thiosulphate						*t*	*u*	2
Zeolite	*c*	*e,f*	*i,j,k*		*o,p*	*t*	*u*	6
Number of compounds	4	8	6	-	7	8	5	

CN: China IN: India VN: Vietnam ID: Indonesia TH: Thailand BD: Bangladesh PH: Philippines
[†] Lower case letters refer to publications listed in Table 1.

In the majority of the studied countries, zeolites were used to remove ammonia and other nitrogenous compounds in the water column, even though the efficacy of this practice in brackish water systems has been questioned due to its lower efficacy (Boyd, 1995a; GESAMP, 1997). Many of these water and sediment treatment compounds are relatively innocuous inorganic substances with a short environmental persistence, and are not expected to result in toxic effects to aquatic organisms when applied according to recommendations (Boyd and Massaut, 1999). They are, however, likely to alter, at least temporarily, water quality parameters of ecosystems such as alkalinity, hardness and pH.

2.2. Fertilizers

Fertilizers are used to induce algal blooms and to increase the natural productivity of ponds. Organic fertilizers such as cow, pig and chicken manure are commonly used in extensive and in integrated livestock-fish farms (Table 3). This practice has raised food safety and environmental concerns because manure may increase the risk of bacterial proliferation in the culture system and also may be a source of residues of antibiotics applied in poultry and livestock production, which can also contribute to the development of antimicrobial-resistant bacteria (Petersen and Dalsgaard, 2003). Intensive and semi-intensive cultures often rely on the use of inorganic nitrogenous and phosphate combinations (Table 3), especially during the early stages of the production cycle, when recently stocked fish and shrimps are not able to feed on pelleted feed. The high organic matter discharges and consequent risks of eutrophication in receiving waters due

to inputs of feeds and fertilizers has been one of the main criticism to the aquaculture industry all over the world (Naylor et al., 2000; Primavera, 2006). The impact of organic matter and nutrient discharges on receiving aquatic ecosystems has been described in detail elsewhere (Costanzo et al., 2004; Tello et al., 2010 and references therein) and consequently will not be further discussed in the present review.

Table 3. Fertilizers used in Asian aquaculture[†].

Fertilizers (n = 14)	CH	IN	VN	ID	TH	BD	PH	No. Countries
Inorganics								
Ammonium phosphate							*u,w*	1
Ammonium sulphate		*e*					*u,w*	2
Calcium ammonium nitrate		*e*						1
Calcium nitrate							*u*	1
Diammonium phosphate			*i*				*w*	2
NPK		*f*	*i*		*o,p*		*u*	4
Super phosphate		*e*			*p*			2
Triple super phosphate		*e*		*N*		*r*		3
Organics								
Horse manure							*w*	1
Chicken manure		*e,f*		*N*	*o*		*u,w*	4
Cow manure		*e,f*			*p*		*u,w*	3
Molasses (sugar waste)							*w*	1
Pig manure							*u,w*	1
Urea		*e,f*	*i*	*N*	*o*	*r*	*u,w*	6
Number of fertilizers	-	8	3	3	5	2	11	

CN: China IN: India VN: Vietnam ID: Indonesia TH: Thailand BD: Bangladesh PH: Philippines
[†] Lower case letters refer to publications listed in Table 1

2.3. Pesticides

Twenty-nine different pesticides were identified (Table 4). These compounds are mainly used to treat fungal and parasitic infections in the cultured species, to kill unwanted organisms entering the system within the inflow water, and to kill pests and predators when ponds cannot be drained completely before stocking. The main fungicides mentioned in the reviewed publications were malachite green, copper sulphate, methylene blue and trifluralin (Table 4). Malachite green is also used as a powerful bactericide (Hernando et al., 2007), but its use in food producing activities has been recently banned in many countries (e.g. China, Vietnam, EU, USA, Canada) due to its attributed carcinogenic properties (Yang et al., 2007; Pérez-Estrada et al., 2008). Organic fungicides have been demonstrated to be from moderately to highly toxic to aquatic invertebrates, fish and primary producers depending on their specific mode of action (Maltby et al., 2009). Inorganic fungicides such as copper sulphate have been demonstrated to be highly toxic to planktonic organisms (De Oliveira-Filho et al., 2004), and its over-use in aquaculture may result in human health issues due to copper bioaccumulation in the cultured species (Li et al., 2005). Insecticides are mainly used to control ectoparasitic infections, in finfish aquaculture, and to kill crustaceans remaining before stocking ponds in shellfish aquaculture. The most common insecticides used are the organochlorine endosulfan, the organophosphate trichlorfon, and its by-product dichlorvos (Table 4). Several studies (Ludwig, 1993; Qin and Dong, 2004) have assessed the toxicity of trichlorfon on zooplankton species (rotifers, cladocerans and copepods) showing that these organisms are highly sensitive to the concentrations recommended to be used in finfish treatments (300 μg/L) in countries like Thailand (Tonguthai, 2000) and China (Yulin, 2000). The sensitivity of different taxonomic groups of aquatic organisms has been investigated for a range of insecticides comparing toxicity data derived in laboratory and semi-field experiments, demonstrating that aquatic crustaceans and insects are the most sensitive taxa of non-target

organisms potentially affected by residual concentrations of these compounds, followed by fish (Maltby et al., 2005).

Table 4. Pesticides used in Asian aquaculture[†].

Pesticides (*n* = 29)	CH	IN	VN	ID	TH	BD	PH	No. Countries
Fungicide								
Acriflavine					*o*	*t*		2
Copper sulphate	*a,b*	*e*	*g,k,m*		*p*			4
Fentin acetate				*n*			*w*	2
Malachite green[‡]	*a,b,c*	*f*	*i*	*n*	*o,p*	*t*	*u*	7
Methylene blue	*a*			*n*		*r,t*		3
Nystatin	*a*							1
Trifluralin		*e*			*o,p*		*u*	3
Herbicide								
2,4-D		*e*					*u*	2
Dalapon		*e*						1
Diuron		*e*						1
Paraquat		*e*						1
Simazine		*e*						1
Insecticides								
Organochlorines								
Endosulfan		*f*	*i,m*	*n*	*p*		*u,w*	5
Lindane		*e*						2
Organophosphates								
Azinphos-methyl							*u*	1
Chlorpyrifos		*f*						1
Diazinon							*u*	1
Dichlorvos		*e*	*i,m*		*o,p*			3
Dimethoate		*f*						1
Fenitrothion						*s,t*		1
Malathion		*e,f*				*t*		2
Monocrotophos		*f*						1
Trichlorfon	*a,b*		*i,j,m*		*p*	*g,t*	*w*	5
Pyrethroids								
Deltamethrin			*i,m*					1
Piscicides								
Aluminium phosphide						*g*		1
Nicotine							*u,w*	1
Oil cake		*e*						1
Rotenone		*e*	*g,j,m*			*g*	*u*	4
Saponin (teaseed cake)		*e*	*g,j,m*	*n*	*o,p*	*g*	*u,v,w*	6
Number of compounds	5	18	8	5	8	9	11	

CN: China IN: India VN: Vietnam ID: Indonesia TH: Thailand BD: Bangladesh PH: Philippines
[†] Lower case letters refer to publications listed in Table 1
[‡] Currently banned for use in aquaculture in most Asian countries

A few piscicides such as rotenone and saponin compounds are commonly used in shrimp culture to kill fish entering with the inflow water before stocking, while saponin also has been used to induce moulting in shrimp production (GESAMP, 1997; Boyd and Massaut, 1999). Ling (2003) highlighted that the application of rotenone to kill fish in aquaculture ponds often causes a significant decline of zooplankton abundance, with cladocerans and copepods being the most sensitive invertebrate groups. The use of herbicides to kill submerged and floating weed has only been reported in India (Table 4).

2.4. Disinfectants

A wide range of disinfectants are used in intensive aquaculture, particularly in finfish and shrimp hatcheries and grow-out ponds, to disinfect the culture facilities and equipment, to maintain hygiene throughout the production cycle and also to treat bacterial disease outbreaks. The most commonly used disinfectants are formaldehyde, potassium permanganate, chlorine releasing compounds (e.g. calcium hypochlorite, sodium chloride, benzalkonium chloride), and iodine (Table 5). Formaldehyde is frequently used to kill filamentous bacteria in fish and crustaceans grow-out ponds and hatcheries, and is also used at a higher concentrations to treat fungal and ectoparasitic infections of protozoans and trematodes in finfish production (Boyd and Massaut, 1999). Potassium permanganate is a strong oxidizing agent used as a broad-spectrum disinfectant in finfish and shellfish production, but can also be used to treat fungal infections and as a piscicide during pond preparation between production cycles. Chlorine gas and powdered forms such as calcium hypochlorite and sodium hypochlorite are used to disinfect the water supplies in fish and shrimp hatcheries and for water and sediment disinfection between production cycles. Sodium chloride is used for removal of external parasites and fungi from adult fish. Quaternary ammonium compounds such as benzalkonium chloride are used in finfish and shellfish production to treat bacterial, protozoan and monogenean infections and as fungicides in shrimp hatcheries. Iodine and iodophores such as povidone-iodine are frequently used in egg disinfection (Boyd and Massaut, 1999), and ozonation has been recognized as one of the most important methods of disinfection in shrimp hatcheries (GESAMP, 1997).

Table 5. Disinfectants used in Asian aquaculture[†].

Disinfectants (*n* = 21)	**CH**	**IN**	**VN**	**ID**	**TH**	**BD**	**PH**	**No. Countries**
Benzalkonium chloride	*a*	*f*	*i,j,k,l,m*		*o,p*	*g,s,t*	*u*	6
Calcium hypochlorite	*a,b,c*	*e*	*i,k,m*		*o,p*		*u,w*	5
Calcium peroxide	*c*							1
Calcium sulphide							*u*	1
Chlorine	*a*		*i,j,l*	*n*	*o*	*t*		5
Chlorine dioxide	*b,c*							1
Copper chloride	*a*				*p*			2
Copper complex solution	*b*		*i,m*		*o*		*u*	4
DDAB		*f*						1
Formaldehyde	*b,c*	*e,f*	*i,j,m*	*n*	*o,p*	*g,t*	*v,w*	7
Hydrogen peroxide			*i*		*p*	*t*		3
Iodine	*b*	*e,f*	*i*		*o*		*u*	5
Ozone					*p*			1
Potassium monopersulphate			*k*				*u*	2
Potassium permanganate	*a,b*	*e*	*i,j,k,m*	*n*	*o,p*	*g,r,s,t*	*u*	7
Potassium thiosulphate			*i,m*					1
Povidone-iodine	*a,b,c*		*i,k,m*					2
Sodium cyanide							*w*	1
Sodium dichloroisocyanurate	*b*							1
Sodium hypochlorite					*o*		*u*	2
Trichloroisocyanuric acid	*c*							1
Number of compounds	13	6	11	3	11	5	10	

CN: China IN: India VN: Vietnam ID: Indonesia TH: Thailand BD: Bangladesh PH: Philippines
[†] Lower case letters refer to publications listed in Table 1
DDAB: didodecyldimethylammonium bromide

Overall, aquaculture disinfectants are moderately to highly toxic to planktonic and macroinvertebrate species with acute L(E)C50 ranging between 1 to 100,000 µg/L (ECOTOX, 2010). They are, however, characterized by a high solubility and a rather low persistence in the aquatic environment. Tišler and Zagorc-Končan (1997) demonstrated that formaldehyde may be harmful

to phytoplanktonic organisms and crustaceans at concentrations required to treat bacterial infestations in fish (Tonguthai, 2000). In addition, chlorine disinfectants (e.g. calcium or sodium hypochlorite) react with organic matter, giving rise to significant concentrations of organic chlorine compounds such as halogenated hydrocarbons, which are highly toxic for aquatic life and can become persistent environmental contaminants (Emmanuel et al., 2004).

2.5. Antibiotics

The review revealed thirty-six different antibiotics that were used in aquaculture in the seven major production countries (Table 6). Antibiotics are routinely applied in bath treatments or mixed with feed to prevent (prophylactic use) and treat (therapeutic use) bacterial infections. Oxytetracycline and chloramphenicol have been reported to be used in all seven countries in the past years, but the later one has been recently banned for its application in aquaculture in most of the Asian countries. Tetracycline and quinolone antibiotics are the most commonly used antibiotic groups together with sulfonamides, the latter typically potentiated with trimethoprim (Table 6). Vietnam and China showed the highest number of reported types of different antibiotics with 31 and 17 being used respectively. Countries such as Indonesia and Bangladesh, which are characterized by a more fragmented aquaculture industry with a lower degree of intensification in culture practices showed less diverse antibiotic use in terms of number of compounds (Table 6).

The occurrence and effects of antibiotics in aquatic ecosystems has received increasing attention by scientists in the recent years, not only due to aquaculture-induced pollution but also due to their heavy use in other animal husbandry activities and in human medicine (see Halling-Sörensen et al., 1998; Boxall et al., 2004; Sarmah et al., 2006; Kümmerer, 2009; Santos et al., 2010). Antibiotics are most toxic to microorganisms and primary producers (Jones et al., 2002; Zounková et al., 2011). Several toxicological studies have shown that, in general terms, aquaculture antibiotics are not particularly toxic to invertebrates and fish at environmentally relevant concentrations (Wollenberger et al., 2000; Isidori et al., 2005; Park and Choi, 2008; Zounková et al, . 2011). However, Wollenberger et al. (2000) observed long-term effects in invertebrates (i.e., reproduction disruption in daphnids) at relatively low concentrations of oxolinic acid (NOEC = 380 µg/L). Several studies have demonstrated that concentrations toxic to micro algal growth are generally 1 or 2 orders of magnitude below those of invertebrates (Halling-Sörensen, 2000; Isidori et al., 2005; Park and Choi, 2008; Lai et al., 2009a). Among the phytoplanktonic communities, blue-green algae have been found to be the most sensitive taxonomic group to antibiotics (Halling-Sörensen et al., 2000; Robinson et al., 2005; Brain et al., 2008), probably due to their morphologic resemblance with the target bacteria.

Antibiotic pollution may result in indirect effects on both invertebrates and vertebrates since algae constitute the basis of the food chain and a decrease in their production might indirectly affect the entire aquatic food web. In addition, several studies have demonstrated that antibiotic pollution affects water quality parameters and the structure of natural bacterial communities, resulting in effects on endpoints describing functional processes of aquatic ecosystems such as nitrification and organic matter mineralization (Klaver and Mathews, 1994; Näslund et al., 2008; see Tello et al., 2010 for further discussion). On the other hand, an important concern on the use of antibiotics is the development of resistant strains of bacteria, which can compromise treatment effectiveness and public health by means of antibiotic-resistant bacteria transfer to consumers. The studies by Tendencia and De la Peña (2001) and Le et al. (2005) have reported the presence of antibiotic-resistant bacteria in shrimp farms which used antibiotics regularly in Philippines and Vietnam, respectively. Furthermore, the environmental release of large quantities of antibiotics has the potential to result in high levels of bioaccumulated residues through the aquatic food chains, eventually leading to secondary effects in top-predators (e.g. mammals and birds) (Cabello, 2006).

Table 6. Antibiotics used in Asian aquaculture[†].

Antibiotics (*n* = 36)	CH	IN	VN	ID	TH	BD	PH	No. Countries
Aminoglycosides								
Gentamycin	*c*		*h,i,j,m*		*p,q*			3
Kanamycin			*j,k*					1
Neomycin		*e*	*h,j*	*n*				3
Streptomycin	*a*		*i,j*	*n*				3
Beta-lactams								
Amoxicillin			*j,k*			*t*		2
Ampicillin	*c*		*h,j,k,m*					2
Penicillin	*a,b*							1
Macrolides								
Erythromycin	*a,c*		*i*	*n*	*o*		*u,v*	5
Nitrofurans[‡]								
Furaltadone			*h,i,m*					1
Furazolidone	*a*	*e*					*u,v*	3
Nifurpirinol		*e*		*n*			*u*	3
Nitrofurazone		*e*	*i*		*o*			3
Quinolones								
Ciprofloxacin	*c*		*i,j*		*p,q*			3
Enrofloxacin	*c*		*h,i,j,k,m*	*n*	*p,q*			4
Flumequine			*h,j*					1
Norfloxacin	*c*		*h,i,j,m*		*p,q*			3
Ofloxacin			*j*					1
Oxolinic acid	*a,c*		*h,j,m*		*o,p,q*	*g*	*u,v*	5
Sulfonamides								
Sulfachloropyridazine			*h*					1
Sulfadiazine		*e*	*i,j,k*			*t*		3
Sulfadimethoxine			*j*					1
Sulfadimidine			*j*					1
Sulfamethazine	*c*		*j*		*p,q*			3
Sulfamethoxazole	*c*		*h,j,m*			*t*	*u*	4
Sulfamonomethoxine					*o*			1
Tetracyclines								
Tetracycline			*i*		*p,q*		*u*	3
Chlortetracycline	*a,b*	*e*	*j*		*p,q*	*s,t*		5
Doxycycline	*a*	*e*	*j,k*			*g*	*u*	5
Oxytetracycline	*b,c*	*e,f*	*g,i,j,k*	*n*	*o,p,q*	*g,s,t*	*u,v*	7
Others								
Chloramphenicol [‡]	*a,c*	*e,f*	*i*	*n*	*p,q*	*g*	*u,v*	7
Colistin			*h,i,j,k,m*					1
Florfenicol			*j,k*					1
Metronidazole			*m*					1
Rifampicin	*c*						*u*	2
Trimethoprim		*e*	*h,i,j,k,m*		*p,q*	*t*	*u*	5
Number of compounds	17	10	31	7	14	9	11	

CN: China IN: India VN: Vietnam ID: Indonesia TH: Thailand BD: Bangladesh PH: Philippines

[†] Lower case letters refer to publications listed in Table 1

[‡] Currently banned for use in aquaculture in most Asian countries

2.6. Other feed additives, hormones, vaccines, anaesthetics and probiotics

Several compounds were found to be applied mixed with feed to ensure optimal diet quality and improve the immunological status of the cultured species. These include several vitamins and mineral premixes as well as essential fatty acids, amino acids, and polysaccharides with growth-promoting effects (Table 7). In addition, in China and Vietnam, aquaculture farmers frequently use specific local herbs and garlic to enhance the immunological status of the cultured species. Different to other feed additives (e.g. antibiotics), these compounds are expected to be highly metabolized by the cultured species and are not expected to result in significant environmental impacts (Anderson, 1992). The reviewed publications showed that oral administration of the synthetic steroid hormone 17-α-methyl-testosterone in hatcheries to produce monosex tilapia is widely practiced in south-east Asia and their potential endocrine disrupting effects on aquatic organisms receiving effluents of hatcheries still requires further in-depth studies.

The use of vaccines (against Vibrio infections) in shrimp has only been reported in Thailand (Table 7). As anticipated by Grisez and Tan (2005), vaccination is expected to increase in Asian aquaculture in the near future. The use of anaesthetic compounds has been reported in India, however it is limited to a few transport operations of broodstock and fish seed (Pathak et al., 2000). The use of vaccines and anaesthetics are not expected to result in major environmental concerns due their high biodegradability and the low volumes that are used (Grisez and Tan, 2005; Burridge et al., 2010).

Table 7. Immunostimulants, growth promoters, hormone, vaccine, anaesthetic and probiotic use in Asian aquaculture[†].

Groups of compounds	CH	IN	VN	ID	TH	BD	PH	No. Countries
Aminoacids	*c*		*j,k*		*p*		*u*	4
Anaesthetics		*e*						1
Fatty acids		*f*	*i,j*				*u*	3
Hormones					*o*		*u*	2
Local herbs	*b*		*g,i,j,k*					2
Minerals		*e,f*	*i,j*				*u*	3
Polysaccharids	*c*		*h,i,k*		*o*	*t*		4
Probiotics	*c,d*	*f*	*k*	*n*	*p*	*t*	*u,w*	7
Vaccines					*o*			1
Vitamins		*f*	*i,j,k*	*n*	*o,p*	*s*	*u,w*	6

CN: China IN: India VN: Vietnam ID: Indonesia TH: Thailand BD: Bangladesh PH: Philippines
[†] Lower case letters refer to publications listed in Table 1

The use of probiotic products has been reported in the seven investigated countries (Table 7). During the last years, the use of probiotics or 'beneficial' micro-organisms has been widespread over aquaculture producing countries in order to improve water quality and the immunological status of the cultured species and has been considered as a more environmentally safe alternative to the prophylactic use of antibiotics (Decamp et al., 2008; Wang et al., 2008). Qi et al. (2009) provided a detailed review of the current status of the probiotics used in aquaculture in China and identified at least six different groups ranging from photosynthetic and nitrifying bacteria to micro-organisms for nutritional and enzymatic contribution to the digestion in the cultured species. The use of probiotics or biological products is likely to interfere with the natural bacterial composition in aquatic ecosystems. However, due to their relatively recent introduction in Asian aquaculture, their specific chemical composition and potential environmental impacts have not been extensively investigated.

2.7. Control of chemical use in Asian aquaculture

During the last decade, the responsible use of chemicals in Asian countries has been questioned. Several studies have pointed out that some farmers frequently lack trained work-force and institutional support on how to use chemicals. The insufficient knowledge on disease diagnosis and mode of action of these compounds, especially among smallholders, is of major concern (Pathak et al., 2000; Holmström et al., 2003; Faruk et al., 2005). In many cases, this is related to the fact that the information displayed on chemical labels is insufficient (e.g. leaving out the name of the active ingredient or the recommended dosage) or written in foreign languages, and several farmers have reported problems understanding them (Gräslund et al., 2003; Faruk et al., 2005). This often makes farmers reliant on information obtained by discussions with fellow farmers rather than the recommendations prescribed by chemical companies (Le and Munekage, 2004; Faruk et al., 2005). Furthermore, several studies have shown that in many rural areas aquaculture farmers are highly influenced by extensive promotional programmes carried out by chemical retailers/agents in order to increase their sales (Tonguthai, 2000; Faruk et al., 2008), contributing to an excessive and inappropriate use of chemicals.

The misuse of several aquaculture drugs (in particular antibiotics) has resulted in several export bans established by EU and US to farmed aquatic products from some Asian countries such as

China, Bangladesh, Indonesia and Vietnam (Hernández Serrano, 2005). In most cases, residual concentrations of banned compounds (i.e., chloramphenicol, nitrofurans, malachite green) were detected in shrimp (Vass et al., 2008). The economic losses for international exporters and governments and the recognized human health hazards of consuming antibiotic residues has supported the establishment of several food safety controls at the National level as well as in the private sector. This trend has encouraged farmers to search for reliable alternatives to antibiotic use, such as for example probiotics (Wang et al., 2008) and vaccines (Grisez et al., 2005), and/or has resulted in more cautious use of antibiotics i.e., respecting the withdrawal periods, use of chemicals that are not banned in importing countries due to food safety issues. Although there is a logical trend to prevent humans being exposed to residues of antibiotics, considerable amounts of these compounds are expected to be released in surrounding ecosystems during and after the treatment period (Anh et al., 2010) and, hence, their potential environmental hazard still requires further investigations.

The aquaculture industry of Asia has grown faster than the associated development of legal instruments regulating production and importation of aquaculture chemicals. To date, regulatory frameworks concerning aquaculture chemicals in countries of Asia are very diverse. Authorisation of compounds frequently relies on tests assessing the efficacy and safety of the active ingredients on the cultured species, the safety of the chemical to human health (e.g. establishing Maximum Residue Limits) and, in a few cases, evaluating the toxicological effects of the compound using a limited number of non-target standard test species (Van Houtte, 2000). The division of tasks and responsibilities among different agencies (e.g. public health and food safety, agriculture, animal health services, environment) and the lack of a standardized management programme covering the chemical registration and further evaluation processes contribute to the weakness of their legal and institutional frameworks (Van Houtte, 2000). The sustainability of the aquaculture sector requires the development of new national policies and regulations, and the continuous monitoring and enforcement of such regulations at a farm level. Aside from the national regulations, government initiatives, private institutions and NGOs have developed national and international certification schemes to ensure environmentally and socially responsible aquaculture production (see Corsin et al., 2007). Farmers participating in certification programmes must comply with standards that frequently include criteria on chemical storage, usage, disease management, and occupational health and safety, as well as mitigating measures of environmental pollution (e.g. establishment of waste-water treatment systems). Inspections carried out by certification schemes play an important role in controlling that veterinary drugs and chemicals are used according to manufacturer's instructions, with particular attention to withdrawal periods and the use of banned compounds. However, these certification schemes have been developed for a reduced number of high-value species and the costs of paying the application fees and/or meeting some of the established standards are often unaffordable by small holders (Anh et al., 2011). Therefore, they are principally established in medium to large-scale farms and/or farms with considerable export shares (Boyd et al., 2005; Corsin et al., 2007).

In the latter years, several international organizations have organized workshops and delivered technical reports to support the increase of social awareness on the impacts of aquaculture chemicals on the environment and human health (e.g. Barg and Lavilla-Pitogo, 1996; GESAMP, 1997; Hernández Serrano, 2005; WHO, 2006; Bondad-Reantaso, 2010). However, the relationship between current chemical use patterns, Asian aquaculture scenarios and potential effects on aquatic ecosystems health has not been properly investigated, and is not considered as part of the chemical registration procedures in developing countries. To achieve this, there is a need to involve industry, regulatory authorities, and the academic community in the development and implementation of environmental risk assessment (ERA) tools that allow the identification of the environmental risks posed by different (old and new) chemical applications in aquaculture production. In contrast, in several developed regions such as North-America and Europe, the potential environmental risks posed by the use of chemical products in aquaculture production have been largely recognized. In the next section, a brief introduction to the regulatory

classification and risk assessment methods used in EU for substances applied in aquaculture production will be provided.

3. ERA for aquaculture chemicals in EU

3.1. Regulatory classification of aquaculture chemicals

Chemical regulatory frameworks established in developed countries (i.e., EU countries, USA), generally classify environmentally hazardous chemicals according to their intended mode of use. For instance, the EU Directive 2001/82/EC (EU, 2001) on the marketing authorisation for veterinary medicines defines Veterinary Medicinal Products (VMPs) as any substance or combination of substances used to prevent or treat diseases and/or to modify physiological functions in farmed animals. VMPs therefore include antibiotics, parasiticides, anaesthetics, vitamins, growth promoters and medical disinfectants. Conversely, other chemicals used in animal husbandry, disinfectants and pesticides aiming at killing unwanted organisms in the equipment or in culture facilities, which are not intended to act directly in or on the cultured species are regulated according to the Biocides Directive (98/8/EC) in the EU (EU, 1998).

Given the potential environmental risks of these two groups of compounds, ERA studies are nowadays implemented in the regulatory frameworks by incorporating a scientific weight-of-evidence process that relates chemical use patterns and aquaculture practices with potential risks for surrounding aquatic ecosystems (Redshaw, 1995; Costello et al., 2001; Koschorreck et al., 2002). Thus, market authorizations are provided to those compounds that ensure maximal efficacy for treating or preventing cultured species diseases and are not expected to result in unacceptable human health and environmental risks. ERA guidelines have been set for VMPs and biocides. The reader is referred to ECB (2003) for a guidance document on the ERA of biocides. ERA guidelines for aquaculture VMPs will be described below. Other compounds such as fertilizers, probiotics, oxidizing compounds, and liming compounds applied with the intention of modifying the environmental conditions of the culture media are not expected to result in toxicological risks, but can, however, be considered as precursors of ecological effects. Water quality parameters such as pH, dissolved oxygen, Biological Oxygen Demand (BOD), nitrogen and phosphorus concentrations, need to be monitored in order to assess the farm compliance with the environmental quality standards set for aquaculture effluents (see Boyd, 2003).

3.2. ERA guidelines for aquaculture VMPs

In support of the registration of VMPs on the market, the International Cooperation on Harmonization of technical requirements for Authorization of Veterinary Medicinal Products (VICH), a collaboration by EU, Japan, USA, Canada, Australia and New Zealand, has issued two international ERA guidance documents (Phase I and Phase II), with a specific branch dedicated to aquaculture (VICH, 2000 and 2004). Some years later, the Committee for Medicinal Products for Veterinary use of the European Medicines Agency released a Technical Guidance Document (EMEA, 2007), which provides more specific guidance information in accordance to the recommendations presented by the VICH guidelines. The VICH Guidelines Phase I consists of a straightforward decision tree aimed at estimating the environmental exposure of the substance to adjacent aquatic ecosystems. According to this decision tree, the application of products resulting in a low environmental exposure level (i.e., substances extensively metabolized in the treated animal) will be considered as having low direct environmental risk and have their ERA terminated at Phase I. This might, for example, be the case for vitamins and some injected vaccines. On the other hand, compounds applied in non-confined aquaculture facilities, like parasiticides, and those for which the recommended dose results in a water concentration exceeding the cut-off value of 1 µg/L require evaluation at the second phase.

Phase II is divided into two tiers (Tier A and B) and aims to determine risk quotients (RQ), which are calculated by dividing the predicted environmental concentration (PEC) by the predicted no effect concentration (PNEC). Tier A requires the evaluation of a dataset comprising physical-chemical properties of the compound (e.g. water solubility, K_{ow}), environmental fate studies (e.g. degradation in aquatic ecosystems) and acute effect studies on at least three aquatic organisms (i.e., one algae, one Daphnid and one fish species) belonging to different taxonomic groups. RQs are calculated for every taxonomic group and if any RQ is ≥ 1, guidelines are given to calculate a refined PEC by considering several environmental processes and circumstances (e.g. adsorption to sediment, temperature and salinity effects). If the RQ remains higher than one, the substance needs further evaluation under Tier B (VICH, 2004).

In Tier B, RQs are calculated with the refined aquatic PECs calculated in Tier A and PNECs derived from chronic toxicity studies performed with crustaceans, fish and algae within the environment under study (i.e., freshwater or saltwater). In addition, if the RQ for invertebrates in the Tier A is ≥ 1, guidance is provided on how to calculate PECs for the sediment and a long-term toxicity study on benthic aquatic organisms is required. For highly hydrophobic substances (log Kow > 4), environmental fate studies on bioconcentration in fish are also investigated. Finally, if the calculated sediment or aquatic RQs are still ≥ 1 and/or the bioconcentration factor for fish is ≥ 1000, the Technical Guidance Document (EMEA, 2007) introduces the possibility to refine the exposure assessment by (1) assessing the degradability of the active ingredient in the actual environment under consideration, (2) including toxicokinetic studies, and/or (3) using reliable monitoring data, when available. The VICH guidelines assume that non-relevant metabolites (excreted metabolites that represent ≤ 10% of the administered dose and do not form part of biochemical pathways) are equal or less toxic than the parent compound and, hence, the focus on the parent compound will provide a conservative estimation. De Knecht et al. (2009) pointed out that studies assessing the environmental fate and effects of metabolites of VMPs are very limited mainly due to economic and technical reasons, and they supported the use of alternative methods such as QSARs (quantitative structure-activity relationships) and QSPRs (quantitative structure property relationships) for these purposes.

Even though specific guidance on how to refine the effect assessment on surrounding aquatic ecosystems has not been provided, the possibility of using toxicity data from other non-target organisms and from ecosystem studies is indicated in these guidelines. Probabilistic approaches like the species sensitivity distribution concept which include toxicity data for a wide range of species have been used in the refinement of PNECs for a wide array of chemicals (Posthuma et al., 2002). Microcosm and mesocosm experiments have been identified as promissory tools in the derivation of higher-tier threshold concentrations for VMPs since they allow testing ecological impacts under more realistic exposure patterns, considering direct and indirect effects and recovery of initial effects, not only at a species level, but also at a community level (Van den Brink et al., 2005). On occasion, higher-tier marine investigations have been performed by using highly limited and controlled amounts of aquaculture chemicals under full commercial use conditions (Telfer et al., 2006). This allows a range of field investigations including measurements of water quality parameters and environmental concentrations and distribution of the parent compound and their primary metabolites in a number of sentinel organisms. In addition, the short and long term effects of chemicals on pelagic and/or benthic communities can also be assessed (Willis et al., 2005; Telfer et al., 2006).

4. Research needs for the ERA of aquaculture chemicals in Asia

4.1. Information on chemical use

Only nine of the twenty-three (39%) reviewed studies report quantitative information on dosage and frequencies of chemical application (Table 1). The available data are mainly based on recommendations provided by chemical producing companies rather than actual application

dosages, and focus on pond grow-out systems, whereas hatcheries, nurseries and cage-based farming systems are misrepresented. Furthermore, most of the information presently available was collected several years ago and is of limited relevance given the fast development of the sector and the new trends in aquaculture production (e.g. displacement of antibiotic treatments by biosecurity preventive measures or probiotics). Moreover, in the context of vibrant chemical supplies, the changing attitudes of Asian aquaculture famers towards chemical use are not well evidenced. It is necessary to collect updated and accurate information on (1) farmers knowledge and attitudes towards the use of chemicals, (2) names of active ingredients that are currently used, their mode of application and their actual quantities applied by farmers as well as scenario-specific description parameters (e.g. water volume of ponds, dilution factors to the surrounding environment), and (3) information on aquaculture management practices (e.g. frequency of effluent discharges, pre-treatment or post-treatment of effluents). This information is crucial for developing exposure scenarios for aquatic ecosystems and performing site-specific risk assessment studies.

4.2. Environmental exposure assessment

Environmental models that aim at estimating the environmental fate and distribution of chemical residues released from different aquaculture production systems are essential tools in prospective risk assessment studies. Whereas a number of modelling tools for calculating release and environmental distribution of particulate organic matter and veterinary medicines or chemicals used in marine cage aquaculture have been developed (e.g. Henderson et al., 2001; SEPA, 2003; Mente et al., 2006), those for inland pond and cage aquaculture systems are scarce. The VICH guidelines for assessing environmental risks of VMPs described above provide useful guidance to conduct exposure assessments. They are, however, not developed in detail, and specific characteristics of different aquaculture production systems are not properly addressed. Metcalfe et al. (2009) provide detailed technical advice and a useful list of simple algorithms for the calculation of lower-tier PECs of veterinary medicines applied in several production systems (i.e., baths or in medicated feeds in self-contained facilities or ponds, net pens and cage systems, flow-through systems, and recirculating systems). Phong et al. (2009) have developed a useful model based on mass-balance equations for estimating concentration of antibiotics (i.e., oxytetracycline and oxolinic acid) in fish ponds by adapting a pesticide fate model designed for rice paddy fields to typical aquaculture practices of south-east Asia. In a broader scale study, Rose and Pedersen (2005) simulated the environmental behaviour and fate of oxytetracycline in the water column and sediments of streams receiving effluent discharges from fish hatcheries in the US using the mass-balance simulation model WASP (Ambrose et al., 1993). Improved modelling tools should be developed that can include specific characteristics of different aquaculture scenarios of Asia (e.g. higher temperatures) as well as the temporal and spatial variability of environmental discharges in the calculation of peak and time-weighted average environmental concentrations.

Furthermore, the potential mixture of several compounds in the environment and the metabolism of aquaculture chemicals in the cultured species need to be considered when developing higher-tier exposure assessments. Reimschuessel et al. (2005) have constructed a database of drug metabolism studies in aquatic species based on more than 400 publications which facilitates the search of pharmacokinetic data for, among others, different species, culture environments, chemical dosages and modes of administration. This database, however, is mostly populated with studies performed with antibiotics in aquatic species cultured in temperate-cold regions (e.g. salmon, channel catfish and rainbow trout). The use of pharmacokinetic parameters calculated for different species and/or under different environmental conditions, such as water temperature and salinity, may lead to inaccurate approximations. Therefore, there is a need of developing more studies on the metabolism of aquaculture drugs in the warm-water species cultured in Asia, with especial emphasis on those used as antiparasitic and antimicrobial treatments.

Most of the available degradation and fate data for chemicals that is needed for developing exposure assessments have been derived using standard guidelines proposing the use of environmental conditions typical of temperate regions (e.g. temperatures of 20°C). However, tropical aquatic ecosystems are characterized by higher temperatures, radiation intensity and microbial activity (Daam and Van den Brink, 2010). Higher temperatures are expected to result in higher dissipation rates of chemicals since solubility, hydrolysis and vaporization are potentiated (see Sánchez-Bayo and Hyne, 2011 for pesticides). In addition, higher photodecomposition and microbial activity are also expected to contribute to a faster degradation of chemicals under tropical conditions. The study by Lai et al. (2009b) suggests that some antibiotics (i.e., oxolinic acid and flumequine) may degrade faster under the intense radiation conditions of tropical regions and, hence, parameters such as irradiation and water turbidity should be considered in the derivation of refined exposure concentrations for (sub)tropical aquatic ecosystems. Choo et al. (1994) found that the half-life time of the antibiotic oxytetracycline hydrochloride was 5 times higher in saltwater than in freshwater, using the same temperature, pH and light regime. These studies support the need of assessing fate and degradation parameters of chemicals for specific environmental conditions of the scenario under study. When such data is not available, at least differences between the environmental conditions of the original experimental set-up and the study area should be taken into account when performing exposure assessment studies.

Environmental monitoring studies of aquaculture chemicals have focused on assessing the environmental concentrations in water and sediments from marine (e.g. Coyne et al., 1994; Capone et al., 1996; Haya et al., 2005; Telfer et al., 2006) and freshwater aquaculture (e.g. Lalumera et al., 2004; Dietze et al., 2005; Boxall et al., 2006a) in temperate regions, with the largest emphasis being on antibiotics compounds and sea-lice treatments used in salmon aquaculture. Only one study was found to be available in the peer-reviewed literature relating chemical use with measured environmental concentrations of those chemicals in adjacent aquatic ecosystems in the (sub)tropical Asian region (Le and Munekage, 2004). Le and Munekage (2004) analyzed concentrations of four antibiotics (trimethoprim, sulfamethoxazole, norfloxacin and oxolinic acid) in drainage canals and shrimp ponds in Vietnam. The peak measured concentrations for these four compounds ranged between 1,040 and 2,500 µg/L in water and between 426 and 2,616 mg/kg in sediments (wet weight). Monitoring studies may provide primary data for developing retrospective risk assessment studies as well as for the validation of exposure models used in prospective analysis. Further research needs to focus on assessing the distribution and fate of aquaculture chemicals in aquatic ecosystems receiving aquaculture-induced chemical pollution in Asian countries with emphasis on aquaculture pond and cage systems with considerable water exchange.

4.3. Biological effects assessment

According to the VICH guidelines, lower-tier safe environmental concentrations for aquatic ecosystems must be derived from acute and chronic toxicity data for algae, crustaceans and fish which are characteristic of the environmental conditions of the region of use. For the calculation of toxicity data, a number of international standardized methods such as OECD guidelines for freshwater experiments (OECD, 1992) and ISO guidelines for saltwater experiments (ISO, 1995 and 1999) are proposed. However, these test procedures have been mainly developed for a limited number of aquatic standard test species and most toxicity databases are currently populated with toxicity data for aquatic species characteristic of temperate ecosystems. The feasibility of the temperate-tropical extrapolation of toxicity data has been studied by several authors comparing sensitivity of tropical and temperate aquatic ecosystems to mostly pesticides using results of laboratory and semi-field experiments (see Rico et al., 2011 and references therein). These studies support the initial use of temperate toxicity data for the ecological risk assessments in the tropics. However, due to the limited number of ecotoxicological studies available for aquaculture disinfectants and antibiotics, a large uncertainty remains for these compounds and further research should address these data gaps. Furthermore, most available

data on the toxicity of aquaculture chemicals to aquatic life is for freshwater organisms (Gräslund and Bengston, 2001), and their use in ecological risk assessments for marine environments may be disputable. Wheeler et al. (2002) compared species sensitivity distributions of freshwater and saltwater species and showed that, for pesticides and narcotic compounds, saltwater species tended to be more sensitive than freshwater organisms. On the contrary, the comparison of species sensitivity distributions comparisons performed by De Zwart (2002) and Maltby et al. (2005) based on toxicity data of crustaceans for several insecticides did not demonstrate significant differences. Since marine toxicity datasets are also mainly composed by temperate organisms, a research gap remains in assessing whether temperate marine toxicity data will result in a sufficient protection level for tropical marine ecosystems.

In order to get a better estimation of the potential toxicological risks of aquaculture chemicals for aquatic ecosystems adjacent to Asian aquaculture farms, further ecotoxicological studies should focus on the assessment of the effects of aquaculture chemicals on indigenous species. For this, international guidelines should be adapted to the environmental characteristics of the ecosystems under study (e.g. Satapornvanit et al., 2009; Rico et al., 2011), and sensitive species suitable for the monitoring of aquaculture-induced chemical pollution should be identified. Additionally, model ecosystem studies including sensitive endpoints relating to the specific mode of action of the studied compounds (e.g. studies on microorganism communities and primary producers for antimicrobials, studies on invertebrate communities for parasiticides) are recommended in order to get a better understanding on the potential effects of these chemicals on the structure and functioning of tropical ecosystems. These studies will contribute to the derivation of refined threshold concentrations, which in turn may serve to validate the use of lower-tier PNECs calculated with single-species toxicity data. Furthermore, since aquaculture farmers often rely on more than one chemical per production cycle (e.g. Gräslund et al., 2003) or use products that contain a combination of two or more different active ingredients, aquatic ecosystems are likely to be exposed to mixtures of compounds, potentially becoming more complex in aquaculture clustered areas. Christensen et al. (2006) studied the combined ecotoxicological effects of mixtures of antibiotics used in aquaculture, showing potential synergistic and additive effects on freshwater algae, activated sludge microorganisms and luminescent bacteria for several binary combinations of antibiotics and supporting the need to take mixtures into account in environmental hazard assessments. Drainage canals and streams receiving aquaculture effluents are likely to be exposed to different stressors co-occurring at the same time (e.g. veterinary medicines and nutrients co-existing with situations of dissolved oxygen depletion) and to stressors occurring in successive intervals (e.g. veterinary medicines) (Tello et al., 2010). Future research must consider multi-stress situations in aquatic ecosystems, also considering other pollutants (e.g. agricultural, industrial and urban chemical residues) in areas that are not only impacted by aquaculture activities.

5. Conclusions

The present study shows that there is a long list of chemicals and biological products that have been used over the last years in aquaculture production in Asia. Among them, disinfectants, pesticides and antibiotics have been demonstrated to be the most environmentally hazardous compounds due to their high toxicity to non-target organisms and/or potential for bioaccumulation over tropic chains, and can potentially affect biodiversity and functioning of adjacent aquatic ecosystems. Government and public awareness about chemical use in Asian aquaculture currently focus on risk-benefit analysis dominated by economic and food safety issues, whereas the potential risks for ecosystem health are not properly addressed. As aquaculture production is expanding and intensifying in Asia, it is envisaged that environmental concerns about the impacts of aquaculture chemicals in surrounding ecosystems will increase in the coming years, constituting a key issue in the assessment of the environmental sustainability of the sector. It is the responsibility of governments to integrate ERA studies in the market registration of potentially hazardous chemicals, as already implemented in several developed

countries. In this regard, the VICH guidelines described in the present study may serve as starting point for assessing environmental impacts caused by aquaculture-induced chemical pollution in Asia. Industry, academia and regulators have a shared responsibility to work together in the development and expansion of the knowledge which underpins the ERA for chemicals used in Asian aquaculture. There is now an urgent need to collect up-to-date quantitative information on chemical use associated to several aquaculture practices that can influence the distribution and fate of chemicals in the environment. Environmental risk assessment modelling tools need to be developed for specific production systems, with especial emphasis on pond and cage aquaculture, and should take into account specific aquaculture production scenarios (e.g. clusters or isolated farms), their interaction with the outside environment (e.g. relation between chemical applications and effluent discharges), and the effect of specific environmental characteristics interfering with the distribution and behaviour of chemicals in the aquatic ecosystems (e.g. temperature, salinity, dilution in adjacent water bodies). Simultaneously, laboratory and semi-field toxicity studies should assess the effects of aquaculture chemicals on indigenous non-target organisms, while chemical and biological monitoring programs should be implemented to allow an empirical evidence of the distribution and impacts of aquaculture pollutants on local aquatic ecosystems.

Acknowledgments

The authors are indebted to Liu Liping and Lin Ronghua for their contribution to an early version of this chapter.

Chapter 3

Use of veterinary medicines, feed additives and probiotics in four major internationally traded aquaculture species farmed in Asia

Andreu Rico, Tran Minh Phu, Kriengkrai Satapornvanit, Jiang Min, A. M. Shahabuddin, Patrik J.G. Henriksson, Francis J. Murray, David C. Little, Anders Dalsgaard, Paul J. van den Brink

Abstract

This study reports the outcomes of a survey on the use of chemical and biological products in 252 grow-out aquaculture farms and 56 farm supply shops in four countries in Asia. The survey was conducted between 2011 and 2012, and included nine aquaculture farm groups: Penaeid shrimp farms in Bangladesh, China, Thailand and Vietnam; *Macrobrachium* prawn farms, and farms producing both Penaeid shrimps and *Macrobrachium* prawns in Bangladesh; tilapia farms in China and Thailand; and Pangasius catfish farms in Vietnam. Results were analysed with regard to the frequencies of use of active ingredients and chemical classes, reported dosages, and calculated applied mass relative to production. A range of farm management and farm characteristics were used as independent variables to explain observed chemical use patterns reported by farmers within each group. Sixty different veterinary medicinal ingredients were recorded (26 antibiotics, 19 disinfectants, and 15 parasiticides). The use of antibiotic treatments was found to be significantly higher in the Vietnamese Pangasius farms. However, total quantities of antibiotics, relative to production, applied by the Pangasius farmers were comparable or even lower than those reported for other animal production commodities. Semi-intensive and intensive shrimp farms in China, Thailand and Vietnam showed a decrease in the use of antibiotic treatments. These farm groups utilised the largest amount of chemicals relative to production, with feed additives and plant extracts, probiotics, and disinfectants, being the most used chemical classes, mainly for disease prevention. The surveyed farmers generally did not exceed recommended dosages of veterinary medicines, and nationally or internationally banned compounds were (with one exception) reported neither by the surveyed farmers, nor by the surveyed chemical sellers. Factors underlying the observed differences in chemical use patterns differed widely amongst farm groups, and geographical location was found to be the only factor influencing chemical ingredient application patterns in the majority of the studied farm groups.

This chapter has been published in *Aquaculture* 2013, 412/413: 231-243.

1. Introduction

The Asian aquaculture sector has grown at a rapid pace during recent decades, and nowadays accounts for nearly 90% of the global aquaculture production (FAO, 2012a). Intensification of aquaculture practices in Asian aquaculture has often been accompanied by more frequent outbreaks of infectious diseases that require therapeutic treatment (Bondad-Reantaso et al., 2005). Natural and synthetic chemicals such as antibiotics, disinfectants, parasiticides, probiotics, and other feed additives have become indispensable inputs to treat and prevent bacterial and parasitic diseases, to improve water quality, and/or as growth promoters. The use of these substances has contributed to the productivity and growth of the Asian aquaculture sector, but has also attracted criticism. Chemical residues in the cultured organisms constitute a potential hazard for human consumers (Sapkota et al., 2008; Heuer et al., 2009), and indicate the fallibility of national and international food safety controls when they exceed food safety standards (Love et al., 2011). Moreover, the continued application of compounds such as antibiotics has been associated with the development of drug-resistant bacteria both inside and outside of aquaculture facilities (Le et al., 2005), and environmental residues of highly toxic substances, such as disinfectants or parasiticides, can exert toxic effects on non-target organisms, contributing to a potential degradation of ecosystems receiving aquaculture effluents (Rico et al., 2012a).

The current information on the use of chemicals and biological products applied by Asian farmers is very limited, or even unavailable for some important aquaculture species (Rico et al., 2012a). The Food and Agriculture Organization (FAO) reports the outcomes of a survey performed during 2009 on the use of aquaculture medicinal products on 12 different aquatic species groups, with special focus on four major aquaculture-producing countries in Asia (China, Philippines, Thailand and Vietnam) (Alday-Sanz et al., 2012). The outcomes of this survey show high frequencies of use for some Asian species groups (e.g. Pangasius, shrimp), and a greater availability of veterinary medicinal products in Asian markets compared to other regions (Alday-Sanz et al., 2012). However, with some exceptions, these data and other published data (see review by Rico et al., 2012a) have limited scope for species and country-specific comparisons, since information was collected from different sources and actors in different years, and fail to provide detailed descriptions of dosages and volumes applied. The collection of detailed information on the use of antimicrobials and other chemical inputs in Asian aquaculture is of crucial importance to evaluate their potential risks for human health and for the environment, as well as to evaluate the prudent use of such compounds, and their effectiveness for preventing and treating disease outbreaks.

In the current study we assessed the use of veterinary compounds, feed additives and probiotics for four internationally traded aquatic species based on a systematic survey of 252 grow-out farms and 56 farm supply shops. The survey covered nine aquaculture farm groups with different levels of production intensity, thus potentially showing different chemical use patterns. These were: (1) Penaeid shrimps in Bangladesh, China, Thailand and Vietnam, (2) *Macrobrachium* prawns, and concurrent shrimp and prawn production systems, in Bangladesh, (3) tilapia in China and Thailand, and (4) *Pangasius* catfish in Vietnam. This mix of countries and species was selected mainly due to their recent great increase in production and trade, both by volume and value (Fig. 1). The objective of the present study was two-fold. First, to quantitatively assess the current use of veterinary compounds, feed additives and probiotics in the aforementioned aquaculture farm groups and to compare them in terms of active ingredients used, actual *vs* recommended application dosages, and mass of chemicals applied relative to production. The second objective was to try to explain the differences in chemical use patterns observed in each of the studied aquaculture farm groups, in order to identify a potential relationship between the chemical use patterns and management characteristics of each aquaculture farm group. This was done by correlating data on farm-level aquaculture management practices and farm characteristics, with the data on chemicals and active ingredients used in the farms. The dataset provided by the current study offers the most extensive source of quantitative information on volumes and

dosages of chemicals applied in Asian aquaculture, and constitutes a basis for on-going studies aimed at: (1) assessing the appropriateness of the chemical use practices to treat and prevent disease outbreaks, (2) identifying occupational health hazards, and (3) performing human health and environmental risk assessments in each of the studied farm groups.

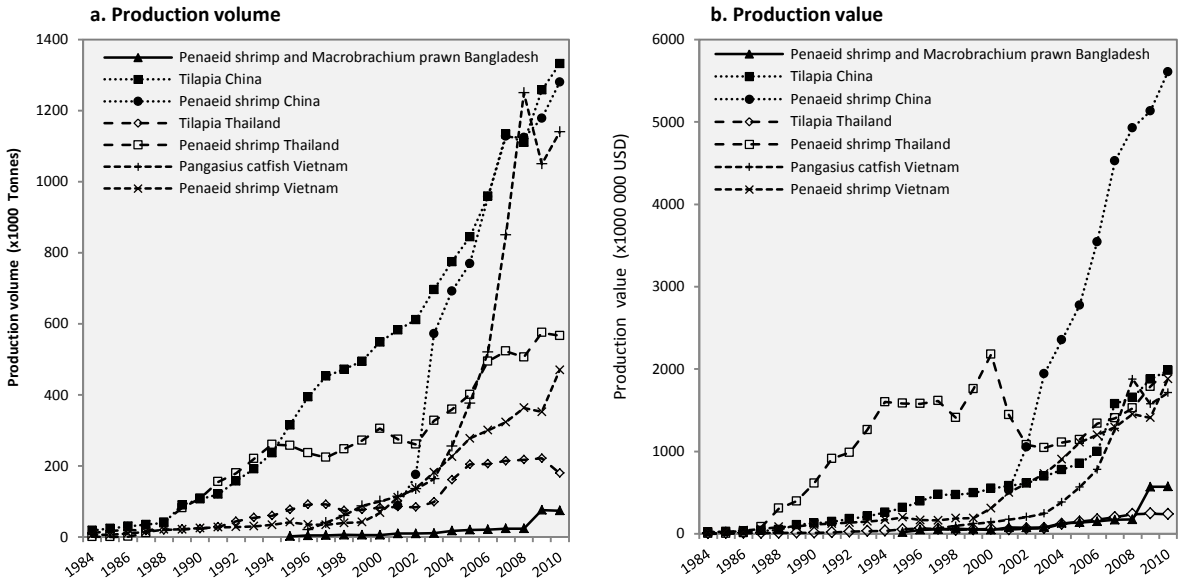


Figure 1. Trends of (a) production volume and (b) production value over the last decades for the aquaculture species included in the present study (Data source: FAO, 2012b).

2. Materials and methods

2.1. Chemical use interviews

Information on the application of veterinary compounds, feed additives and probiotics was collected between 2011 and 2012 through structured interviews conducted with farm owners, managers or technical staff of 252 aquaculture grow-out farms (Table 1). These farms were selected as a sub-sample of more than 1,600 farms for which baseline data on aquaculture management practices, social and economic aspects had previously been collected in the SEAT project Primary Survey (Murray et al., 2013). The studied sub-set of aquaculture farms was selected taking into account species group, farm-scale and geographical location as selection variables (see Murray et al., 2013 for rationale). This farm selection was directed towards a wide representation of different aquaculture practices with a wide geographical spread within the study areas. Interviews were also carried out with staff managing aquaculture farm supply shops, co-located in the areas where the farms were situated, thereby being likely points of purchase for those farmers. The objectives of these interviews were: (1) to be used as a validation step for chemicals that may have been under reported, (2) to build a chemical sales inventory to facilitate triangulation of the information provided by the farmers, and (3) to collect information on recommended dosages displayed on product labels. The characteristics of the surveyed aquaculture farm groups, geographical areas, dates of the interviews as well as number of farms and farm supply shops interviewed are detailed in Table 1.

The interviews with the grow-out farmers were conducted using structured questionnaires by local staff members experienced with aquaculture and with previous training in collection of chemical use data. The questionnaire comprised three sections: (1) respondent characteristics, (2) farm characteristics and management practices, and (3) chemical use. For each of the veterinary compounds, feed additives and probiotic products used, information was collected with regards

to: percentage of the main active ingredient(s) in the formulation used, application purposes and methods, dosage, pond water depth and cultured species biomass at the time of application, treatment duration, and average number of treatments per culture cycle.

Table 1. Characteristics of the interviewed farms and chemical use interviews.

Country	Main species	Main production system [a]	Region / Province(s)	Interview dates	Number of farms	Number of supply shops
Bangladesh	Shrimp (*Penaeus monodon*)	Improved extensive (brackish water ponds)	South-west / Khulna	June 2011 to November 2011	24	19 [f]
	Shrimp and Prawn (*Penaeus monodon* and *Macrobrachium rosenbergii*)	Improved extensive concurrent with rice [b] (brackish/freshwater ponds)	South-west / Khulna	June 2011 to January 2012	22	
	Prawn (*Macrobrachium rosenbergii*)	Improved extensive concurrent with rice [b] (freshwater ponds)	South-west / Khulna	November and December 2011	20	
China	Tilapia (*Oreochromis niloticus*)	Intensive and semi-intensive polyculture (freshwater ponds) [c]	South-east / Maoming	August and September 2011	25	5
	Shrimp (*Litopenaeus vannamei*)	Intensive monoculture and polyculture (brackish water ponds) [d]	South-east / Zhanjiang	October 2011	30	5
Thailand	Tilapia (*Oreochromis niloticus*)	Intensive and semi-intensive monoculture and polyculture (freshwater ponds) [e]	Central / Suphanburi, and Nakhon Pathom; East / Chachoengsao	September 2011 to March 2012	31	5
	Shrimp (*Litopenaeus vannamei*)	Intensive monoculture (brackish water ponds)	East / Chachoengsao and Chanthaburi; South / Surat Thani	October and December 2011	34	4
Vietnam	Catfish (*Pangasianodon hypophthalmus*)	Intensive monoculture (freshwater ponds)	South / An Giang Province, Can Tho, Dong Thap and Tra Vinh	October 2011 to February 2012	32	9
	Shrimp (*Penaeus monodon*)	Intensive and semi-intensive monoculture (brackish water ponds)	South / Soc Trang and Bac Lieu	December 2011 to February 2012	34	9
Total					252	56

[a] For a definition of the production systems see Murray et al. (2013)
[b] Tipically co-cultured with fish species (e.g. *Hypophthalmichthys molitrix, Catla catla, Labeo rohita*)
[c] All the interviewed farms cultured tilapia in combination with carps.
[d] The 27% of the interviewed farms combined the culture of shrimps with pompano (*Trachinotus ovatus*), mud crab (*Scylla serrata*) or prawns (*Macrobrachium rosenbergii*).
[e] The 61% of the interviewed farms cultured tilapia in combination with carps.
[f] The records obtained from the farm supply shops in Bangladesh could not be exclusively attributed to specific species as they are produced in the same area.

2.2. Data analysis

2.2.1. Compound classification

The chemical and biological products were classified into five categories: (1) antibiotics, (2) disinfectants, (3) parasiticides, (4) feed additives and plant extracts, and (5) probiotics. The parasiticide group also included compounds with biocidal properties (e.g. insecticides), used to kill unwanted organisms entering the aquaculture production systems with the inflow water. The main active ingredient(s) in each of the reported products was often recorded for antibiotics, disinfectants and parasiticides. When it was not available, it was identified by searching the reported product name in the sales inventories, and/or by cross checking with published literature. Products in the category of feed additives and plant extracts were classified as amino acids, herbs, minerals, polysaccharides and vitamins. However, due to the complexity of these formulations, the active ingredients were not further identified. For the same reason, the bacterial composition of the probiotic formulations was only qualitatively described.

2.2.2. Comparison of reported and recommended dosages

Reported dosages of antibiotic, disinfectant and parasiticide compounds were compared with recommended dosages. As farmers typically report dosages in mass of formulated product per area unit, the reported dosages were recalculated into standard dosage units (e.g. mg a.i. L^{-1}, for compounds applied directly to water, or mg kg^{-1} body weight of cultured organism, for compounds applied mixed with feed) and compared with the recommended dosages recorded from the labels of the products sold in the farm supply shops. Where the recommended dosages were unavailable, additional information on dosages was obtained from the literature (e.g., Noga, 1996; Arthur et al., 2000).

2.2.3. Calculation of chemical mass inputs

The chemical mass applied per average tonne of harvested produce in each farm group was calculated for each active ingredient in the antibiotic, disinfectant and parasiticide categories, for each product class in the feed additives and plant extracts category (i.e., amino acids, herbs, minerals, polysaccharides, vitamins and other feed additives), and for probiotics. This was done by calculating the mass applied of each chemical or product relative to production for each individual farm, and multiplying this value by the frequency of farmers that reported their use within the farm group. The methodology used for the calculation of the chemical or product mass used relative to production for each individual farm is described in the Supporting Information. The calculated chemical mass applied was compared with literature data available for other aquaculture (salmon) and non-aquaculture commodities.

2.2.4. Multivariate analysis

Multivariate analyses were performed in order to assess the differences in active ingredients and chemical categories of compounds used among the studied farm groups. A dataset containing all the chemicals reported (dependent variables) was used to test the differences between different aquaculture farm groups (independent variables) in their chemical use practices. Multivariate analyses were also used to explore correlations between the respondent and farm characteristics, and the reported differences in chemical use at farm level. Specific chemical use datasets (dependent variables) for each of the studied farm groups were prepared in order to test the significance of each of the 16 descriptive parameters (independent variables) shown in Table 4 on the prevalence of chemical use. These 16 parameters were selected considering their potential to influence chemical and biological product management practices. Significance of the correlation between the independent variables and the variance in the chemical use datasets was tested by performing 499 Monte Carlo permutations by Redundancy Analysis (RDA) using the CANOCO 5 software package (Ter Braak and Smilauer, 2012). The correlation of the tested independent variable was considered significant when $p \leq 0.05$, and marginally significant when $0.05 < p < 0.1$. Individual biplots were constructed for each chemical use dataset including only those independent variables that resulted in significant or marginally significant correlations.

3. Results

3.1. Chemical use in grow-out farms

3.1.1. Antibiotics

The studied farms reported use of a total of 20 different antibiotic compounds. The percentage of shrimp farms that reported to use antibiotics in Thailand (2.9%), Vietnam (2.9%) and China (6.7%) and the percentage of tilapia farms that reported to use antibiotics in Thailand (9.7%) and China (16%) markedly contrast with the frequency of use in the Vietnamese Pangasius farms (100%)

(Fig. 2; Table 2). The use of antibiotics in Bangladesh was only reported in one out of the 66 interviewed farms. Oxytetracycline, amoxicillin, florfenicol and enrofloxacin were reported in at least two countries. Overall, a maximum of two antibiotics were reported to be applied on any individual farm, with the exception of Pangasius farms in Vietnam. Here, 17 different antibiotic compounds belonging to 10 different antibiotic classes were used (penicillins, aminoglycosides, cephalosporins, quinolones, tetracyclines, amphenicols, polymyxin, diaminopyrimidines, rifamycins and sulfonamides), with enrofloxacin (69% of farmers used it), florfenicol (63%), sulfamethoxazole potentiated with trimethoprim (44%), and doxycycline (34%) being the most common ones (Fig. 3). Pangasius farmers reported use of three different antibiotics on average, and 10% of the interviewed farms reported use of five or six different antibiotics. It must be noted, however, that some antibiotic formulations used by Pangasius farmers contained a mix of two different active ingredients (sulfadimethoxine and ormetoprim; sulfamethoxazole and trimethoprim; apramycin and levofloxacin). In all cases, antibiotics were reported to be applied once a day mixed with feed for a period ranging between three and eight days. Most farmers reporting antibiotic use applied them to treat disease outbreaks, and only 5% of cases reported prophylactic use.

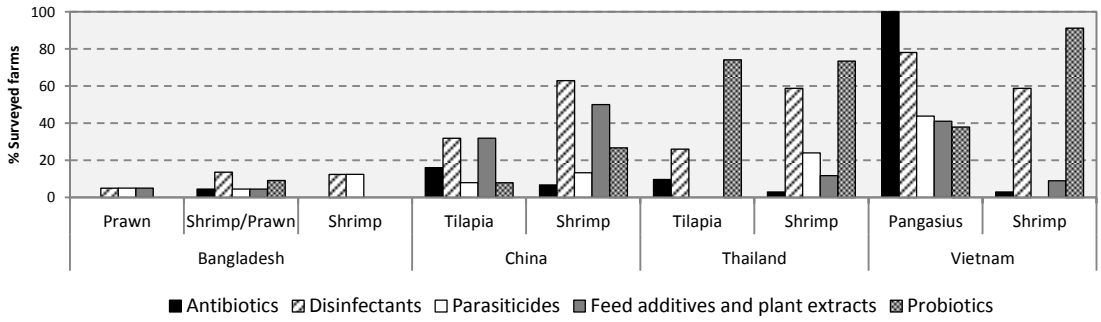


Figure 2. Percentage of farmers that use antibiotics, disinfectants,, parasiticides, feed additives and plant extracts, and probiotics in each of the studied farm groups. The background data of this figure is provided as Supporting Information (Table S1).

3.1.2. Disinfectants

Fifteen different disinfectants were reported to be applied for water disinfection and disease prevention (89% of the reported applications), as disease treatment (10%), and to disinfect equipment used during aquaculture operations (1%). Disinfectants were most commonly used among *Pangasius* farmers (78% of farms), which were applied regularly to the pond water to control bacterial proliferations, followed by shrimp farmers of China (63%), Thailand (59%) and Vietnam (59%) (Fig. 2; Table 2). The majority of the disinfectant applications done by these shrimp farmers were done for disease prevention, being applied directly to water during the culture cycle (65%) or prior to stocking (14%), and only 11% of the reported disinfectant applications were done to treat disease outbreaks. Overall, the most commonly used disinfectants were single doses of iodine solutions (iodophors such as povidone-iodine), chlorine and chlorine-releasing compounds (benzalkonium chloride, calcium hypochlorite), and potassium permanganate (Fig. 3), with intervals between applications ranging from seven days to months.

3.1.3. Parasiticides

A total number of 13 compounds were found to be used to treat (external and internal) parasite and fungal infections in the cultured species, and to kill unwanted organisms in the culture ponds. There were marked differences in compounds used and application purposes among countries and species. The highest frequency of application was found for the *Pangasius* farms (44% of the farms applied parasiticides) (Fig. 2; Table 2). Treatments with copper sulfate (25%) applied directly

to the pond water and in-feed medications of praziquantel (25%) were the most common parasiticide treatments, followed by in-feed applications of ivermectin (6.3%) (Fig. 3). About one fourth of the surveyed shrimp farmers of Thailand used the insecticides trichlorfon and/or dichlorvos (21%), or copper sulfate (6%), for killing unwanted organisms entering the ponds with the in-flow water during pond preparation prior to stocking. In-feed medications with avermectins and/or water treatments with fungicides (mebendazole and zinc sulfate) were reported to be applied in 13% of the surveyed shrimp farms in China, and the fungicide methylene blue was used in 13% of the shrimp farms in Bangladesh (Fig. 3).

Table 2. Summary data on the use of antibiotics, disinfectants and parasiticides in the surveyed farms: total number of recorded compounds (*n*), percentage of farms that use them (% use), and number of compounds used per farm (*n* per farm; median, minimum-maximum).

		Antibiotics			Disinfectants			Parasiticides			Total		
		n	% use	n per farm	*n*	% use	*n* per farm	*n*	% use	*n* per farm	*n*	% use	*n* per farm
Bangladesh	Prawn	0	0	0 (0-0)	1	5.0	0 (0-1)	1	5.0	0 (0-1)	2	10	0 (0-1)
	Shrimp/Prawn	1	4.5	0 (0-1)	2	14	0 (0-1)	2	4.5	0 (0-2)	5	14	0 (0-4)
	Shrimp	0	0	0 (0-0)	2	13	0 (0-2)	1	13	0 (0-1)	3	17	0 (0-2)
China	Tilapia	2	16	0 (0-1)	5	32	0 (0-1)	2	8.0	0 (0-2)	9	44	0 (0-3)
	Shrimp	2	6.7	0 (0-1)	8	63	1 (0-2)	4	13	0 (0-3)	14	63	1 (0-4)
Thailand	Tilapia	2	9.7	0 (0-1)	3	26	0 (0-1)	0	0	0 (0-0)	5	29	0 (0-2)
	Shrimp	2	2.9	0 (0-2)	6	59	1 (0-3)	4	24	0 (0-2)	11	79	1 (0-3)
Vietnam	Pangasius	17	100	3 (1-6)	6	78	2 (0-3)	3	44	0 (0-2)	26	100	6 (1-9)
	Shrimp	1	2.9	0 (0-1)	5	59	1 (0-4)	0	0	0 (0-0)	6	68	1 (0-4)

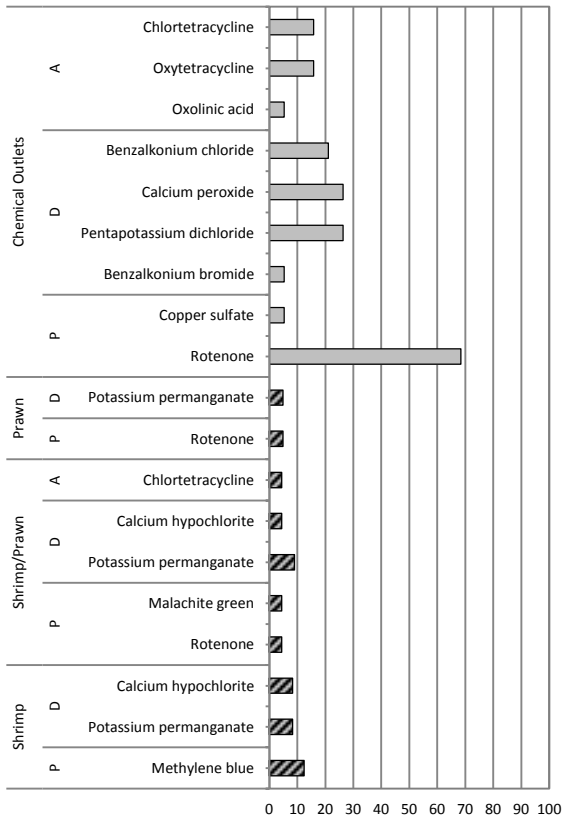
3.1.4. Feed additives and plant extracts

Feed additives and plant extracts, directly applied to the water or mixed with feeds, were predominantly used in Chinese shrimp farms (50% of farms), Vietnamese Pangasius farms (41%), Chinese tilapia farms (32%) (Fig. 2). Of the feed additives used in Chinese shrimp farms, the most common ones were vitamin premixes (38%), amino acids (24%), medicinal herbs and root extracts with antibacterial properties such as *Artemisia annua*, *Rheum rhabarbarum*, *Radix curcumae* and *Radix isatidis* (22%), as well as polysaccharides (11%). Pangasius farmers used predominantly herb extracts (61%) (e.g. *Combretum dasystachyum* Kurz) for pond water disinfection and feed supplements such as vitamin premixes (28%), amino acids (11%), and polysaccharides (11%). Chinese tilapia farmers only reported the use of medicinal herbs (54%) for disease treatment and mineral premixes (46%) as feed additives.

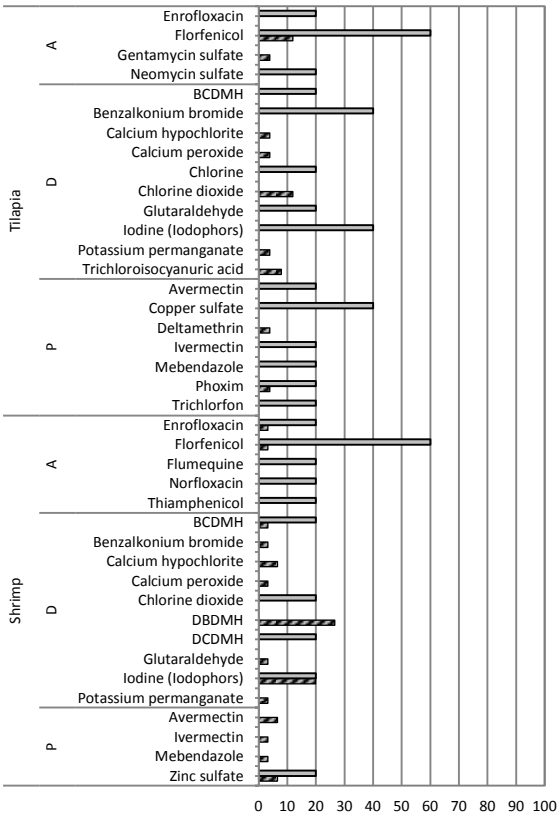
3.1.5. Probiotics

The use of probiotics was found to be highest in Vietnamese shrimp farms (91%), followed by the tilapia (74%) and shrimp (74%) farms in Thailand, Vietnamese Pangasius farms (38%), shrimp farms in China (27%), concurrent shrimp-prawn farms in Bangladesh (9%) and Chinese tilapia farms (8%) (Fig. 2). Probiotics were not reported to be used in the surveyed prawn and shrimp farms in Bangladesh. The probiotic products applied included a broad variety of formulations such as photosynthetic bacteria (e.g. *Rhodopseudomonas* spp.), microorganisms for nutritional and enzymatic contribution to digestion (e.g. *Bacillus* spp. and yeasts), and bacteria for improving water quality (e.g. *Nitrosomonas* spp., *Nitrobacter* spp.). The main bacterial genera were normally listed on the labels, but the specific species and their concentration in the products were most often not declared. Of the interviewed farmers, 84% reported to apply the probiotic products directly to water in order to improve the water quality and to reduce stress in the cultured species; whereas the other 16% reported to apply probiotics mixed with feeds as a nutritional supplement to improve food digestibility and the health conditions of the cultured species.

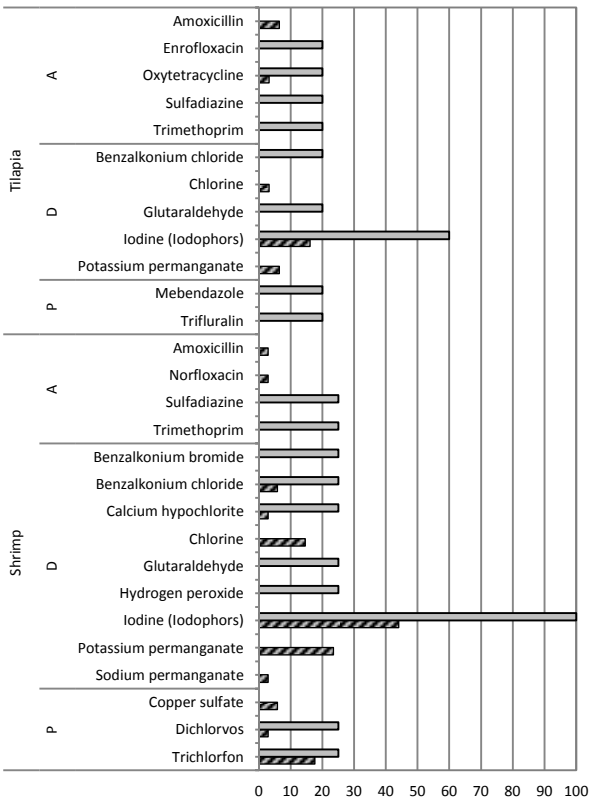
a. Bangladesh



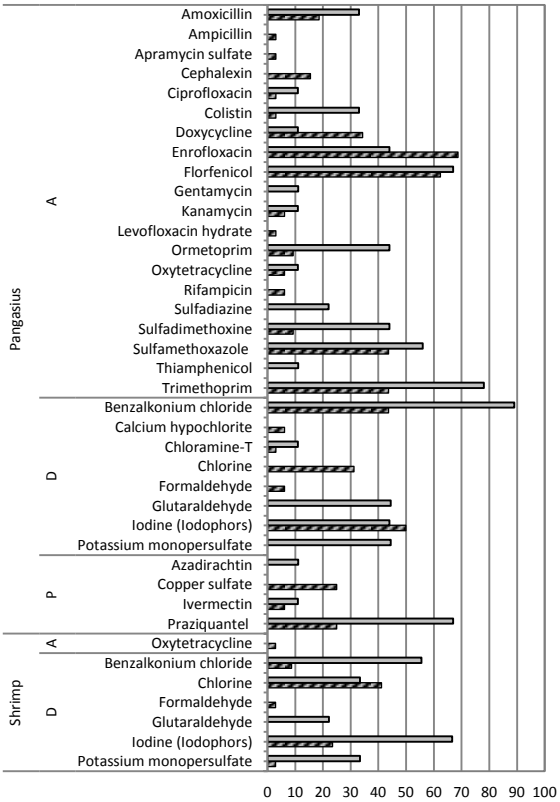
b. China



c. Thailand



d. Vietnam



% of outlets that sell it % of surveyed farms that use it

Figure 3. Percentage of farmers using antibiotics (A), disinfectants (D) and parasiticides (P) in each of the studied farm groups. The records obtained from the farm supply shops in Bangladesh could not be exclusively attributed to specific species as more than one species were produced in the same area. The background data of the figure is provided as Supporting Information (Table S2,S3). (BCDMH: 1-Bromo-3-chloro-5,5-dimethylhydantoin; DBDMH: 1,3-Dibromo-5,5-dimethylhydantoin;DCDMH: 1,3-Dichloro-5,5-dimethylhydantoin).

3.2. Chemicals sold in farm supply shops

The percentage of the surveyed farm supply shops in which the different compound groups were available are shown in Table 3, and the availability of the different antibiotics, disinfectants or parasiticides in Figure 3. Chemical compounds available in the supply shops that were not reported by the interviewed farmers ranged between 1 and 9 compounds per farm group (Table 3).

Table 3. Percentage of the surveyed farm supply shops that sell antibiotics, disinfectants, parasiticides, feed additives and plant extracts, and probiotics in the four studied countries. The right column shows the compounds available in the surveyed shops that were not reported by the interviewed farmers.

		A	D	P	Feed additives and plant extracts	Probiotics	Chemicals not reported by the surveyed famers
Bangladesh	Shrimp and Prawn	16%	63%	74%	37%	26%	A: oxytetracycline, oxolinic acid; D: benzalkonium choride, benzalkonium bromide, calcium peroxide, pentapotassium dichloride
China	Tilapia	80%	40%	40%	100%	100%	A: enrofloxacin, neomycin sulfate; D: BCDMH, benzalkonium bromide, chlorine, glutaraldehyde, iodine; P: avermectins, copper sulfate, mebendazole
	Shrimp	60%	80%	40%	100%	100%	A: Flumequine, norfloxacin, thiamphenicol; D: chlorine dioxide, DCDMH
Thailand	Tilapia	40%	60%	20%	60%	100%	A: enrofloxacin, sulfadiazine, trimethoprim; benzalkonium chloride, glutaraldehyde; P: mebendazole, trifluralin
	Shrimp	25%	100%	25%	0%	100%	A: sulfadiazine, trimethoprim; D: benzalkonium bromide, glutaraldehyde, hydrogen peroxide
Vietnam	Pangasius	89%	100%	78%	56%	78%	A: gentamycin, sulfadiazine, thiamphenicol; D: glutaraldehyde, potassium monopersulfate; P: azadirachtin
	Shrimp	0%	100%	0%	44%	100%	D: glutaraldehyde

A: antibiotics; D: disinfectants; P: parasiticides; BCDMH: 1-Bromo-3-chloro-5,5-dimethylhydantoin; DCDMH: 1,3-Dichloro-5,5-dimethylhydantoi

3.3. Comparison of reported and recommended chemical application dosages

The comparison between reported and recommended chemical application dosages for antibiotics, disinfectants and parasiticides is shown in Figure 4 as ratios between reported and maximum recommended dosages. The majority (77%) of the reported single application dosages fell below the maximum recommended application dosages (ratios below 1). Cases in which the reported dose exceeded the recommended dose by a factor of three or more (ratios above 3) were only reported in the shrimp farms in China (*n*=1), Thailand (*n*=6) and Vietnam (*n*=7), and on a single Pangasius farm in Vietnam (*n*=1), accounting for 11% of the all the evaluated cases. These cases mainly corresponded to applications of chlorine or chlorine releasing compounds (80% of the cases) for disinfection during pond preparation.

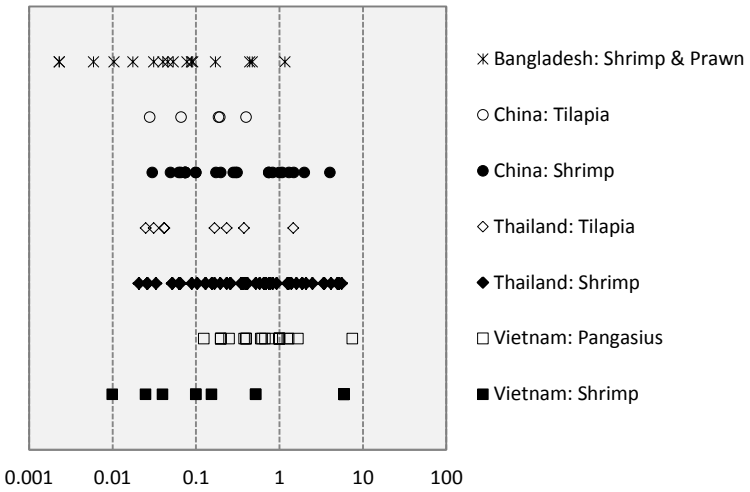


Figure 4. Ratios between calculated dosages based on the information reported by the farmers and maximum recommended dosages of antibiotics, disinfectants and pesticides. The figure only displays the data entries for which enough information was available to calculate the actual applied dosages.

3.4. Chemical mass inputs

The estimated amounts of each chemical class per average tonne of harvested produce are shown in Figure 5, and the disaggregated data (the amounts per active ingredient) for antibiotics, disinfectants, and parasiticides in Table S5. The highest mean amount of chemical inputs per tonne of harvested produce was calculated for the Chinese shrimp farms (18.8 kg tonne^{-1} harvested produce), followed by the Thai shrimp farms (18.2 kg tonne^{-1}), the Vietnamese shrimp farms (16.0 kg tonne^{-1}), and the Thai tilapia farms (13.5 kg tonne^{-1}) (Table S4). The total chemical inputs was dominated by probiotics in Thai shrimp and tilapia production (77 and 94%, respectively), and by probiotics, and feed additives and plant extracts, in the case of shrimp farms in China (50 and 46%, respectively) and Vietnam (35% for both compound categories). The largest amounts of veterinary compounds and biocides applied per tonne of harvested produce were calculated for the Vietnamese shrimp farms (4.8 kg tonne^{-1} harvested produce), followed by the Thai shrimp farms (4.1 kg tonne^{-1} harvested produce). In both cases this amount was dominated by disinfectants. Parasiticides contributed the most to the total applied mass in the Bangladeshi prawn farms (43%), followed by the Bangladeshi shrimp farms, and the Vietnamese Pangasius farms (5.6% for both). The marked difference observed in the Bangladeshi prawn farms, in respect to the other farm groups, can be explained by the low harvest yields and the high contribution of rotenone-containing plants and potassium permanganate (Table S5), which require high dosages for pond preparation and disease treatment in comparison to other (more toxic) parasiticides or biocidal ingredients (Table S2). The contribution of the antibiotics to the total chemical mass applied per tonne of harvested produce was markedly higher in the Vietnamese Pangasius farms (21%) in comparison to the other studied farm groups (Fig. 5b). It was estimated that Vietnamese Pangasius farmers, on average, used 93 grams of antibiotics per tonne of harvested fish (Table S4). The antibiotics that had the highest contribution to this amount were: sulfamethoxazole, cephalexin, amoxicillin, florfenicol and enrofloxacin (Table S5). In line with this, the antibiotic classes with the highest estimated application amount relative to harvested Pangasius biomass were, in decreasing order, sulfonamides, cephalosporins, penicillins, amphenicols, and quinolones (Table S6).

3.5. Factors related to chemical use

The results of the multivariate analyses showed that the use of chemicals by the different aquaculture farm groups was significantly different, as displayed in Figure 6. Antibiotics, probiotics and disinfectants were the most prominent chemical groups. This confirms the

relatively high use of antibiotics in the Vietnamese Pangasius farms in comparison to the other farm groups. It also shows a relatively high prevalence of probiotics use in the Vietnamese and Thai shrimp farms, and of disinfectants in the Vietnamese Pangasius farms, followed by the shrimp farms in Vietnam and Thailand.

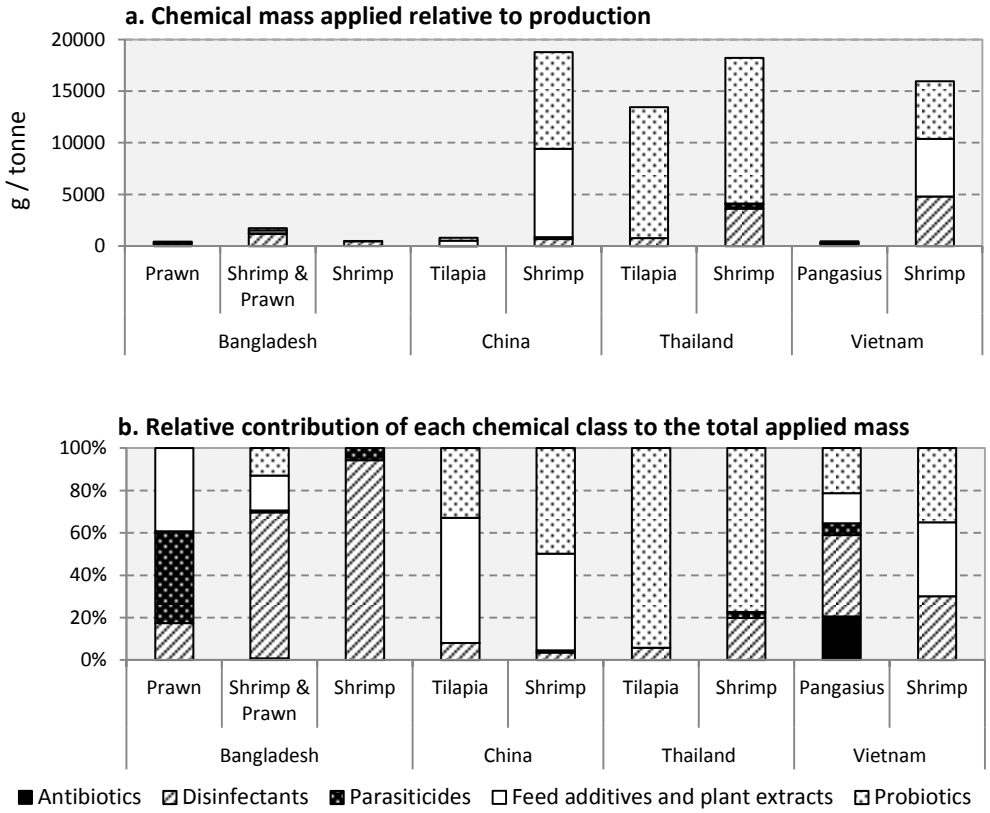


Figure 5. Calculated mass of each chemical and biological product group relative to production (a), in grams per tonne of harvested produce, and relative contribution of the applied mass of chemical or biological product category to the total applied mass (b). The mass of antibiotics, disinfectants and parasiticides is expressed in grams of active ingredient. The mass of feed additives and plant extracts, and probiotics is expressed in grams of formulated product. The background data on the calculated mass of each chemical and biological product group relative to production is provided as Supporting Information (Table S4).

The characteristics of the surveyed farms are shown in Table 4. The results from the Monte Carlo tests for each of the studied aquaculture farm groups, and the resulting biplots, are shown in the Supporting Information (Tables S7 and S8, and Figure S1). Although the number of chemicals reported to be applied in the surveyed Bangladeshi farms was very low, a trend was observed towards the use of a high number of compounds in the concurrent shrimp-prawn farms that reported the highest survival rates (survival shrimp: p = 0.01; survival prawn: p = 0.04). Also shrimp farms with higher reported stocking densities (p = 0.05) and longer crop durations (p = 0.05) tended to use a greater range of chemical inputs, and a similar trend was observed for larger prawn farms (p = 0.06), and those that reported the highest survival rates (p = 0.09).

In the surveyed Chinese tilapia farms, the frequencies of use of probiotics, feed additives and plant extracts, and parasiticides were marginally significantly higher for larger farms (p = 0.06), compared to smaller ones. For this farm group, higher frequencies were also positively correlated to the level of formal education of the respondents (p = 0.002). Farmers with university degrees were more likely to use them. In Chinese shrimp farms, a significant positive correlation was observed between the use of therapeutants, feed additives and probiotics, and increasing stocking densities (p = 0.04), annual yields (p = 0.02), and shrimp mortalities (p = 0.02). There was

also a trend towards greater use of chemicals and biological products in intensive monoculture systems (p = 0.07), compared to polyculture systems.

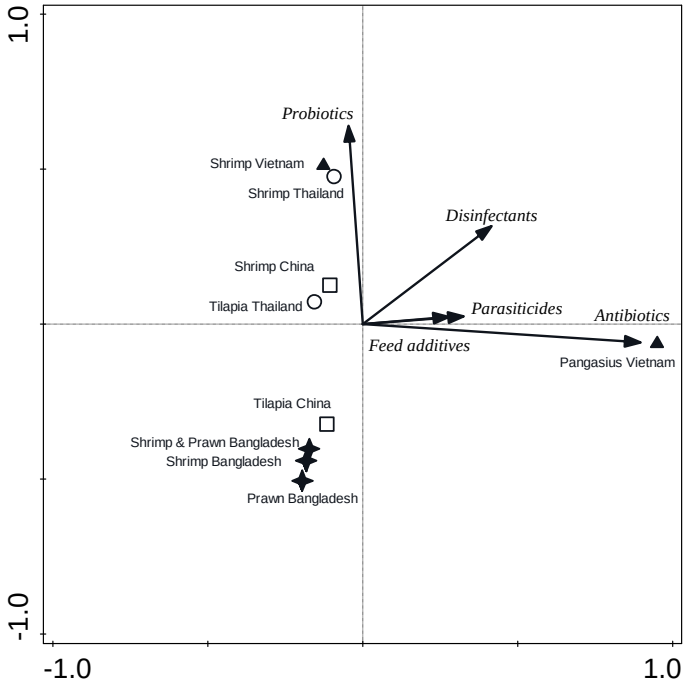


Figure 6. Ordination diagram (redundancy analysis; RDA) indicating the different use of chemical classes in the studied farm groups. The label 'feed additives' represents the compound category: feed additives and plant extracts. Of all the variance observed in the chemical use dataset, 57% can be explained by the different aquaculture farm groups. Of this explained variance, 50% is displayed on the first axis (x) and 6.1% on the second axis (y). The results of the Monte Carlo permutations indicate that a significant part of the variance on chemical use is explained by the different aquaculture farm groups (p = 0.002).

The chemical use data for Thai tilapia farms reflects a tendency towards differences in active ingredients used in different provinces (p = 0.06), and a higher frequency of chemical use by farmers that had received formal training on aquaculture production (p = 0.04). For the surveyed shrimp farms in Thailand, farm scale was found to be significantly correlated with reported chemical use patterns (p = 0.02), indicating that large farms, compared to the medium and small scale farms, tended to use more biocidal compounds as preventive measure for pond preparation (trichlorfon, dichlorvos, copper sulfate). On the contrary, use of probiotics and disinfectants was more commonly reported for medium-scale farms, and use of antibiotics and feed additives was most common in small-scale farms. The certification schemes adopted by shrimp farmers in Thailand had a marginally significant correlation with the compound classes used by them (p = 0.06). This points towards a relationship between the large farms, certified with the Thai Code of Conduct (CoC), and higher use of biocidal compounds.

The variance in the different active ingredients applied by the Pangasius surveyed farms could be significantly explained by the provincial distribution of the farms (p = 0.05), the adoption of the Global GAP certification scheme (p = 0.03), and the stocking density (p = 0.01). However, differences in compound classes used by different Pangasius farmers could only be marginally attributed to location (province) (p = 0.08) and the respondent's education level in aquaculture (p = 0.09). The latter indicating a link between no, or little, formal aquaculture training among farmers in the An Giang province, and high frequency of use of antibiotic compounds. None of the studied parameters resulted in significant effects on the chemical use reported by the shrimp famers in Vietnam, with only a marginal trend (p = 0.07) observed towards the highest use of disinfectants in the Soc Trang province, and the highest reported frequency of use of probiotics and feed additives in the Bac Lieu province.

Table 4. Parameters tested in the multivariate analysis, and characteristics of the respondents and surveyed farms in relation to each of the tested parameters.

		Bangladesh			China		Thailand		Vietnam	
		Shrimp (n=24)	Shrimp & Prawn (n=22)	Prawn (n=20)	Tilapia (n=25)	Shrimp (n=30)	Tilapia (n=31)	Shrimp (n=34)	Pangasius (n=32)	Shrimp (n=34)
Respondent characteristics	Role in the farm [a]	O (17); M (4); NR (3)	O (21); M (0); NR (1)	O (18); M (1); NR (1)	O (24); M (1); NR (0)	O (28); M (1); NR (1)	O (20); M (1); NR (0)	O (27); M (6); NR (1)	O (25); M (6); NR (1)	O (31); M (2); NR (1)
	General educational level [b]	NO (1); PS (4); SS (19); U (0); NR (0)	NO (2); PS (3); SS (17); U (2); NR (0)	NO (3); PS (5); SS (11); U (0); NR (1)	PS (5); SS (17); U (2); NR (1)	PS (6); SS (19); U (0); NR (0)	PS (15); SS (12); U (3); NR (1)	PS (10); SS (7); U (13); NR (4)	PS (5); SS (18); U (9); NR (0)	NO (8); PS (12); SS (9); U (5); NR (0)
	Aquaculture education [c]	NO (20); WT (4); U (0); NR (0)	NO (17); WT (5); U (0); NR (0)	NO (14); WT (3); U (0); NR (3)	NO (20); WT (3); U (1); NR (1)	NO (19); WT (11); U (0); NR (0)	NO (9); WT (22); U (0); NR (0)	NO (8); WT (21); U (5); NR (0)	NO (17); WT (7); U (8); NR (0)	NO (15); WT (12); U (1); NR (6)
Farm geographical location	Farm cluster [d]	Cluster A (6); Cluster B (6); Cluster C (1); Cluster D (6); Cluster E (5)	Cluster A (7); Cluster B (8); Cluster C (7)	Cluster A (7); Cluster B (6); Cluster C (7)	Cluster A (5); Cluster B (7); Cluster C (4); Cluster D (2); Cluster E (7)	Cluster A (12); Cluster B (6); Cluster C (12)	Cluster A (1); Cluster B (13); Cluster C (1); Cluster D (8); Cluster E (1); Cluster F (7)	Cluster A (2); Cluster B (3); Cluster C (5); Cluster D (1); Cluster E (1); Cluster F (6); Cluster G (3); Cluster H (8); Cluster I (5)	Cluster A (4); Cluster B (4); Cluster C (6); Cluster D (6); Cluster E (1); Cluster F (3); Cluster G (2); Cluster H (6)	Cluster A (1); Cluster B (1); Cluster C (6); Cluster D (6); Cluster E (11); Cluster F (9)
	Province	Khulna (24)	Khulna (22)	Khulna (20)	Maoming (25)	Zhanjiang (30)	Nakhon Phatom (17); Chachoengsao (14)	Chantaburi (10); Surat Thani (18); Chachoengsao (6)	An Giang (16); Dong Thap (9); Can Tho (5); Ben Tre (1); Tra Vinh (1)	Soc Trang (25); Bac Lieu (9)
Adopted certification schemes	Certification: YES or NO [e]	NO (24); YES (0)	NO (22); YES (0)	NO (20); YES (0)	NO (24); YES (1)	NO (30); YES (0)	NO (14); YES (11); NR (6)	NO (0); YES (33); NR (1)	NO (27); YES (5)	NO (34); YES (0)
	Certification scheme [f]	NO (24)	NO (24)	NO (24)	NO (24); PF (1)	NO (30)	NO (11); GAP (14)	NO (0); GAP (26); GAA/ACC/GAP (1); GAP/CoC (4); GAA/ACC/GAP/CoC (1); CoC (1); NR (1)	NO (23); PGGAP (4); GGAP (5)	NO (34)

Table 4. (continued)

		Bangladesh		China			Thailand		Vietnam	
		Shrimp (n=24)	Shrimp & Prawn (n=22)	Prawn (n=20)	Tilapia (n=25)	Shrimp (n=30)	Tilapia (n=31)	Shrimp (n=34)	Pangasius (n=32)	Shrimp (n=34)
Farm characteristics	Farm scale [g]	S (9); M (9); L (6)	S (13); M (9); L (0)	S (9); M (11); L (0)	S (15); M (8); L (2)	S (13); M (15); L (2)	S (19); M (11); L (1)	S (13); M (14); L (7)	S (15); M (11); L (6)	S (10); M (18); L (6)
	Aquaculture production system [h]	IE (24)	IE (22)	IE (20)	IP (4); IL (21)	IM (22); IP (7); SIP (1)	SIM (12); SIP (18); IM (1)	IM (34)	IM (32)	IEA (2); SIM (21); IM (11)
	Surface area (ha) [i]	14 (0.2-79)	0.5 (0.1-1.1)	0.4 (0.2-1.0)	3.3 (0.3-19)	3.2 (0.2-22)	4.4 (0.3-22)	16 (1.0-75)	2.9 (0.2-11)	2.7 (0.7-11)
	Number of grow-out ponds [i]	1	1	1	3.8 (1.0-17)	3.8 (1.0-16)	3.1 (1.0-13)	14 (0.6-80)	4.3 (1.0-15)	6.1 (2.4-17)
Farming records	Crops per year [i]	1	1	1.1 (1.0-1.5)	1.4 (1.0-2.4)	2.2 (1.7-3.0)	1.2 (1.0-2.3)	2.6 (1.9-4.0)	1.4 (1.0-1.5)	1.1 (1.0-2.0)
	Average crop duration (months) [i]	3.0 (2.5-4.7)	Shrimp 3.2 (3.0-4.0); Prawn 6.9 (4.0-12)	6.3 (5.0-8.5)	6.2 (3.6-12)	3.0 (2.1-3.8)	7.9 (4.7-12)	3.5 (2.7-5.1)	7.3 (6.0-8.9)	5.3 (3.0-7.1)
	Stocking density (individuals/m^2) [i]	7.4 (2.6-20.5)	Shrimp 3 (0.6-9.0); Prawn 1.7 (0.2-4.4)	2.8 (0.9-5.7)	3 (1.8-4.5)	154 (26.7-465)	2.5 (0.3-7.0)	64 (38-104)	52 (24-88)	25 (7.6-44)
	Annual yield (tons/ha/crop) [i]	NA	NA	NA	14.6 (4.4-26)	11 (1.4-32)	NA	NA	295 (76-509)	NA
	Survival (%) [i]	36 (13-84)	Shrimp 36 (7.0-77); Prawn 54 (15-85)	48 (19-80)	89 (78-100)	62 (39-80)	57 (40-85)	81 (70-96)	77 (61-88)	72 (33-92)

All information showed in this table was collected during the chemical use interviews, with the exception of the geographical location (cluster and province level classification), farm scale, aquaculture production system, survival rates, and the information on the categories of farm characteristics and farming records for the Thai farms, which was retrieved from the SEAT project Primary Survey (Murray et al., 2013).

[a] O: Owner; M: Manager; NR: Not reported.

[b] NO: None; PS: Primary school; SS: Secondary school; U: University studies; NR: Not reported.

[c] NO: None; WT: Workshop or training course in aquaculture; U: University studies on aquaculture; NR: Not reported.

[d] Farm cluster classification performed according to Murray et al. (2013).

[e] NO: Do not belong to any certification scheme; YES: Belong to one or more certification schemes; NR: Not reported.

[f] NO: None; PF: Pollution-free (Chinese national standard); GAP: Good Aquaculture Practices (Thai national standard); GAA/ACC: Global Aquaculture Alliance and Aquaculture Certification Council joint certification standard; CoC: Code of Conduct (Thai national standard); PGGAP: In preparation for obtaining the Gobal GAP standard; GGAP: Global GAP; NR: Not reported.

[g] S: Small; M: Medium; L: Large. For a description of the farm scale classification see Murray et al. (2013).

[h] IE: Improved extensive; IP: Intensive polyculture; IL: Intensive or semi-intensive integrated with livestock; IM: Intensive monoculture; SIP: Semi-intensive polyculture; SIM: Semi-intensive monoculture; IEA: Improved extensive alternate. For a description of the production system categories see Murray et al. (2013).

[i] Mean (95% Confidence Interval); NA: Not available.

4. Discussion

4.1. Chemical use in the surveyed farm groups

Sixty different veterinary medicinal ingredients were recorded including 26 antibiotics, 19 disinfectants and 15 parasticides. Based on the farm groups studied, semi-intensive and intensive shrimp production in China, Thailand and Vietnam were found to rely most heavily on chemical and biological product inputs per tonne of harvested produce, in comparison to the extensive shrimp and prawn systems of Bangladesh (Fig. 5a). Thus, a positive correlation was found between the estimated total amount of chemicals and biological products used by shrimp/prawn aquaculture farmers, and their production intensity. For shrimp farmers in Thailand, Vietnam and China, our results revealed relatively low frequencies of antibiotic use, while displaying more frequent use of disinfectant treatments (Table 2). This contrasts with the outcomes of similar surveys conducted during the last decade in shrimp farms in Thailand (Holmström et al., 2003) and Vietnam (Le and Munekage, 2004; Tu et al., 2006). Several factors that could explain these differences, and the apparent decline in the use of preventive and therapeutic antibiotic treatments in shrimp production, include: (1) the replacement of the black tiger shrimp (*P. monodon*), in Thailand and China, and nowadays starting to take place in Vietnam, by the white leg shrimp (*L. vannamei*), a species with higher growth rates and less vulnerable to specific diseases (Lebel et al., 2010); (2) the use of specific pathogen free (SPF) larvae; (3) the introduction of several biosecurity measures including the reduction in the occurrence of pathogens, parasites and predators by the regular use of several antibacterial agents and biocides for water (pre-)treatment and disinfection of the equipment and production systems; (4) the use of a wide range of prebiotic and probiotic formulations for improving the water quality and the health status of the cultured animals; (5) the development of microbial resistance to several antibiotics; (6) the pressure by national and international organizations to reduce their use due to food safety reasons and potential market restrictions. In line with these results, notifications from the EU Rapid Alert System for Food and Feed (RASFF) on food safety standard violations from antibiotic residues on shrimp products from these three countries have seen a considerable reduction in the last five years (Murray et al., 2011).

Amongst the fish producing farm groups, tilapia farmers from Thailand reported the largest amount of chemical inputs relative to production (mainly consisting of probiotics), followed by tilapia farmers in China, and Pangasius farmers of Vietnam (Fig. 5a). The highest prevalence of veterinary compounds use was observed in Vietnamese Pangasius farms (Table 2, Fig. 5b). For this farm group, a total number of 32 different ingredients were recorded, including those reported by farmers and those available in the farm supply shops, with farmers reporting an average use of 6 different veterinary compounds. The percentage of antibiotic use, as well as the most commonly recorded active ingredients, are in accordance with the results of the survey performed by Phuong (2010) during 2008, suggesting that the antibiotic use has been stable during the last few years. Antibiotics applied to Pangasius are mainly used for treatment of bacillary necrosis and the red spot disease, which are reported to be caused by *Edwardsiella ictaluri* and *Aeromonas* spp., respectively. The regular application of antibiotics and the use of doses below the therapeutic effective dose has resulted on the development of bacterial resistance and a consequent loss on the efficacy of some antibiotics (Dung et al., 2008, 2009; Bartie et al., 2012). The prevalence of antibiotic resistant bacteria in Pangasius fish ponds and the potential horizontal gene transfer from fish pathogens and other aquatic bacteria to humans requires further attention by local authorities. Nowadays, there are a total of 28 antibiotic ingredients authorized for use in aquaculture in Vietnam (Tai, 2012), and this list must be revised with especial attention to their resistance potential, excluding antibiotics that are also used in human medicine (e.g. penicillins, cephalosporins or rifampicin). The use of biosecurity measures to prevent bacterial diseases becomes almost impossible in Pangasius ponds due to the high fish densities and the required high water exchange rates (Phan et al., 2009). Thus, the main alternative to extensive antibiotic

use is the introduction of vaccines, as already done in the European salmon industry (Gudding, 2012). Research is currently dedicated to the development and testing of vaccines against *Edwardsiella ictaluri,* showing promissory results (Thinh et al., 2009; Dung, 2011).

The dataset generated through the interviews conducted in the farm supply shops was found to correspond reasonably well with the dataset generated in the farm interviews. Compound-specific discrepancies were not further investigated, as they could simply be related to the fact that farmers purchased their products in different shops or through feed retailers. The comparison between recommended dosages and those reported by the farmers demonstrates that there is no evidence to affirm that the surveyed aquaculture farmers overdose their cultured animals, but rather the opposite. In our study, exceeded recommended dosages were mainly attributed to chlorine and chlorine releasing compounds applied for pond disinfection prior to stocking in shrimp farms, and this could be explained by the different effectiveness of these compounds in presence of organic matter and the corresponding variable application dosages (Arthur et al., 2000).

Chemicals banned under national regulations in Bangladesh (BDOF, 2011), China (CMA, 2002), Thailand (Tukwinas, 2002) and Vietnam (VMARD, 2009), and major seafood importing countries, such as EU countries, United States, Canada and Japan (see Love et al., 2011) (such as chloramphenicol, nitrofurans, etc), were generally not reported to be applied by the interviewed farmers, neither were they found to be available in the interviewed farm supply shops. The exception was the parasiticide/fungicide malachite green, which was reported to be applied in only one out of the 66 surveyed farms in Bangladesh, and is currently internationally banned for use in aquaculture due to its attributed carcinogenic properties (Srivastava et al., 2004). Fluoroquinolone antibiotics, which have been recently banned for application in aquaculture in the US and Canada, were reported to be applied in China, Thailand, and Vietnam, with a markedly higher frequency of application in the Vietnamese Pangasius farms (especially enrofloxacin). The high rate of associated veterinary drug violations of Vietnamese aquaculture products, particularly for catfish, in US, Japan and Canada (Love et al., 2011) has forced the Vietnamese government to ban the use of enrofloxacin and ciprofloxacin for use in aquaculture (VMARD, 2012). Hence, it is expected that their use will have recently diminished.

4.2. Comparison with other food producing commodities

The interviewed Pangasius farmers were found to use a wider range of antibiotic ingredients than salmon farmers in European countries, Chile and Canada (Table 5). However, the amount of antibiotics used per tonne of harvested Pangasius produce in Vietnam (93g) did not exceed the most recent estimate for antibiotics used in Canadian salmon production, and is considerably lower than the most recently reported values for salmon production in Chile (Table 5). Estimated amounts of antibiotic used in the other farm groups investigated in the current study are well below the values reported for Pangasius production in Vietnam (Table S4), and are in the range of the most recently reported values for salmon production in Norway and United Kingdom (after the displacement of antibiotic treatments by vaccines). On the other hand, a study by Grave et al. (2010) showed that estimated amounts of antibacterial agents used in poultry and livestock production in European countries varies from 18 to 188 g a.i. tonne^{-1} of produced biomass. This suggests that the use of antibiotics in Pangasius production, and in all other presently studied aquaculture farm groups, fall short of the reported amounts for other important food-producing species. In comparison to the chemical use in salmon production, the surveyed aquaculture farmers, however, rely upon larger amounts of other antimicrobial agents for disinfecting ponds, water and equipment, and chemicals with biocidal properties, most commonly used for killing unwanted organisms entering the ponds with the in-flow water. The quantity of chemicals used to kill external parasites, worms, and fungal infestations (excluding other biocides) in our studied farm groups (Table S4), fall short, or in the range of, the quantities used in salmon aquaculture

(Table 5). A few exceptions, however, are concurrent shrimp-prawn farms and the shrimp farms in Bangladesh, and Pangasius farms in Vietnam. For these farm groups, the estimated amounts were one order of magnitude greater due to the application of fungicides, which require higher dosages in comparison to the antihelmintics and insecticides reportedly used in the other farm groups, and the parasiticide compounds traditionally used to control sea-lice infestations in salmon production (e.g. emamectin benzoate, deltamethrin, cypermethrin, hydrogen peroxide) (Burridge et al., 2010; Bravo, 2012).

Table 5. Quantities of chemicals used in Atlantic salmon aquaculture (g a.i. tonne^{-1} harvested fish).

Country	Compound class (year)	Mean (min-max)	No. of compounds	Reference
Norway	Antibiotics (1980-1989)	464 (163-864)	NA	NIPH (2009) [a]
	Antibiotics (1990-1999)	71.6 (1.28-257)	NA	
	Antibiotics (2000-2011)	1.42 (0.54-2.29)	4	
	Sea-lice treatments (2001-2008)	0.20 (0.15-0.26)	5	
	Antihelmintics (2001-2008)	0.31 (0.10-0.72)	2	
	Fungicides (2001-2008)	0.71 (0.52-0.82)	2	
	Sum all parasiticides (2001-2008)	1.21 (0.87-1.43)	9	
Chile	Antibiotics (1999-2003)	252 (210-280)	7	Bravo (2012)
	Antibiotics (2007-2008)	580 (640-520)	7	
	Parasiticides (1999-2003)	0.22 (0.14-0.28)	4	
	Parasiticides (2007-2008)	1.20 (0.88-1.52)	3	
UK	Antibiotics (2007)	11.7	NA	Burridge et al. (2010)
	Sea-lice treatments (2007)	1.50	NA	
Canada	Antibiotics (2007)	175	NA	Burridge et al. (2010)
	Sea-lice treatments (2007)	0.16	NA	

[a] Data for antibiotic use between 2009 and 2011 was obtained from: R. Gudding, Norwegian Veterinary Institute, Pers. Comm., 2013.
NA: Not available

4.3. Factors influencing chemical use patterns

The analysis of determinants on chemical use patterns indicated that the observed variability within the studied aquaculture farm groups cannot be explained by the same factor or group of factors (Table S7, S8). The farm characteristics influencing chemical use patterns were found to correspond with the culture intensity. For instance, the analysis showed a trend towards higher reported survival rates in the concurrent shrimp-prawn farms, and prawn farms that used them. This suggests that the introduction of chemical and biological treatments in these extensive systems, mainly as a preventive measure, could have contributed to increased survival (Fig. S1).

On the other hand, the Chinese shrimp farms with intensive monoculture practices showed a tendency towards a greater reliance on disinfectants, antibiotics and probiotics (Fig. S1). This trend was negatively correlated to the survival rates (Fig. S1), suggesting that these farms might have reached a maximum in terms of stocking and production intensity, which could be related to the need to use antimicrobial and probiotic treatments in order to control disease outbreaks. Chemical use patterns in Thai shrimp farms, however, showed a clear correlation between the chemical groups used and the size of the farms (Table S7). Thus indicating that larger farmers with better investment possibilities, and well established aquaculture management practices, tend to use greater amounts of biocidal products during pond preparation as a preventive biosecurity measure instead of antibiotic or expensive probiotic treatments. In comparison to the Chinese shrimp farmers, Thai farmers generally held lower stocking densities (Table 4) and also used less chemicals for disease treatment. Interestingly, the province in which a farm was located was the only factor that showed a clear correlation with the type of active ingredient(s) used in all the farm groups evaluated (Table S8). This suggests that regional chemical marketing strategies as well as the recommendations by fellow farmers are key drivers explaining variability in chemical use practices. On the other hand, the results of this study show that the application of

certification schemes in the surveyed tilapia and Pangasius farms does not imply a reduction on the use of antibiotic or other environmentally hazardous compounds in comparison to non-certified farms (Table S7). Conversely, large-scale shrimp farmers of Thailand that obtained the Thai Code of Conduct certification showed a trend towards more frequent use of biocidal compounds during pond preparation (i.e., biosecurity) than non-certified small- and medium-scale farmers (Table S7, Fig. S1).

4.4. Study limitations and recommendations

Given the limited number of interviewed farmers within each farm group, generalizations (e.g. to a species-country level) should carefully take into account the characteristics and type of production systems included in each of the studied farm groups. Moreover, further studies should also focus on chemical application patterns in hatcheries, nurseries and broodstock production, in order to get a comprehensive overview of the amounts of chemicals applied throughout the entire life cycle of aquaculture products. The majority of the grow-out farmers visited in the present study were producing for international markets, with the exception of some tilapia farms in Thailand. Future research should therefore look at different market segments, as export oriented products of Asian countries may often need to comply to more strict regulations than those aimed for domestic markets. Another challenge faced with some of the interviewed farm groups, was polyculture of more than one aquatic species, making it difficult to distinguish the main target species for which the chemical treatment was used for. In such cases, we assumed that significant investments would only be made for the primary (most valuable) species. Given this, and a number of other assumptions made during the calculation of the chemical application volumes, the final calculated values must be considered as estimations, and the variability observed in the results should be taken into account when using the calculated values for follow-up studies. Moreover, future studies investigating factors related to chemical use patterns should also preferably consider variability in disease occurrence and diagnostic capacity, in relation to therapeutic treatments, as important potential drivers for chemical use. The chemical inventories generated in the farm supply shops were found to be a useful source of information to get a broader picture on the status on chemical use, identify substances that are being introduced in the market, and for confirming the active ingredients commonly used by farmers. We therefore recommend such an approach for further surveys aimed at identifying chemical ingredients and evaluating chemical use dosages by aquaculture farmers in Asia.

Acknowledgements

The authors of this study would like to express our extreme gratitude to all the interviewed farmers and chemical sellers for their time and kind responses. We are indebted to the staff members of Kasetsart University (Thailand), Can Tho University (Vietnam), Bangladesh Agricultural University (Bangladesh), and Shanghai Ocean University (China) for conducting the surveys and for their collaboration in data entry.

Supporting Information

The Supporting Information of this chapter can be downloaded from:
http://dx.doi.org/10.1016/j.aquaculture.2013.07.028.

Chapter 4

Modelling environmental and human health risks of veterinary medicinal products applied in pond aquaculture

Andreu Rico, Yue Geng, Andreas Focks, Paul J. van den Brink

Abstract

A model called ERA-AQUA was developed to assess the risks posed by the use of veterinary medicinal products applied in aquaculture ponds for the targeted produce, surrounding aquatic ecosystems, consumers, and the trade of the aquaculture produce. The model calculates risks by following a risk quotient approach, by calculating predicted exposure concentrations (exposure assessment) and predicted no effect concentrations (effect assessment) for the endpoint under study. The exposure assessment is performed by combining information on the environmental characteristics of the aquaculture pond, characteristics of the cultured species, aquaculture management practices and physico-chemical properties of the compound under study. The model predicts concentrations of veterinary medicines in the pond water, pond sediment, cultured species, and in the watercourse receiving pond effluent discharges by mass balance equations. The effect assessment is performed by combining (eco)toxicological information and food safety threshold concentrations for the studied compound. In the present study, the scientific background, strengths and limitations of the ERA-AQUA model are presented together with a sensitivity analysis and an example showing its potential applications.

This chapter has been published in *Environmental Toxicology and Chemistry* 2013, 32: 1196-1207.

1 . Introduction

Veterinary Medicinal Products (VMPs) have been reported to be applied worldwide in aquaculture production to combat parasites, prevent and treat bacterial diseases, and as growth promoters (Lyle-Fritch et al., 2006; Burridge et al., 2010; Rico et al., 2012a). VMPs applied in aquaculture may be released into the environment through continuous or intermittent effluent discharges (Lalumera et al., 2004; Le and Munekage, 2004), posing a potential risk for the biodiversity and functioning of surrounding aquatic ecosystems. Moreover, some veterinary medicines can accumulate in medicated aquatic animals and might pose a potential risk for consumers and the trade of the aquaculture produce when residual concentrations in harvested animals exceed human health threshold concentrations and Maximum Residue Limits (MRLs) established in food safety controls, respectively (Albabouch et al., 2005).

Several models have been developed to assess the environmental fate and dispersion of veterinary medicines applied in marine cage aquaculture (Henderson et al., 2001; SEPA, 2003). However, the great bulk of the global aquaculture production is dominated by freshwater and coastal pond systems (Boyd et al., 1998), mainly distributed along the Asian continent and other tropical and sub-tropical regions (Bostock et al., 2010). Available environmental risk assessments for VMPs applied in aquaculture ponds are limited to a number of developed countries with rigorous regulatory frameworks (i.e., EU countries, US, Japan) and often rely on simple algorithms for the calculation of environmental exposure concentrations (e.g. Boxall et al., 2006a; Metcalfe et al., 2009). Such simplified risk assessment do not allow an efficient implementation of detailed aquaculture scenarios and often tend to overestimate risks by neglecting important chemical dissipation processes (e.g. sorption to sediment) and operating characteristics (e.g. water exchange dynamics). In addition, environmental fate assessments of VMPs applied in aquaculture farms rarely include a dynamic and realistic description of the pharmacokinetics and bioaccumulation of the studied drug in the cultured species (Rico et al., 2012a).

The ERA-AQUA model was developed to perform prospective risk assessments of VMPs applied in pond aquaculture. In this study, the ERA-AQUA model is introduced and the main processes and assumptions considered during the modelling process are described. A sensitivity analysis was performed in order to identify the most important parameters to take into account when developing exposure scenarios for VMPs applied in aquaculture ponds. Moreover, the potential of the model is shown through an example of a risk assessment performed for two different VMPs, oxytetracycline and benzalkonium chloride, applied in a striped catfish scenario developed for the Mekong Delta region, Vietnam.

2. The ERA-AQUA model

The ERA-AQUA model is able to estimate risks posed by the use of VMPs applied in aquaculture ponds for four different endpoints: the targeted produce, aquatic ecosystems receiving aquaculture effluents, consumers, and the trade of the harvested aquatic animals. An exposure assessment and an effect assessment are performed for each of the studied endpoints following a conservative approach. Finally, the risk assessment is performed using a risk quotient approach (i.e., by dividing a predicted exposure concentration by a predicted safe concentration) and showing the potential for exceedance of predicted safe VMP concentrations. The ERA-AQUA model (version 2.0) is incorporated in a graphical user interface in Microsoft Excel that allows the calculation of risks for a wide range of aquaculture scenarios and aquaculture VMPs and is freely available at www.era-aqua.wur.nl. In this section the exposure assessment, effect assessment and risk assessment calculations performed by the ERA-AQUA model are briefly described. For a detailed explanation of the equations used by the ERA-AQUA model the reader is referred to Rico et al. (2012b).

2.1. Exposure assessment

The exposure assessment calculations are performed by integrating 34 scenario parameters and 26 drug-related parameters describing the environmental characteristics of the pond under study and the watercourse receiving aquaculture effluents, physiological and feeding characteristics of the cultured species, aquaculture management practices, drug application schemes, physico-chemical properties of the applied drug and consumer's information (Table 1). The ERA-AQUA model predicts the dynamics of the concentrations of veterinary medicines in four different compartments: pond water, pond sediment, the cultured species, and the water layer of the watercourse receiving effluent discharges. The model includes fifteen drug transfer and dissipation processes modelled by mass balance equations (Fig. 1). The process-level equations included in the ERA-AQUA model are a combination of equations drawn from the literature and those formulated especially for the model.

Table 1. Minimum scenario and drug-related parameters required to perform a complete risk assessment with the ERA-AQUA model (version 2.0). Default value parameters and constant value parameters are not included.

Symbol	Description	Symbol	Description
Scenario Parameters			
Aquaculture pond characteristics		*Feed administration to the cultured species*	
A	Area of the pond	SFR	Daily specific feeding rate
$h_{pond\text{-}water}$	Initial water depth	w_{SFR}	Individual's weight of SFR
ss	Mass concentration of suspended solids in water	$p_{CH2,\ food}$	Lipid fraction of feed
$m_{om,ss}$	Fraction of organic matter in suspended solids	FCR	Feed conversion ratio in the cultured species
$h_{pond\text{-}sediment}$	Depth of the top sediment layer	FE	Fraction of eaten feed
f_{om}	Mass fraction of organic matter in sediment		
φ	Sediment porosity	*Water exchange management*	
ρ	Sediment bulk density	$IRRI$	Average daily water supply rate
T	Average ambient temperature	$DRAIN$	Average daily drainage rate
$PERC$	Average daily percolation rate	$t_{effluent}$	Duration of the effluent discharge
$RAIN$	Average daily rainfall rate		
$EVAP$	Average daily evaporation rate	*Watercourse characteristics*	
		$v_{watercourse}$	Flow velocity
Cultured species characteristics		$h_{watercourse}$	Water depth
w_0	Initial organism weight	b	Bottom width
w_{max}	Maximum organism weight	s	Side slope (horizontal/vertical)
ρ_{cs}	Initial cultured species density		
p_{CH2}	Lipid fraction of cultured organisms	*Consumer's characteristics*	
$MORT$	Mortality fraction during the culture period	$Cons$	Daily consumption of the cultured species
		bw	Consumer's body weight
Planned stocking/harvest time			
$Stock$	Planned stocking day	*Simulation period*	
$Harvest$	Planned harvest day	$Simulation$	Duration of the simulation period
Drug-related Parameters			
Drug application		*Drug pharmacokinetics*	
DA	Drug administration method	$BioT_{1/2(Mref,Tref)}$	Biological half-life in the cultured species
D	Drug dose applied per day	$w_{ref,BioT1/2}$	Individual's weight of $BioT_{1/2(Mref,Tref)}$
C_{IRRI}	(Dissolved) Drug concentration in irrigation water	$T_{ref,BioT1/2}$	Temperature of $BioT_{1/2(Mref,Tref)}$
Physico-chemical characteristics		*Toxicity data for the cultured species*	
M	Relative molecular mass	$EC50_{cs}$	Acute EC50 for cultured species
k_{ow}	Octanol/water partition coefficient		
k_{oc}	Sorption coefficient on organic carbon	*Toxicity data for non-target aquatic organisms*	
$SOL\ (T_{ref})$	Solubility in water at reference temperature	$EC50_{acute\text{-}algae}$	EC50-72h for algae
T_{refSOL}	Reference temperature of $SOL(T_{ref})$	$EC50_{acute\text{-}inv}$	Acute EC50-48h for *Daphnia sp.*
$VP(T_{ref})$	Vapour pressure at reference temperature	$LC50_{acute\text{-}fish}$	Acute LC50-96h for fish
T_{refVP}	Reference temperature of VP(T_{ref})	$NOEC_{chronic\text{-}algae}$	NOEC-72h for algae
$DT50_{water}$	Half-life degradation in water	$NOEC_{chronic\text{-}inv}$	Chronic NOEC-21d for *Daphnia sp.*
T_{refw}	Reference temperature of $DT50_{water}$	$NOEC_{chronic\text{-}fish}$	Chronic NOEC-28d for fish
$DT50_{sediment}$	Half-life degradation in sediment		
T_{refs}	Reference temperature of $DT50_{sediment}$	*Food safety data*	
		ADI	Acceptable daily intake
		MRL	Maximum residue limit

EC50: Median effective concentration; LC50: Median lethal concentration; NOEC: No observed effect concentration.

2.1.1. Calculation of the drug transfer and dissipation rate coefficients

The modelling calculations start by deriving a series of transfer and dissipation coefficients of the drug in the water and sediment compartments and correcting them for the ambient temperature of the modelled scenario. These are the volatilization rate coefficient, the degradation rates in water and sediment, and the desorption rate from sediment. The volatilization coefficient is calculated according to the method described by Mackay and Leinonen (1975), based on the relative molecular mass and the Henry's coefficient of the substance, the mass transfer coefficient of carbon dioxide in water, and the mass transfer coefficient of water in air. The degradation rate in water and sediment are calculated based on the empirical half-life of the compound in water and sediment respectively, assuming first-order kinetics. The desorption rate from sediment is calculated according to the hindered diffusion model described in Birdwell et al. (2007), which is based on the aqueous diffusivity of the compound in water, the sorption coefficient of the compound on organic carbon and sediment characteristics (i.e., porosity, bulk density, organic matter content). Subsequently, the drug uptake and elimination rate coefficients in the cultured species (i.e., absorption from water, assimilation rate from feed, excretion with water and egestion rate constants) and the individual's growth rate constant are calculated. Drug uptake and elimination rate coefficients in the cultured species are calculated according to the OMEGA bioaccumulation model described in Hendriks et al. (2001), and are derived based on the octanol-water partition coefficient of the drug, feed characteristics (e.g. lipid content) and cultured species characteristics (e.g. organism weight, lipid fraction, feed ingestion and assimilation rates). The individual's growth rate constant is calculated using the equation described in Hendriks et al. (2001), which takes into account the food ingestion by the cultured organisms and the assimilation rate, the later one being derived in the present model from the feed conversion ratio (FCR) of the feed in the cultured organisms. The biotransformation rate of the compound in the cultured species is calculated according to Van der Linde et al. (2001) from the difference between the experimentally derived elimination rate constant, previously corrected for the organism weight and the ambient temperature in the modelled scenario according to Arnot et al. (2009), and the estimated minimum elimination. The latter is the sum of the excretion, egestion and individual's growth rate constants.

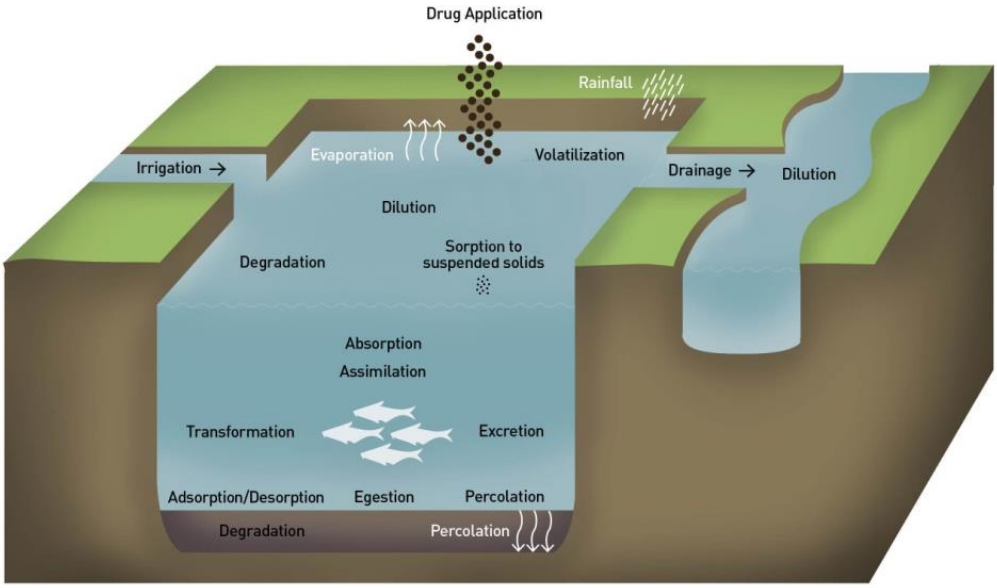


Figure 1. Drug transfer and dissipation processes included in the exposure assessment. From Rico et al. (2012b).

2.1.2. Calculation of the water balance and cultured species mass balance

The water balance in the aquaculture pond consists of water additions by irrigation and precipitation and water withdraws by effluent discharges, percolation and evaporation. The

modelling of the cultured species biomass includes potential mortality during the simulation period and biomass growth in the cultured species. The individual organism's growth is modelled according to the Von Bertalanffy's asymptotic growth equation (Von Bertalanffy, 1938), taking into account the initial organism's weight, the maximum organism's weight and the growth rate constant.

2.1.3. Calculation of predicted exposure concentrations

The ERA-AQUA model includes two possible drug administration methods: application mixed with feed (i.e., in-feed treatments) or application directly to water (i.e., bath treatments). For drugs applied mixed with feed, the drug mass that is ingested by the cultured species is assumed to be automatically up-taken after application, and the fraction that is not consumed is assumed to be instantaneously dissolved into the pond water. For drugs applied directly to water, an instantaneous mixing of the applied substance in pond water is assumed. Concentrations in the pond water, pond sediment and aquaculture species compartments are simultaneously calculated by three differential equations (Eq. 1-3). The pond water compartment consists of water and suspended solids, and the drug is assumed to reach an instantaneous equilibrium between both phases, determined by the sorption coefficient of the drug to organic carbon. The variation of the drug concentration in the water compartment depends on the adsorption/desorption to the sediment compartment; absorption to and excretion from the cultured species (i.e., physical transport with water); water irrigation, drainage and percolation; drug volatilization; and biochemical degradation processes (Fig. 1; Eq. 1). Moreover, the model provides the possibility of extra drug additions into the aquaculture pond via irrigation water, allowing the possibility to perform risk assessments for chemicals applied in up-stream aquaculture ponds or non-aquaculture chemicals entering the aquaculture pond via irrigation.

$$\frac{\partial M_{pond\,water}}{\partial t} = M_{adsorption/desorption} + M_{excretion} + M_{irrigation} - M_{drainage} - M_{percolation} - M_{volatilization} - M_{degratation} - M_{absorption}$$

Eq. 1

$M_{pond\ water}$ = drug mass in the pond water (M)
$M_{adsorption/desorption}$ = drug mass addition/loss due to adsorption/desorption from sediment (MT^{-1})
$M_{excretion}$ = drug mass addition due to excretion by the cultured species (MT^{-1})
$M_{irrigation}$ = drug mass addition with irrigation water (MT^{-1})
$M_{drainage}$ = drug mass loss by effluent discharge (MT^{-1})
$M_{percolation}$ = drug mass loss by percolation (MT^{-1})
$M_{volatilization}$ = drug mass loss by volatilization (MT^{-1})
$M_{degradation}$ = drug mass loss due to biochemical and photochemical degradation (MT^{-1})
$M_{absorption}$ = drug mass loss due to absorption from water by the cultured species (MT^{-1})

Drug concentrations in the top-layer of the pond sediment are modelled including adsorption/desorption dynamics, water percolation, biochemical degradation, and cultured species drug egestion, assuming that faeces are instantaneously settled into the pond sediment (Eq. 2).

$$\frac{\partial M_{pond\,sediment}}{\partial t} = M_{percolation} + M_{egestion} + M_{adsorption/desorption} - M_{degradation}$$

Eq. 2

$M_{pond\ sediment}$ = drug mass in the pond sediment (M)
$M_{percolation}$ = drug mass addition/loss by percolation (MT^{-1})
$M_{egestion}$ = drug mass addition due to egestion in the cultured species (MT^{-1})
$M_{adsorption/desorption}$ = drug mass addition/loss due to adsorption/desorption from sediment (MT^{-1})
$M_{degradation}$ = drug mass loss due to degradation processes in the sediment (MT^{-1})

The calculation of drug concentrations in the cultured species relies on five different processes: absorption from dissolved drug fraction in water, assimilation from feed, excretion, egestion, and biotransformation (Eq. 3).

$$\frac{\partial M_{cultured\ species}}{\partial t} = M_{absorption} + M_{assimilation} - M_{excretion} - M_{egestion} - M_{transformation}$$

Eq. 3

$M_{cultured\ species}$ = drug mass in the cultured species (M)
$M_{absorption}$ = drug mass absorption from water by the cultured species (M)
$M_{assimilation}$ = drug mass assimilation from food by the cultured species (MT^{-1})
$M_{excretion}$ = drug mass excretion in the cultured species (MT^{-1})
$M_{egestion}$ = drug mass egestion in the cultured species (MT^{-1})
$M_{transformation}$ = drug mass transformation in the cultured species (MT^{-1})

The above differential equations are numerically solved through a Visual Basic code for a given simulation period (i.e., 10 to 365 days) with time steps of one minute assuming that there is a maximum of one drug application per day and that the water exchange in the aquaculture pond starts 2h after the drug application. Finally, drug concentration dynamics in the effluent discharge point are calculated by taking into account the pond water concentration at the moment of the effluent discharge, the effluent's flow and the water flow in the watercourse. The latter being used to predict the dilution of the pond water concentration in the environment. The effluent's flow and the water flow in the watercourse are calculated from data on water exchange management (i.e., average daily water drainage rate and duration of the effluent discharge) and physical characteristics of the watercourse (i.e., water depth, bottom width, side slope and water flow velocity), respectively.

After drug concentration time series for each compartment have been calculated, the model calculates the highest momentary predicted concentration in the pond water (Peak Predicted pond Water Concentration: PeakPWC), the drug concentration in the cultured species in the moment of harvest (Predicted Cultures species Concentration: $PCC_{harvest}$), the highest momentary concentration in the effluent discharge point (Peak Predicted Environmental Concentration: PeakPEC), and time weighted average concentrations in the effluent discharge point for a time period of 3, 21 and 28 days (TWA3, TWA21 and TWA28). The later ones are conservatively derived by selecting the highest value of the time weighted average concentrations of those calculated for all of the time periods possible in the whole simulation period. In order to assess risks for consumers, the Estimated Daily Intake (EDI) of the studied compound for humans is calculated from the $PCC_{harvest}$, the estimated daily consumption of the cultured species and the consumer's body weight.

2.1.4. Limitations of the approach

Like many other models developed to assess the environmental exposure of chemicals, the ERA-AQUA model has some limitations that must be acknowledged. For instance, both the pond sediment and cultured species are treated as single and homogeneous compartments. Thus, drug losses from the top sediment layer due to diffusive transport to bottom layers are not included and pharmacodynamics of the drug in the cultured organisms are not taken into account. The first could result in differences between modelled concentrations in the top sediment layer (i.e., assumed to be subject to instantaneous mixing) and measured concentrations depending on the sediment depth fraction considered. The second may lead to differences between modelled and measured drug concentrations in the cultured species, depending on the sampled organ or tissue, and the metabolic routes used by the cultured species to detoxify the modelled compound.

Partitioning of VMPs in the water and sediment compartment in the ERA-AQUA model are related to the compound's hydrophobicity and the organic matter content in the sediment and

suspended solids. However, many VMPs (e.g. antibiotics) are ionizable weak acids across environmentally relevant pH gradients, and the pH and the cation exchange capacity of the absorption sites can have a major influence on their behaviour in terrestrial and aquatic environments (Brooks et al., 2012). Therefore, for substances with a logarithmic acid dissociation constant (*pKa*) in the range of the pH of the modelled aquaculture compartment the model could over estimate exposure. In a similar way, the uptake and elimination of ionizable pharmaceutical compounds by aquatic organisms is very sensitive to changes in pH (Meredith-Williams et al., 2012). Fu et al. (2009) argued that ionization typically decreases the bioaccumulation potential of organic chemicals in fish. But, for some moderate acids and bases there is an ion trap effect (i.e., dissociation of the compound inside the organism and slowing down the biomembrane crossing process) that can increase or decrease the biological accumulation depending on the pH gradient between the organism's body and the surrounding environment (Fu et al., 2009). In the ERA-AQUA model the uptake and elimination calculations are performed according to the OMEGA model (Hendriks et al., 2001), which largely depends on the octanol-water partition coefficient of the compounds and has only been validated for neutral organic substances (e.g., brominated flame retardants, organochlorines). Hence, the use of the ERA-AQUA model overlooks the influence of pH on partitioning, uptake and bioaccumulation, and will result in over- or underestimations of exposure concentrations for ionizable substances. Further development of ERA-AQUA will consider the scientific state-of-the-art of uptake models for ionizable substances in aquatic organisms.

2.2. Effect assessment

The effect assessment consists of determining safe exposure concentrations for each of the studied endpoints. A Predicted No Effect Concentration for the cultured species ($PNEC_{cs}$) is derived by dividing the short-term median effective concentration (EC50) for the cultured species (e.g. assessing mortality or growth inhibition) by an assessment factor. An assessment factor of 10 is used by the model as default value to extrapolate from the EC50 value to the No Observed Effect Concentration (NOEC) in the cultured species. In order to assess risks of the applied drug for adjacent aquatic ecosystems, acute and chronic PNEC's for algae, invertebrates and fish are calculated. Acute PNEC's for algae, invertebrates and fish (i.e., $PNEC_{acute\text{-}algae}$, $PNEC_{acute\text{-}invertebrates}$, $PNEC_{acute\text{-}fish}$) are derived by dividing the EC50-72h for algae (i.e., growth inhibition), the EC50-48h for *Daphnia* sp. (i.e., immobilization) and the LC50-96h for fish (LC50: median lethal concentration) by an assessment factor. The default assessment factor used by the model for the derivation of acute PNECs is 100. Similarly, chronic PNEC's for these three taxonomic groups (i.e., $PNEC_{chronic\text{-}algae}$, $PNEC_{chronic\text{-}invertebrates}$, $PNEC_{chronic\text{-}fish}$) are derived by dividing the NOEC-72h for algae (i.e., growth inhibition), the NOEC-21d for *Daphnia* sp. (i.e., reproduction), and the NOEC-28d for fish by an assessment for of 10. The ecological effect assessment approach included in the ERA-AQUA model is based on the use of standard toxicity test species and recommended assessment factors according to international guidelines for assessing environmental risks of aquaculture VMPs (VICH, 2004). However, recent research has shown that, for some pharmaceuticals including antibiotics, responses in alternative end points such as histological changes, biochemical responses, behavioural effects or gene regulation, occur in concentrations that are orders of magnitude lower than the concentrations derived by standard ecotoxicological test methods (VICH, 2004). Thus, it is increasingly recognized that the selection of appropriate test species and endpoints for the ecological risk assessment of VMPs must be based on the mode of action of the evaluated substance and the presence of pharmacological target pathways across different taxa and life stages (Boxall et al., 2012; Ankley et al., 2007). Keeping this in mind, the selection of the appropriate toxicity values and assessment factors in the derivation of PNECs can be freely changed by the user, also allowing the possibility to introduce threshold concentrations based on toxicity data for a larger number of species (i.e., derived from species sensitivity distributions) or model ecosystem studies.

In order to assess risks for consumers and trade the Acceptable Daily Intake (ADI) for humans and the Maximum Residue Limit (MRL) used in food safety controls are introduced as input values (see Table 1). When the ADI is not available, it is estimated by the ERA-AQUA model from the no observed adverse effect level for mammals (e.g. rats) and applying an extrapolation factor that can be chosen by the user. It should be noted that the selection of antimicrobial-resistant microorganisms in the cultured species and in the pond environment by VMP residues is not taken into account by the ERA-AQUA model, but will be considered in follow-up studies.

2.3. Risk assessment

Risk quotients are calculated for each of the studied endpoints by dividing the relevant exposure concentrations by the safe concentrations derived in the effect assessment. The risk quotient for the cultured species is calculated by: PeakPWC/$PNEC_{cs}$. Acute risk quotients for non-target aquatic organisms (i.e., $RQ_{acute\text{-}algae}$, $RQ_{acute\text{-}invertebrates}$, $RQ_{acute\text{-}fish}$) are calculated by dividing the PeakPEC by the respective acute PNEC's. Chronic RQ's for algae, invertebrates and fish (i.e., $RQ_{chronic\text{-}algae}$, $RQ_{chronic\text{-}invertebrates}$, $RQ_{chronic\text{-}fish}$) are calculated by TWA3/$PNEC_{chronic\text{-}algae}$, TWA21/$PNEC_{chronic\text{-}invertebrates}$ and TWA28/$PNEC_{chronic\text{-}fish}$, respectively. Risk quotients for the targeted produce and for aquatic ecosystems are calculated by default from the total drug water concentrations (i.e., sum of dissolved fraction and sorbed fraction to suspended solids). However, the dissolved and sorbed concentrations are also provided by the program, allowing the possibility to perform refined risk assessments considering only one of these two drug fractions. Risk quotients for consumers and trade are calculated by EDI/ADI and $PCC_{harvest}$/MRL, respectively. The risk assessment approach followed by the model is based on international guidelines for assessing human health (Benford, 2000; EMEA, 2000) and environmental risks of VMPs applied in aquaculture (VICH, 2004). If the resulting RQs are between one and ten, the model indicates exceedance of the predicted safe exposure value. For RQs higher than ten, a large exceedance of the predicted safe concentration is indicated by the model.

2.4. Sensitivity analysis

A global sensitivity analysis of the chemical fate sub-model of the ERA-AQUA model was performed in order to identify which scenario and drug-related parameters account for most of the variation in the model output. To study the variation in the model output, a sample of scenario parameters and drug-related parameters was generated by Monte Carlo permutations using the Latin Hypercube Sampling (LHS) technique (Iman and Conover, 1980) with the software GenStat 15th Edition (VSN International Ltd., Hemel Hempstead, UK). One thousand samples of the 14 scenario parameters showed in Figure 2 were generated based on uniform distributions built with minimum and maximum parameter values grounded on literature data or assumptions and trying to represent the variability observed in aquaculture pond scenarios. One thousand samples of the 8 drug-related parameters showed in Figure 3 were generated from log-normal distributions built with a list of physico-chemical properties for 30 aquaculture VMPs, including antibiotics, disinfectants and pesticides. The datasets of scenario and drug-related parameters (formed of 1000 combinations each) were run twice for each drug administration method (i.e., application mixed with feed and application directly to water), yielding a total number of 4000 model runs. TWA concentrations in the pond water, pond sediment and cultured species on day 1, 4, and 28 after drug administration were recorded as endpoints for the sensitivity analysis of the model. A detailed description of the datasets used in the sensitivity analysis and characteristics of the model runs is provided in the Supporting Information (Table S1-8).

The sensitivity of the model output towards the variation in the input parameters was assessed for each pond compartment separately by non-parametric regression using smoothing splines (Hastie and Tibshirani, 1990) of third order. The Top Marginal Variance (TMV) and the Bottom Marginal Variance (BMV) of each input parameter were used as sensitivity measures. The TMV represents how the considered model endpoints (TWACs for the studied compartments) can be

approximated by a function that depends only on the input parameter under study. The BMV represents how the considered model output can be approximated by a function that depends on all the other input parameters, i.e. excluding the parameter under study (for rationale the reader is referred to Jansen et al., 1994, and Saltelli et al., 2000). Both, the TMV and the BMV, are expressed as the percentage of the total variance of the model output and, hence, the higher the percentage, the higher the sensitivity of the model output to the studied parameter (Jansen et al., 1994). The TMV and BMV for each of the studied scenario and drug-related parameter towards the pond water, the pond sediment and the cultured species TWACs in the four datasets (i.e., scenario parameters assessed in in-feed and in bath treatments and the drug-related parameters in in-feed and in bath treatments) were calculated using the procedures described in Jansen et al. (1994) with the GenStat 15th Edition software (VSN International Ltd., Hemel Hempstead, UK). Previous to the calculation of the TMVs and BMVs, the input and output data were log-transformed in order to maximize the amount of the model output variance explained by the spline regression analysis.

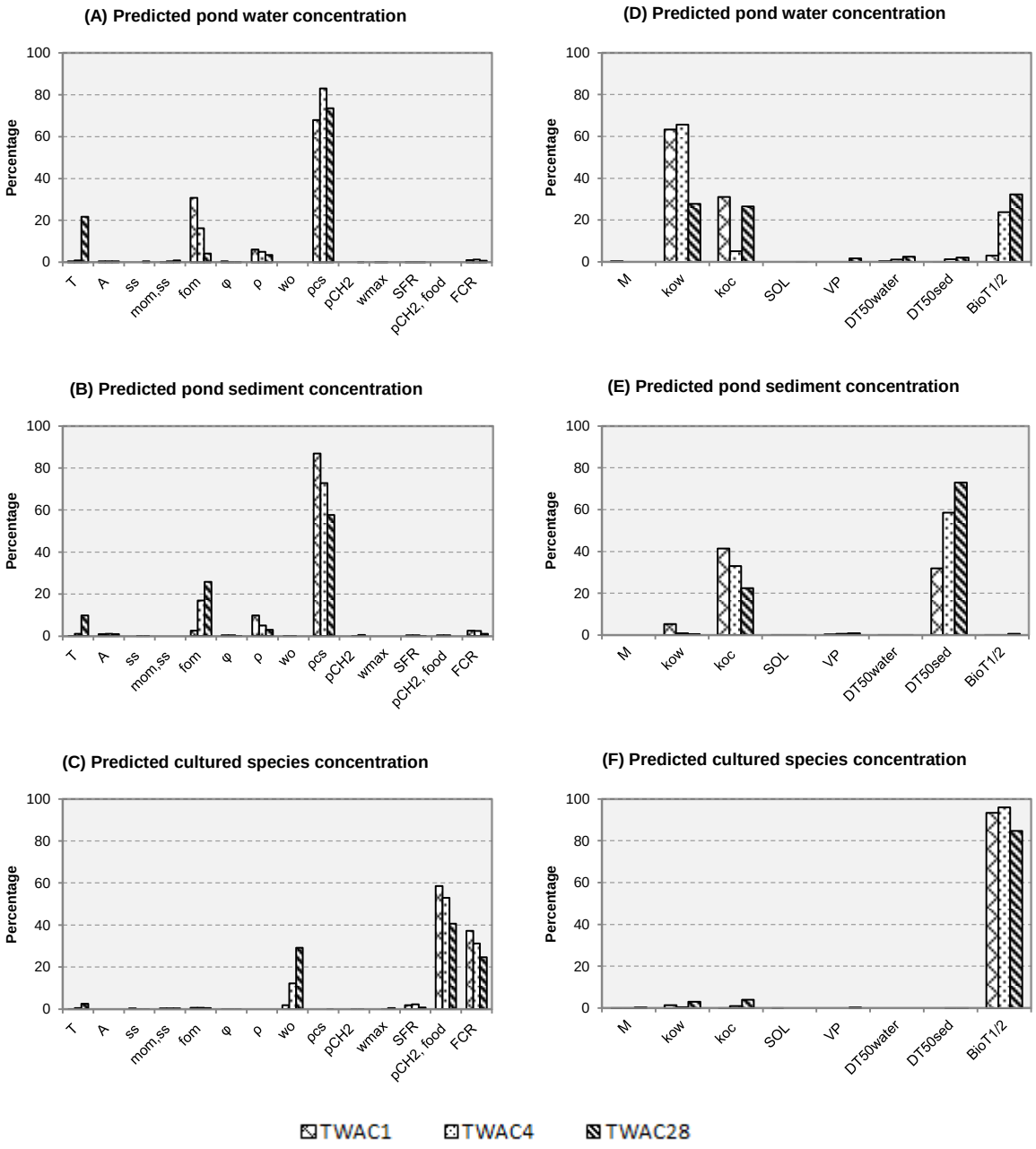


Figure 2. Results of the sensitivity analysis performed for drugs applied mixed with feed. Top marginal variances calculated for the scenario parameters and the time weighted average concentrations calculated for day 1, 4 and 28 after drug application (TWAC1, TWAC4, TWAC28) in the (A) Predicted Pond Water Concentration, (B) Predicted Pond

Sediment Concentration, and (C) Predicted Cultured Species Concentration. Top marginal variances calculated for the drug-related parameters and TWAC1, TWAC4, TWAC28 in the (D) Predicted Pond Water Concentration, (E) Predicted Pond Sediment Concentration, and (F) Predicted Cultured Species Concentration. See Table 1 for a description of the input parameter symbols.

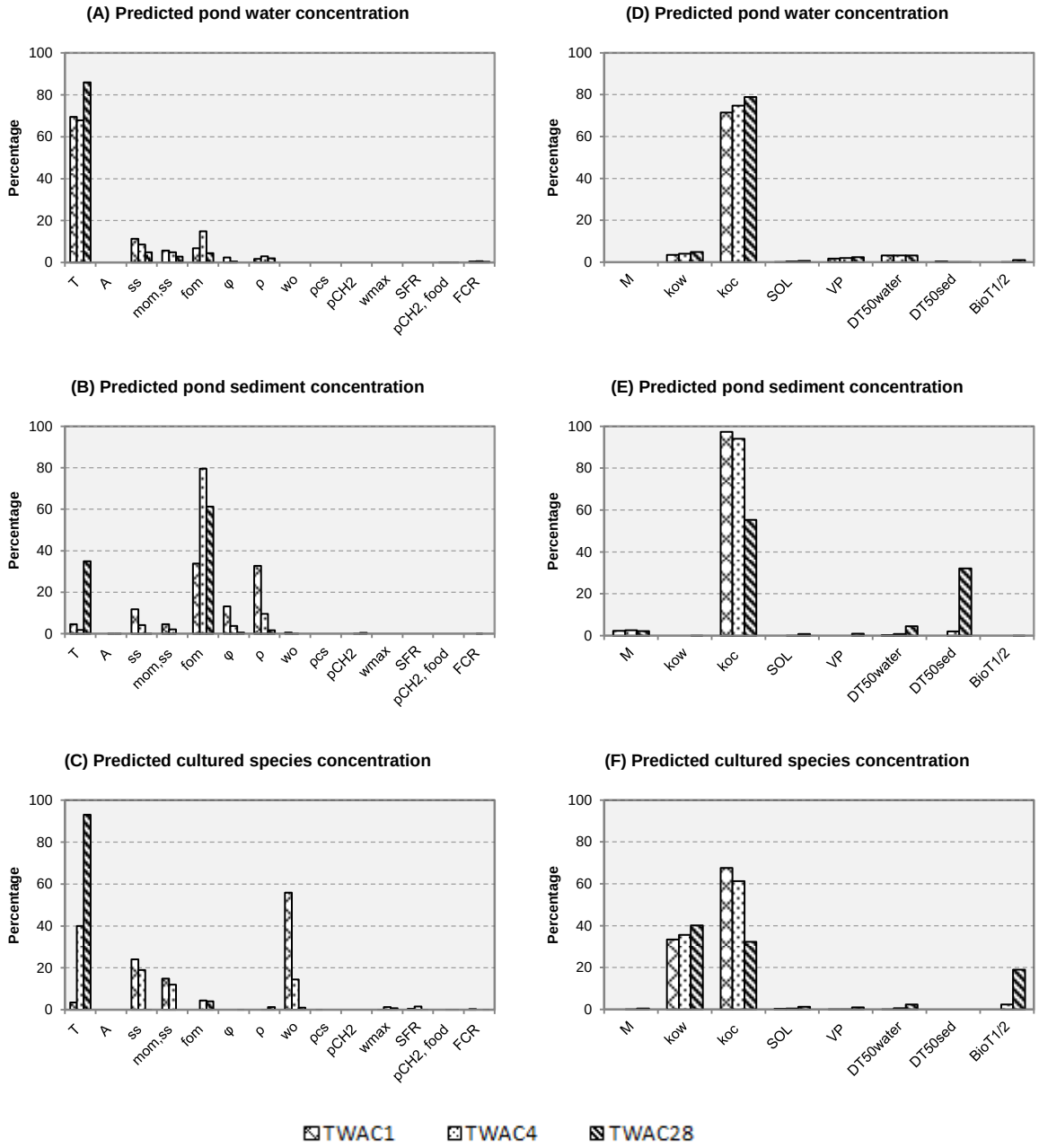


Figure 3. Results of the sensitivity analysis for drugs applied directly to water. Top marginal variances calculated for the scenario parameters and the time weighted average concentrations calculated for day 1, 4 and 28 after drug application (TWAC1, TWAC4, TWAC28) in the (A) Predicted Pond Water Concentration, (B) Predicted Pond Sediment Concentration, and (C) Predicted Cultured Species Concentration. Top marginal variances calculated for the drug-related parameters and TWAC1, TWAC4, TWAC28 in the (D) Predicted Pond Water Concentration, (E) Predicted Pond Sediment Concentration, and (F) Predicted Cultured Species Concentration. See Table 1 for a description of the input parameter symbols.

The percentage of the model output variance explained by the input parameters (i.e., adjusted regression coefficient) ranged between 96.5% and 99.8% for the scenario parameter sensitivity analysis and between 77.3% and 99.8% and for the drug-related parameter sensitivity analysis. This indicates that the spline regression analysis successfully described the relation between parameter variation and the output of the different endpoints. Individual input-parameter TMVs did not differ substantially to the BMVs (Mean: 0.6; SD: 1.1), demonstrating a low correlation

among the studied input parameters. Given the similarity between the calculated TMV and BMV values, only the TMV values are showed to describe the sensitivity analysis results. TMVs for the model input parameters calculated for the TWA1, TWA4, and TWA28 concentrations for drugs applied mixed with feed and in bath treatment are displayed in Figure 2 and 3, respectively. The results of the sensitivity analysis show that for drugs applied mixed with feed the most important scenario parameters are the density of cultured species, the parameters related to the drug assimilation in the cultured species (i.e., organism weight, lipid content of the feed and feed conversion ratio), and the fraction of organic matter in sediment (Fig. 2A,B,C). The most important drug-related parameters for this administration method are the biological half-life in the cultured species, the half-life in sediment, the octanol-water partition coefficient and the organic carbon sorption coefficient (Fig. 2D,E,F). For drugs applied directly to water, the main scenario parameters influencing the model output are the temperature, the fraction of organic matter in sediment and the organism weight at the start of the simulation period (Fig. 3A,B,C). The main drug-related input parameters for drugs applied directly to water were found to be the sorption coefficient to organic carbon, the octanol-water partition coefficient and the half-life of the substance in sediment (Fig. 3D,E,F). For a detailed description of the calculated TMV and BMV values see the Supporting Information (Tables S9-S12).

3. Example of the application of the ERA-AQUA model

As an example of the application of the ERA-AQUA model, we assessed the risks posed by the use of the antibiotic oxytetracycline (OTC) and the disinfectant/parasiticide benzalkonium chloride (BKC) in a realistic striped catfish (*Pangasionodon hypophthalmus*) scenario for the Mekong Delta region (Vietnam). The use of OTC and BKC among striped catfish farmers in the Mekong Delta region have been recently reported by Bosma et al. (2009) and Phan et al. (2009), respectively. The aquaculture pond scenario was built based on assumptions and published data describing typical aquaculture practices of intensive striped catfish production in the region (e.g. Phan et al., 2009). The modelled scenario represents an earthen pond in the last two months of the culture cycle (fish body weight at the start of the simulation period: 0.8 kg) with an area of 0.61 ha, a water depth of 4 m and a cultured species density of 6.72 kg/m^3. Commercial feed with a conversion ratio of 1.69 was daily applied as 3% of the body weight of the cultured organisms, according to the average feeding rates reported by Vietnamese catfish farmers for the last months of the culture cycle (Phan et al., 2009). The average daily water exchange was set to 30% (Phan et al., 2009) and the duration of the effluent discharge was assumed to be of 5 h. The daily irrigation was calculated by subtracting the water addition by rainfall to the water losses by drainage and evaporation in order to keep a daily water balance of zero. No water percolation took place during the simulation period and the top-layer of the depth of the pond sediment layer was set to 5 cm. In the modelled scenario, untreated pond effluents are directly discharged into a drainage canal assuming a dilution factor of approximately 10. A detailed description of the input parameters and assumptions taken for building the aquaculture scenario is provided in the Supporting Information (Table S13).

OTC was daily applied mixed with feed at a dose of 50 mg/kg of fish body weight for a period of 7 days. BKC was applied once per day directly to water at a concentration of 0.4 mg/L for three days with an interval between applications of 4 days. The dosages used in these simulations are based on recommended dosages for chemicals used in Asian aquaculture (Arthur et al., 2000) and prescribed dosages provided by chemical companies. Physico-chemical properties and toxicity data for aquatic organisms for OTC and BKC were collected from online databases, chemical evaluation reports and peer-review literatures (e.g. http://sitem.herts.ac.uk/aeru; US EPA 2006; Kreuzinger et al., 2007). Due to the lack of long-term toxicity data for OTC for fish and for BKC for algae, invertebrates and fish, the chronic risk assessment could not be completed for these endpoints. Due to the lack of pharmacokinetics data for striped catfish, the pharmacokinetics data for OTC and BKC were based on the study of Choo et al. (1995) for red tilapia and QSAR's estimations (US EPA, 2011), respectively. In order to calculate safe exposure levels for consumers,

an ADI of 0.03 mg/kg body weight per day was used for OTC (FAO, 2002) and an ADI of 0.44 mg/kg body weight per day was estimated for BKC based on toxicity data for rats (US EPA, 2006). A consumer's body weight of 60 kg and a daily consumption of 95 grams of fish per day was taken for the calculation of the EDI. The later was based on the average annual consumption of inland fish and other aquatic animals in the Lower Mekong Basin (i.e., 34 kg/per capita/year) (Hortle, 2007). In order to assess potential risks for the trade of the produced catfish, the MRLs of the studied substances for fish imports into EU were used (EU, 2010). For a detailed description of the drug-related parameters used for the model calculations see the Supporting Information (Tables S14-S15). The scenario and drug-related parameter input files used to run the ERA-AQUA model are available upon request.

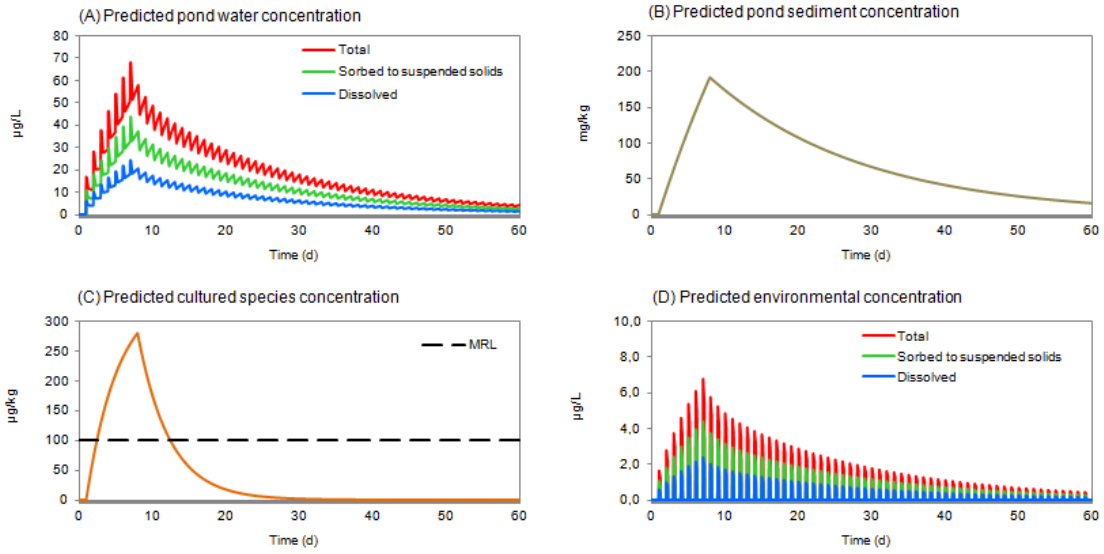


Figure 4. Predicted concentration dynamics for oxytetracycline in (A) pond water, (B) pond sediment, (C) cultured species, and (D) environment, calculated with the ERA-AQUA model. (MRL: Maximum Residue Limit).

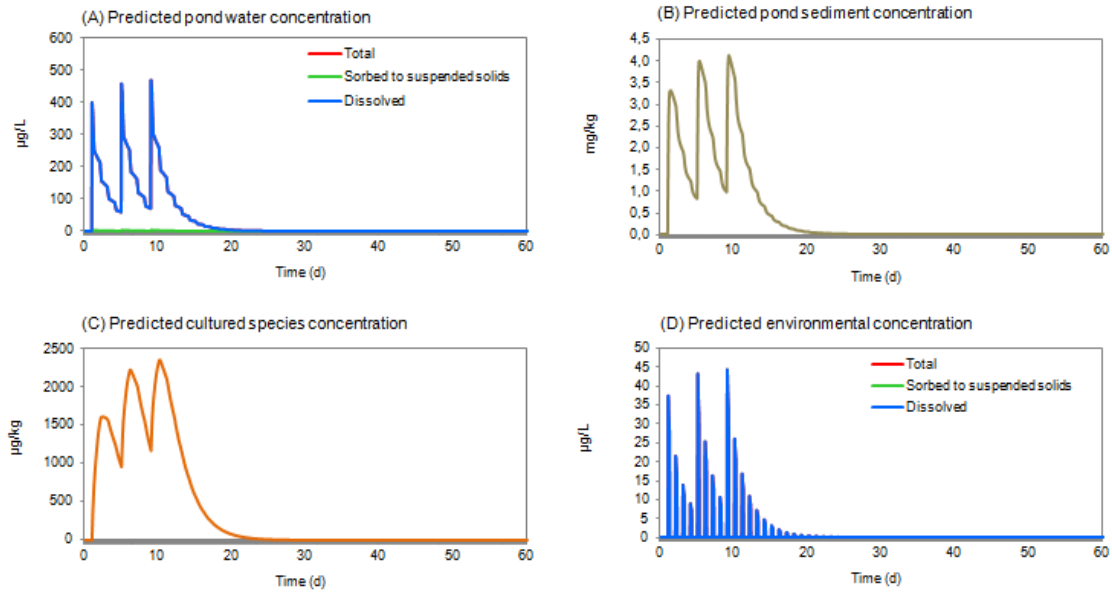


Figure 5. Predicted concentration dynamics for benzalkonium chloride in (A) pond water, (B) pond sediment, (C) cultured species, and (D) environment, calculated with the ERA-AQUA model.

Predicted concentrations in the pond water, pond sediment, cultured species and in the surrounding aquatic ecosystem for OTC and BKC are shown in Figure 4 and 5, respectively. The

model calculations showed that OTC is most likely to remain sorbed to suspended solids and sediment due to its high affinity for organic matter (*Koc* = 102,600 L/kg), and that degradation in sediment and effluent discharges contributed to 76% and 15% of the dissipation of the total applied compound mass from the aquaculture pond, respectively (Fig. 6). After the last drug application, total OTC concentrations in the pond water and in the environment were found to be 68 and 6.8 µg/L, respectively, and dissolved OTC concentrations in the pond water and in the aquatic ecosystem were estimated to be 24 and 2.4 µg/L, respectively. The maximum calculated concentration of OTC in the cultured species was 279 µg/kg. BKC was found to dissipate fast from the water phase and sediment, with water drainage (62% of the applied mass) and biodegradation and chemical break-down in water (25%) being the most important dissipation processes (Fig. 6). Dissimilar to the simulations performed for OTC, most of the BKC mass was predicted to remain dissolved in water, reaching a PeakPWC of 470 µg/L and a PeakPEC of 44 µg/L.

Table 2. Risk assessment for the cultured species, aquatic ecosystems (acute and chronic), consumers and trade for (A) oxytetracycline and (B) benzalkonium chloride calculated with the ERA-AQUA model.

A. Oxytetracycline

Endpoint	Exposure Assessment			Effect Assessment			Risk Characterization		
Cultured Species	PeakPWC	67.9	µg/L	$PNEC_{cs}$	11,600	µg/L	RQ_{cs}	0.01	NE
Aquatic ecosystems (acute)	PeakPEC	6.76	µg/L	$PNEC_{algae}$	3.42	µg/L	RQ_{algae}	1.98	E
				$PNEC_{inv.}$	1020	µg/L	$RQ_{inv.}$	0.01	NE
				$PNEC_{fish}$	1160	µg/L	RQ_{fish}	0.01	NE
Aquatic ecosystems (chronic)	TWA3	1.16	µg/L	$PNEC_{algae}$	18.3	µg/L	RQ_{algae}	0.06	NE
	TWA21	0.77	µg/L	$PNEC_{inv.}$	4,620	µg/L	$RQ_{inv.}$	$1.68 \cdot 10^{-4}$	NE
	TWA28	0.68	µg/L	$PNEC_{fish}$	NA	µg/L	RQ_{fish}	NA	-
Consumers	EDI	$4.47 \cdot 10^{-6}$	mg/(kg x d)	ADI	0.03	mg/(kg x d)	$RQ_{cons.}$	$1.49 \cdot 10^{-4}$	NE
Trade	$PCC_{harvest}$	2.83	µg/kg	MRL	100	µg/kg	RQ_{trade}	0.03	NE

B. Benzalkonium chloride

Endpoint	Exposure Assessment			Effect Assessment			Risk Characterization		
Cultured Species	PeakPWC	470	µg/L	$PNEC_{cs}$	100	µg/L	RQ_{cs}	4.70	E
Aquatic ecosystems (acute)	PeakPEC	44.0	µg/L	$PNEC_{algae}$	0.41	µg/L	RQ_{algae}	107	LE
				$PNEC_{inv.}$	0.18	µg/L	$RQ_{inv.}$	244	LE
				$PNEC_{fish}$	10.0	µg/L	RQ_{fish}	4.4	LE
Aquatic ecosystems (chronic)	TWA3	5.23	µg/L	$PNEC_{algae}$	NA	µg/L	RQ_{algae}	NA	-
	TWA21	2.54	µg/L	$PNEC_{inv.}$	NA	µg/L	$RQ_{inv.}$	NA	-
	TWA28	1.91	µg/L	$PNEC_{fish}$	NA	µg/L	RQ_{fish}	NA	-
Consumers	EDI	$3.65 \cdot 10^{-3}$	mg/(kg x d)	ADI	0.44	mg/(kg x d)	$RQ_{cons.}$	0.01	NE
Trade	$PCC_{harvest}$	2300	µg/kg	MRL	NA	µg/kg	RQ_{trade}	NA	-

PeakPWC: Peak predicted pond water concentration; PeakPEC: Peak predicted environmental concentration; TWA(t): Time weighted average concentration (t = days); EDI: Estimated daily intake; $PCC_{harvest}$: Predicted cultured species concentration at harvest; $PNEC_{cs}$: Predicted no effect concentration for the cultured species; $PNEC_i$: Predicted no effect concentration for the taxonomic group (i); ADI: Acceptable daily intake; MRL: Maximum residue limit; RQ_{cs}: risk quotient for the cultured species; RQ_i: Risk quotient for the taxonomic group (i); $RQ_{cons.}$: Risk quotient for consumers; RQ_{trade}: Risk quotient for trade; NA: Not available; NE: No exceedance; E: Exceedance; LE: Large exceedance.

The risk calculations performed for OTC (Table 2A) showed only moderate risks for algae with a RQ of 1.98 based on total highest predicted concentration, and 0.70 based on the highest predicted dissolved concentration in the watercourse, the later one being more realistic for assessing effects on primary producers (Rico et al., 2012b). The model calculations performed for OTC predicted no exceedance of the safe chemical residue levels for consumers and trade when a drug withdrawal period of 21 days was used. The risk assessment performed for the BKC revealed high expected short-term risks for invertebrates (RQ = 244), algae (RQ = 107) and moderate for fish (RQ = 4.4) in surrounding freshwater ecosystems (Table 2B). Furthermore, the performed calculations showed that their application according to the recommended dosages might induce

mortalities in the cultured species. However, it should be noted that the toxicity data used in the effect assessment for striped catfish is based on extrapolations performed with the LC50-96h calculated for rainbow trout (*Oncorhynchus mykiss*) under laboratory conditions (Dobbs et al., 1995), indicating the need to evaluate the effectiveness of the compound for the treatment of striped catfishes under local conditions. EDI levels for BKC in the cultured species were far from posing a risk, even when fish was harvested after the last application. Risks for commercial export to EU are not relevant due to the lack of MRLs for BKC in EU food safety controls (EU, 2010).

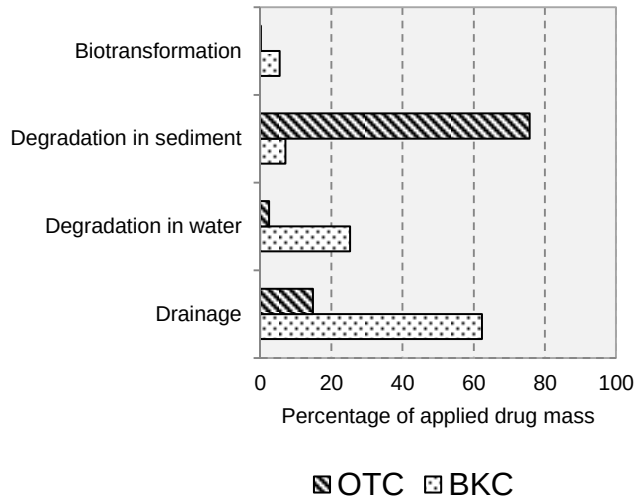


Figure 6. Main dissipation processes for oxytetracycline (OTC) and benzalkonium chloride (BKC) in the modelled aquaculture pond calculated with the ERA-AQUA model.

4. Model applications and conclusions

The ERA-AQUA model was developed to prioritize compounds and environmental process parameters that require refinement in higher-tier risk assessments, as well as to decide on relevant toxicity tests and chemical and biological monitoring campaigns to be included as part of the risk assessment of aquaculture VMPs. For example, the first tier risk assessment performed in the present study for the application of OTC in intensive striped catfish ponds did not reveal significant impacts for non-target aquatic organisms. Given the conservative nature of the risk assessment approach followed here, it is likely that no risks are expected for the use of this compound in the given aquaculture scenario and, hence, the risk evaluation can be terminated here. However, the risk calculations performed for BKC clearly indicated the need of (1) monitoring residual concentrations of BKC in down-stream ecosystems and to assess toxic effects on aquatic biodiversity to refine the effect assessment calculations, including principally invertebrates and primary producers, and (2) assessing the toxic effects of BKC on striped catfish including relevant sub-lethal endpoints such as growth impairment or effects on metabolic functions. On the other hand, the model can be used to study the influence of different chemical application schemes and aquaculture management practices on the predicted environmental exposure and effects of VMPs.

For instance, simulations can be performed in order to evaluate the influence of different drug application schemes and water exchange regimes on the dissipation of VMPs from aquaculture ponds and their environmental release. Simulations can also be performed to derive environmentally sustainable practices aiming at minimizing the impacts of aquaculture effluent discharges in drown-stream aquatic ecosystems, helping to decide on the need of establishing aquaculture effluent treatments (e.g. organic matter settling ponds, waste water treatment systems). Although our model was designed to provide estimates of environmental concentrations of VMPs in the effluent discharge point, larger scale simulations could easily be implemented. Hydrological and ecological models such as WASP (Ambrose et al., 1988) or

AQUATOX (Park et al., 2008) that can incorporate point source pollution into chemical exposure simulations performed at a river scale are a few examples. Moreover, Geographical Information Systems (GIS) could be used to perform watershed level simulations by including for example the possibility to assess environmental risks of individual chemical or chemical mixtures in aquaculture clustered areas by using the ERA-AQUA environmental exposure assessment output.

One of the advantages of the present model over other aquaculture chemical fate models is that the model dynamically predicts the bioaccumulation of VMPs in the cultured organisms. In this way, the model can help to calculate the required withdrawal time of the applied drug in the cultured organisms and to take decisions on the most appropriate harvest moment to comply with food quality standards.

Besides the main applications described above, which focus on assessing risks posed by VMPs that are intentionally applied to treat and/or prevent diseases and parasitic infestations in the cultured species, the ERA-AQUA model can also be used to perform risk assessment studies of compounds entering the aquaculture ponds with the in-flow water. The exposure of aquaculture organisms to potentially toxic compounds such as agricultural (i.e., pesticides) or industrial (i.e., PCBs, PAHs) chemicals can be assessed and, hence, bioaccumulation in the aquaculture produce and potential risks for consumers and trade can be derived. In a similar way, scenarios in which ponds are hydrologically interconnected can be built, helping to identify potential side-effects of VMPs entering non-treated ponds with the in-flow water.

The ERA-AQUA model was designed to assess chemical exposure calculations for a wide range of aquaculture practices, aquatic species and VMPs. However, the model needs to be judiciously parameterized with local data in order to assure a maximum realism of the predictions. To this end, the values of the input parameters that were identified in the present study through the sensitivity analysis must have the priority to be determined precisely through laboratory and field experiments. Even when the model is parameterized with local data, users must be aware that the ERA-AQUA model was built from a series of theoretical (conservative) assumptions concerning the represented aquaculture scenario and the chemical's behaviour in that environment, and hence, must be used as a first tier to assess VMPs risks. Refined, higher tier risk assessments are recommended to be drawn by using a combination of prospective modelling results and chemical and biological monitoring studies. Moreover, monitoring programs can be used to actually measure concentrations, to verify model assumptions and to support or refute model predictions. Although several studies have investigated the occurrence of VMPs inside aquaculture ponds and in the surrounding environment (e.g. Le and Munekage, 2004), these results are hardly comparable to any modelling calculations since they do not present sufficient data on the different aquaculture practices employed and environmental conditions. For this reason, extensive monitoring campaigns or experiments conducted under (semi-)controlled field conditions, including a precise description of the employed aquaculture practices and environmental characteristics, must be undertaken in order to assess the model's accuracy.

In conclusion, the ERA-AQUA model can be considered as a cost-effective tool to identify aquaculture medicinal treatments that may result in a loss of the productivity of the aquaculture pond and/or may pose a risk for external aquatic ecosystems, human health and the trade of the aquaculture produce, helping, for example, to take decisions in the risk evaluation of new or already approved aquaculture medicinal treatments. Other applications include the derivation of environmentally sustainable aquaculture practices related to the use of VMPs, the design of chemical and biological monitoring programs and other higher tier chemical fate and effect studies, and the assessment of risks of non-aquaculture chemicals for the farm's productivity and human's health. Further research studies aimed at calibrating and testing the accuracy of the chemical fate and effect assessments provided by the model are strongly recommended.

Acknowledgments

The authors would like to thank Bryan W. Brooks and one anonymous reviewer for their valuable comments on an earlier version of this chapter.

Supporting Information

The Supporting Information of this chapter can be downloaded from:
http://onlinelibrary.wiley.com/doi/10.1002/etc.2153/suppinfo

Chapter 5

Probabilistic risk assessment of veterinary medicines applied to four major aquaculture species produced in Asia

Andreu Rico, Paul J. van den Brink

Abstract

Aquaculture production constitutes one of the main sources of pollution with veterinary medicines into the environment. About 90% of the global aquaculture production is produced in Asia and the potential environmental risks associated to the use of veterinary medicines in Asian aquaculture have not yet been properly evaluated. In this study we performed a probabilistic risk assessment for eight different aquaculture production scenarios of Asia by combining up-to-date information on the use of veterinary medicines and aquaculture production characteristics. The ERA-AQUA model was used to perform mass balances of veterinary medicinal treatments applied to aquaculture ponds and to characterize risks for primary producers, invertebrates, and fish potentially exposed to chemical residues through aquaculture effluents. The mass balance calculations showed that, on average, about 25% of the applied drug mass to aquaculture ponds is released into the environment, although this percentage varies with the chemical's properties, the mode of application, the cultured species density, and the water exchange rates in the aquaculture pond scenario. In general, the highest potential environmental risks were calculated for parasitic treatments, followed by disinfection and antibiotic treatments. Pangasius catfish production in Vietnam, followed by shrimp production in China, constitute possible hot-spots for environmental pollution due to the intensity of the aquaculture production and considerable discharge of toxic chemical residues into surrounding aquatic ecosystems. A risk-based ranking of compounds is provided for each of the evaluated scenarios, which offers crucial information for conducting further chemical and biological field and laboratory monitoring research. In addition, we discuss general knowledge gaps and research priorities for performing refined risk assessments of aquaculture medicines in the near future.

This chapter has been published in *Science of the Total Environment* 2014, 468/469: 1196-1207.

1. Introduction

During the last decades, the production of aquatic food in the Asian continent has experienced an unprecedented increase and nowadays accounts for about 90% of the global aquaculture production (FAO, 2012a). The great majority of the Asian aquaculture is produced in land-based freshwater or brackish water ponds, which rely on periodic effluent discharges into surrounding water bodies (Bostock et al., 2010). In order to promote optimal health conditions, and to treat and prevent possible disease outbreaks, Asian aquaculture farmers have reported the use of a wide range of veterinary medicines such as antimicrobials, fungicides, anthelmintics and other parasiticides (Bondad-Reantaso et al., 2012; Rico et al., 2012a; Rico et al., 2013a). Residual concentrations of aquaculture medicines used in inland aquaculture farms have been measured in water and sediments of down-stream rivers and estuaries (Le and Munekage, 2004; Zou et al., 2011), and constitute an important source of environmental pollution (Boxall et al., 2004).

Aquaculture pharmaceuticals and other potentially toxic chemicals may result in harmful effects on the biodiversity and functioning of aquatic ecosystems surrounding aquaculture farms, and may compromise the environmental sustainability of the aquaculture sector. To assess the potential ecological effects of veterinary medicines used in aquaculture, environmental risk assessment schemes have been established and implemented in the registration and evaluation procedures of veterinary medicinal products in many developed countries (VICH, 2000; VICH, 2004). Such environmental risk assessment procedures, however, have not yet been adopted in the main aquaculture producing countries of (sub-)tropical Asia, and the number of independent studies investigating the environmental occurrence and ecological risks of aquaculture medicines in the Asian continent is still very limited in comparison to other, temperate, regions. Although concerns have been raised about the potential ecological implications of the intensive use of chemicals in Asian aquaculture (Gräslund and Bengtsson, 2001), consistent data on their environmental fate following use in aquaculture farms and potential ecological risks have never been generated (Thuy et al., 2011; Rico et al., 2012a). Given the large number of chemical treatments used in Asian aquaculture and the wide range of species and aquaculture production scenarios, prioritization approaches need to be used to guide scientific research and economic investments, especially in situations of limited prospective environmental risk regulation and monitoring resources.

In the current study we assessed the potential ecological risks posed by the use of veterinary medicines in eight aquaculture grow-out pond scenarios of Asia. The eight scenarios included in the present study represent shrimp production in Bangladesh, China, Thailand, and Vietnam; prawn production in Bangladesh; tilapia production in Thailand and China; and Pangasius catfish production in Vietnam. These aquaculture farm groups were selected because of their relative increase in production volume over the last decades and the availability of up-to-date data on the use of veterinary medicines and farming practices (Rico et al., 2013a). A probabilistic risk assessment was performed by combining information on the use of veterinary medicines in each of the studied aquaculture scenarios, chemical properties for the evaluated substances, and characteristics of the modelled aquaculture pond scenarios. Risk calculations were performed by using the ERA-AQUA v2.0 model, a mass balance model designed to calculate environmental exposure and risks of veterinary medicines applied in pond aquaculture scenarios (Rico et al., 2012b; Rico et al., 2013b). Chemical exposure profiles in the aquaculture farm effluents were used to calculate acute and chronic risks for primary producers, invertebrates, and fish in adjacent aquatic ecosystems (Fig. 1). The main objectives of the present study were to prioritize compounds, scenarios, and biological taxa that should be targeted in specific ecotoxicological and field monitoring studies, as well as to identify data gaps and research needs that must be addressed in the future in order to perform refined risk assessments for veterinary medicines in Asian aquaculture.

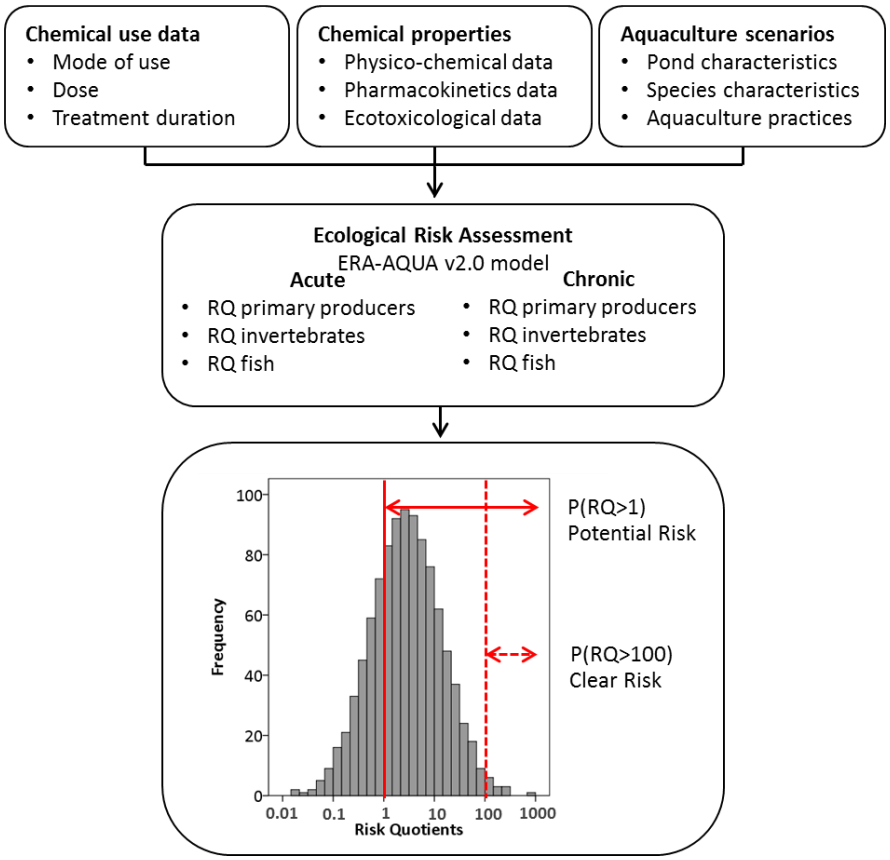


Figure 1. Overview of the information and methodology used in the present study.

2. Material and methods

2.1. Chemical use data

Forty-seven veterinary medicinal treatments were evaluated in the current study including antibiotics, disinfectants and parasiticides. This list of chemical treatments is based on a survey on chemical use performed, during 2011 and 2012, to 252 aquaculture grow-out farmers and 56 farm supply shops corresponding to the countries and aquaculture farm groups included in the current study (for details see Rico et al., 2013a). The list includes chemicals applied directly to water for disinfection of the culture facilities prior to stocking, and chemicals applied either directly to water or mixed with feed for disease treatment or prevention during the culture cycle. The list of chemical treatments evaluated in the present study, mode and frequency of use, recommended dosage, and duration of the treatment period is shown in Table S1. When the recommended dosage and the duration of the treatment were reported as a numeric interval, the highest value was conservatively chosen for the risk assessment calculations.

2.2. Chemical properties

2.2.1. Physico-chemical properties

Information on physico-chemical properties for the veterinary medicinal ingredients was retrieved from online databases (e.g. http://sitem.herts.ac.uk/aeru/vsdb; http://www.chemspider.com), and the literature. Information was collected for the following parameters: molecular mass, octanol-water partition coefficient, sorption coefficient to organic carbon, solubility and reference temperature at which it was determined, enthalpy of dissolution, saturated vapour pressure and temperature at which it was determined, enthalpy of vaporization, half-life degradation of the

substance in water and sediment and temperatures at which they were determined, and molar Arrhenius activation energy. When data was not available for the enthalpy of dissolution, the enthalpy of vaporization, or the Arrhenius activation energy, the default values included in the ERA-AQUA model Rico et al. (2012b) were used. When the required data were not available for the rest of the chemical properties listed above, they were calculated according to the Quantitative Structure-Activity Relationships (QSARs) included in the EPI Suite v4.1 software (US EPA, 2012). The resulting dataset of physico-chemical properties for the evaluated compounds is presented in Table S2.

2.2.2. Pharmacokinetics data

Data on biological half-lives (BioT1/2) in fish and crustaceans for the evaluated compounds were retrieved from the PhishPharm database (Reimschuessel et al., 2005). We only selected studies in which the BioT1/2 values were calculated based on measured concentrations in muscle, and muscle and skin samples, and in which the compound was administered via bath immersion, oral gavage or mixed with feed. This data selection resulted in 218 BioT1/2 data entries. In order to minimize the influence of the experimental set-up in which they were calculated, the BioT1/2 values were normalized to an organism weight of 0.1 kg and a temperature of 20 °C, according to the equation described in Arnot et al. (2009). The normalized BioT1/2 values were classified into four categories according to the aquaculture species group (fish or crustaceans) and the drug administration method (oral administration or administration via water exposure). Subsequently, the available BioT1/2 values within each of these four categories were further classified into chemical classes according to the nature of the active ingredient. The classification yielded 13 different compound classes for antibiotics and anthelmintics applied mixed with feed or in oral gavage (aminoglycosides, aminopenicillins, amphenicols, avermectins, cephalosporins, diaminopyrimidines, heterocyclic acetic acid derivatives, polypeptides, pyrazinaisoquinolines, quinolones, rifampicins, sulfonamides, tetracyclines) and 9 compound classes for disinfectants, ectoparasiticides and other biocides applied directly to water (aldehydes, benzimidazoles, cyanuric acids, dinitroanilines, hydantoins, organophosphates, pyrethroids, other plant derived compounds, and quaternary ammonium compounds).

Finally, one single BioT1/2 value was conservatively chosen to represent each chemical class within each of the four aforementioned categories according to the following procedure:

- When four or more BioT1/2 values were available for a given chemical class, the data was fitted to a log-normal distribution, and the 90^{th} percentile of the resulting distribution was chosen as the representative value for the chemical class. The goodness of fit to a log-normal distribution was evaluated with the Kolmogorov-Smirnov test ($\alpha = 0.05$).
- When the number of BioT1/2 values available for a given chemical class was lower than four, the maximum value was selected as representative for the chemical class.
- When no data was available for a given chemical class, the 90^{th} percentile of the log-normal distribution built with all the available values in all classes of the category (e.g. compounds administered orally to fish) was used as representative for the respective chemical class.

For the category of compounds applied via water exposure to crustaceans, no data was found to be available. Therefore, for all chemical classes in this category, the BioT1/2 values were selected as the 90^{th} percentile of the distribution built with all the BioT1/2 values available for the same administration method to fish. A detailed description of the studies available, the calculated percentiles and distributions is shown in Table S3.

2.2.3. Toxicity data for aquatic organisms

Toxicity data for aquatic organisms was collected from the open literature, toxicity databases (e.g. www.epa.gov/ecotox), toxicity data reviews (e.g. Cunningham et al., 2006), industry reports, and material safety data sheets. A step-wise approach was followed to derive acute and chronic Predicted No Effect Concentrations (PNECs) for primary producers, invertebrates and fish for each of the evaluated compounds. First, acute and chronic PNECs for the three included taxonomic groups were set at the community No Observed Effect Concentration (NOEC) level derived from model ecosystem experiments (i.e., micro- and mesocosms). When no data on model ecosystem experiments was available, and the number of toxicity values calculated from single-species laboratory experiments was equal or higher than 6 for a given taxonomic group, PNECs were derived using the Species Sensitivity Distribution (SSD) concept (Posthuma et al., 2002). Acute exposure SSD curves were constructed for each taxonomic group separately using EC50 values (concentration that affects the 50% of the tested organisms) on immobility or mortality for animals, and (biomass) growth for primary producers. Macrophytes and algal species were pooled together in the primary producers taxonomic group. Only the toxicity data calculated for an exposure period of 2-21 days for fish, 1-7 days for invertebrates, 2-28 days for macrophytes, and 1-7 days for the algae species, was selected (Maltby et al., 2005). Chronic exposure SSD curves for invertebrates and fish were constructed with NOEC values representing effects on reproduction, growth or mortality with exposure periods equal or higher than 21 and 28 days, respectively. For primary producers, chronic SSD curves were constructed using NOECs derived with the same exposure duration and endpoints as evaluated in the acute exposure SSDs. In all cases, the geometric mean was calculated when more than one toxicity value was available for the same species or genus without a specific reference to the species name. SSDs were calculated with the ETX 2.0 software (Van Vlaardingen et al., 2004) by fitting the toxicity data to a log-normal distribution. PNECs were determined as the median Hazardous Concentration for the 5% of species (HC5) of the SSD, calculated according to Aldenberg and Jaworska (2000). When only the acute HC5 was available, chronic PNECs were derived by diving the acute HC5 by an assessment factor of 10 to account for prolonged exposure periods.

When the number of toxicity values was not sufficient to build SSDs, the acute and chronic PNECs for primary producers, invertebrates and fish were calculated by applying assessment factors to single-species toxicity values. Priority was given to the use of standard test species for which standard toxicity testing protocols are available, as recommended in the international guidelines for the environmental impact assessment of veterinary medicines (VICH, 2004). These included the following species of green algae: *Chlorella vulgaris, Scenedesmus subspicatus, Selenastrum capricornutum*; invertebrates: *Daphnia magna*; and fish: *Brachydanio rerio, Cyprinus carpio, Gasterosteus aculeatus, Lepomis macrochirus, Oncorhynchus mykiss, Oryzas latipes, Pimephales pomelas, Poecilia reticulata*. Since some compounds with antimicrobial properties have been demonstrated to exert higher toxicity to blue-green algae and macrophytes than to green-algae (Van der Grinten et al., 2010; Ebert et al., 2011), toxicity data for *Microcystis aeruginosa* (blue-green algae) or *Lemna minor* (macrophyte) were selected when any of these values was lower than the available toxicity value for green-algae. The endpoints used for the calculation of acute and chronic PNECs for primary producers were the EC50 and the NOEC values for biomass or growth, respectively, calculated after an exposure period of 3-4 days. The acute and chronic endpoints used for the derivation of PNECs for invertebrates were the EC50s calculated for mortality or immobilization after an exposure period of 2 days, and the NOEC for growth or reproductive effects after an exposure period of 21 days. The acute and chronic endpoints for fish were based on the LC50 (concentration that kills the 50% of the tested organisms) obtained after an exposure period of 4 days and the NOEC calculated based on effects on reproduction or growth after an exposure period of 28 days. When more than one EC50 or NOEC was available for the same species or a genus without a specific reference to a species name, the geometric mean was calculated. The lowest value was taken when more than one value was available for a specific

taxonomic group. Finally, when only data for non-standard test species was available, toxicity values for those species were also allowed considering the same endpoints and exposure duration as described for the standard test organisms. As a last resort, toxicity data was derived by using the QSARs incorporated in the ECOSAR Class Programme v1.11 (http://www.epa.gov/oppt/newchems/tools/21ecosar.htm) when experimental data was not available. When only acute data meeting the above-mentioned criteria was available for standard or non-standard test species, chronic NOECs for primary producers, invertebrates and fish were derived by dividing the acute EC50 or LC50 values by an extrapolation factor of 10, for primary producers, and 100, for invertebrates and fish. Finally, acute and chronic PNECs derived from single species laboratory toxicity data were calculated by diving the selected toxicity values by an assessment factor of 100 (i.e., accounting for EC50 to no effect extrapolation and for inter-species sensitivity variation) and 10 (i.e., accounting for inter-species sensitivity variation), respectively. The toxicity data origin and extrapolation factors used for the derivation of the PNECs are shown in Table S2.

2.3. Aquaculture scenarios

Model scenarios representing aquaculture grow-out ponds for the mix of aquaculture species and countries included in the current study were generated based on a combination of (1) literature data, (2) survey data on aquaculture production characteristics, and (3) few assumptions. The generated scenarios included information on the duration of the culture cycle, physical characteristics of the aquaculture pond, cultured species characteristics, feed input to the cultured species, local consumer characteristics, and effluent discharge management (Table 1). A detailed description of the data, references, and assumptions made to build the scenarios is provided in the Supporting Information (Fig. S1, and Tables S4 and S5). In order to account for the scenario's variability, 1000 Monte Carlo samples were generated for the scenario parameters that explain the largest variation in the model output, together with the parameters related to water exchange dynamics, which are directly related to the environmental discharge of veterinary medicines. The scenario parameters were selected on the basis of the result of a sensitivity analysis previously performed with the ERA-AQUA model (Rico et al., 2013b). The included variable scenario parameters were: organic matter fraction in sediment, temperature, stocking density of the cultured species, mortality fraction during the culture cycle, percentage of water discharge per event, time interval between water discharge events, and duration of the effluent discharge event. Data distributions for temperature and sediment organic matter fraction for each scenario were obtained from the literature.

Data for the other parameters were retrieved from a database (SEAT project database) on farm characteristics and aquaculture management practices resulting from a survey performed to more than 1,600 aquaculture grow-out farms in south-east Asia (Murray et al., 2013). From this database, only (semi-)intensive monoculture pond-based farms were selected, with the exception of the prawn farms in Bangladesh and the tilapia farms in China, which were represented by polyculture systems, as they combined the culture of the main species with other cyprinids. To each of these datasets, the 18 model distributions available in the @Risk 6.0 software (Palisade corporation, Ithaca, New York, US) were fitted by maximum likelihood estimation of the distribution parameters using the parametric bootstrap technique (with 1000 resamples of the original dataset). Then, the best fitting distribution was selected by the Akaike information criterion (Bozdogan, 1987). Finally, the parameters of the best fitting distribution were used to generate 1000 Monte Carlo samples by Latin hypercube sampling, which were used to represent the variability within each of the aquaculture pond scenarios. The distribution fitting analysis and the Monte Carlo sample generation were performed using the @Risk 6.0 software. The parameters of the fitted distributions and the descriptive statistics of the Monte Carlo sample datasets are provided in Table S6.

Table 1. Risk assessment scenarios used to run the ERA-AQUA model. For a detailed description of the references used and assumptions see Fig. S1, and Tables S4 and S5.

Scenario name	Bangladesh Shrimp	Bangladesh Prawn	China Tilapia	China Shrimp	Thailand Tilapia	Thailand Shrimp	Vietnam Pangasius	Vietnam Shrimp	Data source [b]
Species	***Penaeus monodon***	***Macrobrachium rosenbergii***	***Oreochromis niloticus***	***Litopenaeus vannamei***	***Oreochromis niloticus***	***Litopenaeus vannamei***	***Pangasianodon hypophthalmus***	***Penaeus monodon***	
Duration of the culture cycle (d)	214	230	221	91.0	243	100	216	154	A
Aquaculture pond									
Pond area (m^2)	9422	4130	9805	3933	8423	7890	4437	3850	A
Pond water depth (m)	1.15	2.04	3.21	1.87	1.77	1.99	4.08	1.49	A
Mass concentration of suspended solids in pond water (mg/L)	281	213	114	281	114	281	63.0	134	B
Mass fraction of organic matter in suspended solids (-)	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	C
Top sediment layer depth (m)	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	C
Mass fraction of organic matter in sediment (-) [a]	0.02 ± 0.02	0.02 ± 0.02	0.03 ± 0.01	0.02 ± 0.02	0.03 ± 0.01	0.02 ± 0.02	0.09 ± 0.02	0.02 ± 0.02	B
Sediment porosity (v/v)	0.51	0.51	0.51	0.51	0.51	0.51	0.51	0.51	C
Sediment bulk density (kg/L)	0.76	0.76	0.76	0.76	0.76	0.76	0.76	0.76	C
Average water temperature (°C) [a]	26.4 ± 7.0	26.4 ± 7.0	22.4 ± 7.2	24.4 ± 5.8	28.3 ± 4.2	28.3 ± 4.2	28.0 ± 4.2	28.0 ± 4.2	B
Average percolation rate (mm/d)	5.20	5.20	5.20	0.00	5.20	5.20	0.87	0.87	B
Average rainfall rate (mm/d)	5.14	5.14	4.68	4.51	4.51	4.51	5.36	5.36	B
Average evaporation rate (mm/d)	3.10	3.10	9.04	9.04	3.10	3.10	4.50	4.50	B
Cultured species characteristics									
Organism weight at stocking (g)	0.02	0.02	2.98	0.005	1.20	0.005	19.1	0.01	A
Organism weight at harvest (g)	27.4	61.2	588	12.9	488	15.7	981	31.3	A
Maximum organism weight (g)	60.0	62.0	1070	16.0	790	20.0	1010	60.0	C
Cultured species density at stocking (g/m^2) [a]	0.10 ± 0.09	0.03 ± 0.02	7.97 ± 2.17	1.04 ± 0.75	4.33 ± 3.41	0.31 ± 0.13	792 ± 353	0.14 ± 0.05	A
Lipid fraction of cultured organisms (-)	0.04	0.03	0.03	0.05	0.03	0.05	0.03	0.04	B
Mortality fraction during the culture period (-)	0.69 ± 0.16	0.49 ± 0.21	0.17 ± 0.14	0.34 ± 0.16	0.46 ± 0.16	0.22 ± 0.11	0.24 ± 0.11	0.32 ± 0.18	A
Feed input to the cultured species									
Daily specific feeding rate (SFR) (kg food/kg cultured species · d)	0.08	0.07	0.01	0.05	0.01	0.05	0.06	0.08	B
Organism's weight at which SFR was determined (g cultured species)	0.005	10.0	359	11.0	359	11.0	78.0	0.005	B
Lipid fraction of feed (-)	0.10	0.10	0.06	0.10	0.06	0.10	0.06	0.10	B
Feed conversion ratio in the cultured species (kg food/kg cultured species)	1.83	1.88	1.87	2.00	1.87	2.00	1.69	1.83	B
Fraction of eaten feed (-)	0.90	0.90	0.95	0.90	0.95	0.90	0.95	0.90	B
Effluent discharge management									
Water discharge per event (%) [a]	34.5 ± 14.0	25.6 ± 8.26	12.1 ± 9.60	10.9 ± 8.99	13.9 ± 9.31	14.4 ± 11.7	36.2 ± 16.2	14.4 ± 8.47	A
Time interval between discharge events (d) [a]	8.67 ± 10.5	115 ± 0	20.0 ± 11.8	5.24 ± 7.42	17.1 ± 9.29	43.8 ± 37.9	1.00 ± 0.14	6.84 ± 5.46	A
Duration of the effluent discharge event (h) [a]	9.82 ± 3.49	10.0 ± 6.72	17.9 ± 8.38	3.67 ± 3.01	3.88 ± 3.38	3.33 ± 2.88	6.48 ± 3.05	5.75 ± 6.41	A

[a] Monte Carlo samples were generated based on distribution fitted to the original datasets (see Table S6). Mean ± SD.

[b] A: SEAT project database (Murray et al., 2013); B: literature; C: assumption.

2.4. Chemical fate and risk assessment calculations

The chemical fate and risk assessment calculations for each of the studied farm groups were performed by using the chemicals reported by farmers and those chemicals available in the surveyed chemical shops (see Table S1). This yielded a total of 98 chemical treatment evaluations. All calculations were performed by using the ERA-AQUA v2.0 model (Rico et al., 2013b). The model output was evaluated in terms of chemical dissipation processes and environmental fate as result of the mass balances in the aquaculture pond scenarios, and in terms of acute and chronic Risk Quotients (RQs) for primary producers, invertebrates and fish. For each chemical treatment applied in each scenario, 1000 model runs were performed with time steps of 10 minutes, based on the Monte Carlo scenario samples and the corresponding set of physico-chemical and toxicological data. Treatments reported to be applied for pond preparation (i.e., before stocking of the cultured species) were assumed to start one week before stocking. Treatments applied during the culture cycle (i.e., after stocking of the cultured species) were randomly distributed between the start of the cultured cycle and 30 days before harvest, assuming that farmers will consider a drug withdrawal period between the last drug application and the harvest moment that typically lasts between 21 and 40 days (Hernández-Serrano, 2005). Ecological risks were conservatively calculated for the waste-water discharge point based on the pond's effluent concentration, thus assuming no dilution in the adjacent water body. Acute RQs were calculated by dividing the highest predicted concentration in the pond effluent by the acute PNEC derived for each of the taxonomic groups. Chronic RQs were calculated by dividing the maximum time weighted average concentration in the aquaculture pond effluent calculated for a time period of 3, 21 and 28 days, by the calculated chronic PNECs for primary producers, invertebrates, and fish, respectively. Finally, the probability of exceeding 1 (potential risk) and 100 (clear risk) in the acute and chronic RQ distributions were calculated for each of the evaluated ecological endpoints.

A range of extra model simulations were performed in order to compare scenarios and to perform a sensitivity analysis for the variable scenario parameters mentioned in section 2.3. These simulations were performed separately for each administration method with an average (artificial) compound (Table S2), applied at a dose of 50 mg/kg body weight and 1 mg/L, for applications mixed with feed and directly to water, respectively. The calculated environmental exposure concentrations (i.e., peak concentrations and maximum time weighted average concentrations for 3, 21 and 28 days calculated over the simulation period) and the percentage of the applied drug that is released into the environment through effluent discharges were recorded as endpoints for performing data comparisons and for the sensitivity analysis. The sensitivity analysis was performed according to the method described in Rico et al. (2013b) using smoothing splines of third order, and using the Top Marginal Variance (TMV) as sensitivity measure for the investigated scenario parameters. The TMV represents how well the model output parameter can be approximated by a function that only depends on the scenario parameter under study and, therefore, the higher the TMV the higher the reliance of the model output on the input scenario parameter. TMVs were calculated using the procedures described in Jansen et al. (2005) using GenStat 15th Edition (VSN International Ltd, Hemel Hempstead, UK).

In order to perform a general assessment of the environmental risks posed by the use of veterinary medicines in each aquaculture scenario, a Chemical Risk Index (CRI) was calculated according to Eq. 1 for each endpoint in the evaluated scenarios. The CRI is a function of the calculated probability that a chemical application results in any risk and the probability that the risk actually occurs in the environment. The latter depends on the use or availability of the chemicals in a given scenario and the probability that farmers from each scenario actually exchange water with the environment and an exposure event occurs. Data on frequency of chemical use, availability of chemicals in chemical supply shops, and water exchange in the farm group was collected from Rico et al. (2013a) and the SEAT project database, and are shown in Table S1 and in Fig. S1, respectively.

$$CRI_x = \left[\left(\sum_{i=y}^{n} R_{xy} \cdot U_y\right) + \left(\sum_{i=y}^{n} R_{xy} \cdot S_y\right)\right] \cdot W$$

Eq. 1

CRI_x = chemical risk index for the endpoint *x* (e.g. *x* = acute exposure effects on invertebrates)
RQ_{xy} = probability fraction to exceed a risk quotient of one for the endpoint *x* resulting from the use of the chemical treatment *y*
U_y = fraction of farmers that use the chemical *y*
S_y = fraction of chemical supply shops that sell the chemical *y*
W = fraction of farmers that exchange water with the environment

3. Results and discussion

3.1. Environmental fate and exposure assessment

The applied compound mass that is released unchanged into the environment (i.e., sum of effluent discharge, percolation and volatilization) varied between compounds and scenarios from insignificant values (<0.1%, e.g. dichlorvos in the Thai shrimp scenario), to about 90% (for Chloramine-T in the Pangasius catfish scenario) (Fig. 2; Table S7). Our mass balance indicates that, on average, 25% of the applied compound mass ends up in the environment, with effluent discharge being the most important process (accounting for the 23% of the applied drug mass), followed by percolation (1%) and volatilization (1%). Overall, the main processes that contributed to the dissipation of the applied drugs from the pond environment were degradation in water (37%), water drainage (23%), and degradation in sediment (20%). For compounds applied mixed with feed, the most important dissipation processes were degradation in sediment (29%), drainage (27%), degradation in water (22%), and transformation in the cultured species (13%). For compounds applied directly to water the most important dissipation process was degradation in water (52%), followed by drainage (19%), and degradation in sediment (12%). Our study shows that, on average, 90% of the applied drug dissipates from the pond environment before the end of the culture cycle. The drug mass remaining in the aquaculture pond was mainly attached to sediment (6%), and less than 1% remained bioaccumulated in the cultured organisms at the end of the grow-out culture cycle (Fig. 2).

Figure 2 shows the origin of the data related to the chemical's environmental degradation and pharmacokinetics in the culture species used in this study. Only 57% of the water and 54% of the sediment degradation rates had been derived from experimental studies, and the majority corresponded with degradation rates for antiparasitic compounds such as insecticides or antihelmintics. The pharmacokinetics dataset evaluated in the current study was mostly populated with experiments performed with fish (91%), compared to crustaceans (9%). The 84% of the studies were performed with chemical treatments that had been applied mixed with feed, and the majority of them had been performed with antibiotics (87%). In addition, the 50% of the tested species were salmonids, and tropical fish species were clearly underrepresented. This indicates a substantial lack of available pharmacokinetics data for veterinary medicines in tropical species cultured in Asia (particularly on crustaceans), which is required in order to avoid or reduce uncertainty in extrapolations among species, compound categories, and compound's administration route in future risk assessments.

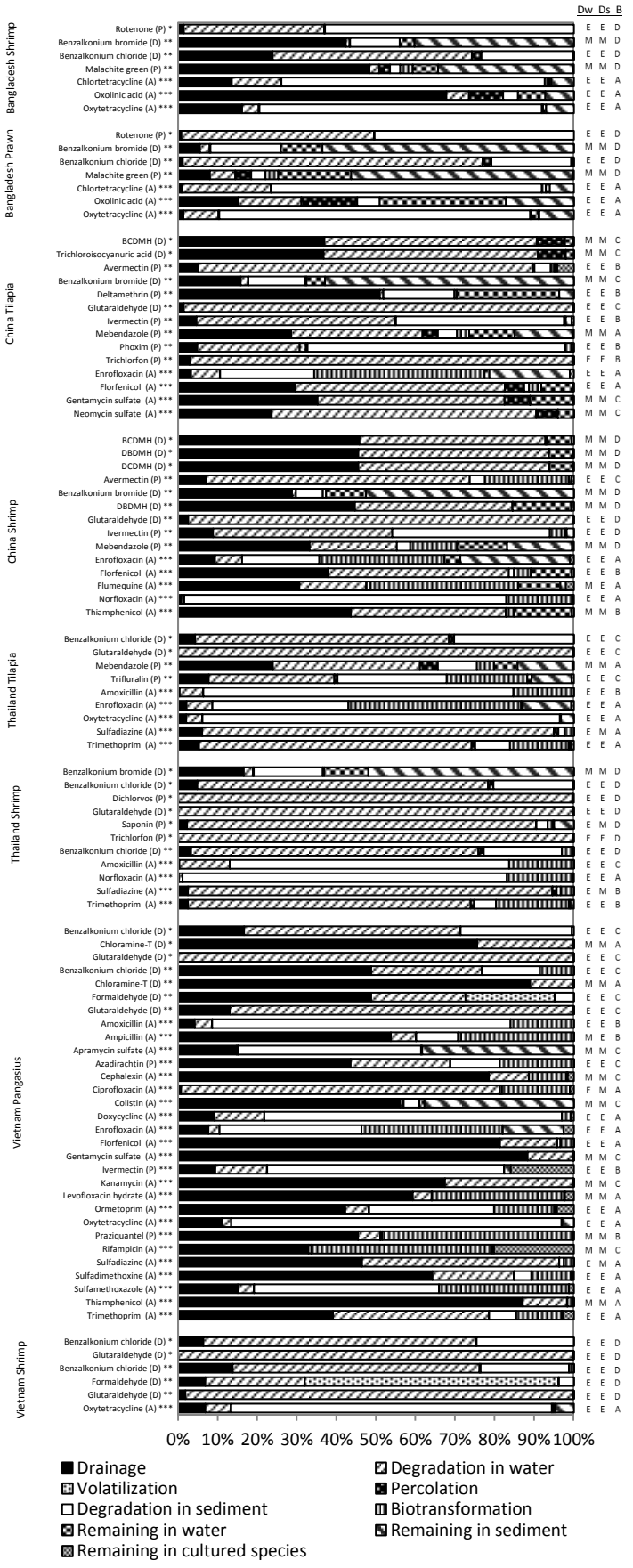


Figure 2. Results of the mass balance performed for each chemical treatment in the evaluated aquaculture pond scenarios. The data is expressed in average percentage of the applied drug mass that dissipates due to different processes or remains in the different aquaculture pond compartments at the end of the culture cycle. Left column: A = antibiotic, D = disinfectant, P = parasiticide, * = chemical applied for pond preparation, ** = chemical applied for disease

management directly to water, *** = chemical applied for disease management mixed with feed. The right hand columns indicate the origin of the water degradation rate (Dw), sediment degradation rate (Ds), and biological half-life (B) used in the model calculations. For the degradation rate in water and sediment the letters indicate: E = experimentally derived; M = based on QSAR model predictions. For the biological half-life the letters indicate the different possibilities described in section 2.2.2: A = ninety percentile of the data distribution derived for the given chemical class in the aquaculture species group; B = maximum value for the specific chemical class in the aquaculture species group; C = ninety percentile of the overall data distribution for all chemical classes with the same mode of application (i.e., directly to water or mixed with feed) in the aquaculture species group (i.e., fish or crustaceans); D = ninety percentile of the overall data distribution for all chemical classes with the same mode of application (i.e., directly to water or mixed with feed) in fish. BCDMH: Bromochlorodimethylhydantoin; DBDMH; Dibromodimethylhydantoin; DCDMH: Dichlorodimethylhydantoin.

Environmental exposure concentrations ranged between the low ng/L range to mg/L depending on the chemical treatment, mode of application, and scenario in which they were applied (Fig. 3). The action limit established by international guidelines above which an aquatic risk assessment has to be conducted for the majority of veterinary medicines is 1 µg/L, except for parasiticides for which the risk assessment always has to be performed (VICH, 2000; VICH, 2004). The percentage of evaluated treatments for which the calculated mean of the distributions of peak PECs and TWAC21 was higher than this limit was 86% and 52%, respectively (Fig. 3).The majority of the water disinfection treatments with benzalkonium chloride and bromide, and hydantoin compounds resulted in peak effluent concentrations higher than 100 µg/L. However, maximum antibiotic concentration in the aquaculture pond effluents were in the majority of the cases below 100 µg/L, with the exception of the aminoglycoside, cephalosporin and sulfonamide antibiotics applied in the Pangasius scenario, which were up to 1,000 µg/L (Fig. 3). According to the typical effluent discharge routes in each of the modelled scenarios, higher dilution of aquaculture effluents is expected to occur in the Chinese shrimp scenario and in the Vietnamese Pangasius scenario, as the majority of the aquaculture farms drain their ponds directly into the sea or into large distributaries of the large Mekong River, respectively (Fig. S1). Measured antibiotic concentrations in coastal areas of China impacted by aquaculture production (Zou et al., 2011) or in the Mekong River have not exceeded the µg/L range (Managaki et al., 2007; Shimizu et al., 2013), suggesting that dilution factors may be up to 1,000 in these areas. Therefore, in order to capture worst-case exposure and effects, monitoring must focus on farms draining into coastal creeks, small-scale streams or drainage canals, particularly in the Vietnamese Pangasius and Chinese shrimp scenarios.

Peak PECs were between 6 and 144 times higher than calculated TWAC21 values (Fig. 3), indicating much higher occurrence of pulsed exposure patterns than chronic exposure concentration in the discharge point of aquaculture effluents, and the acute/chronic ratios were generally lower for the antibiotic treatments in the Pangasius scenario due to the length of the administration period (3-7 days) and the frequent water exchange events (almost every day). The calculated exposure profiles, however, notably differed between farms within each scenario and among aquaculture scenarios depending on the magnitude, frequency and duration of the exposure pulses (e.g. Fig. 4).

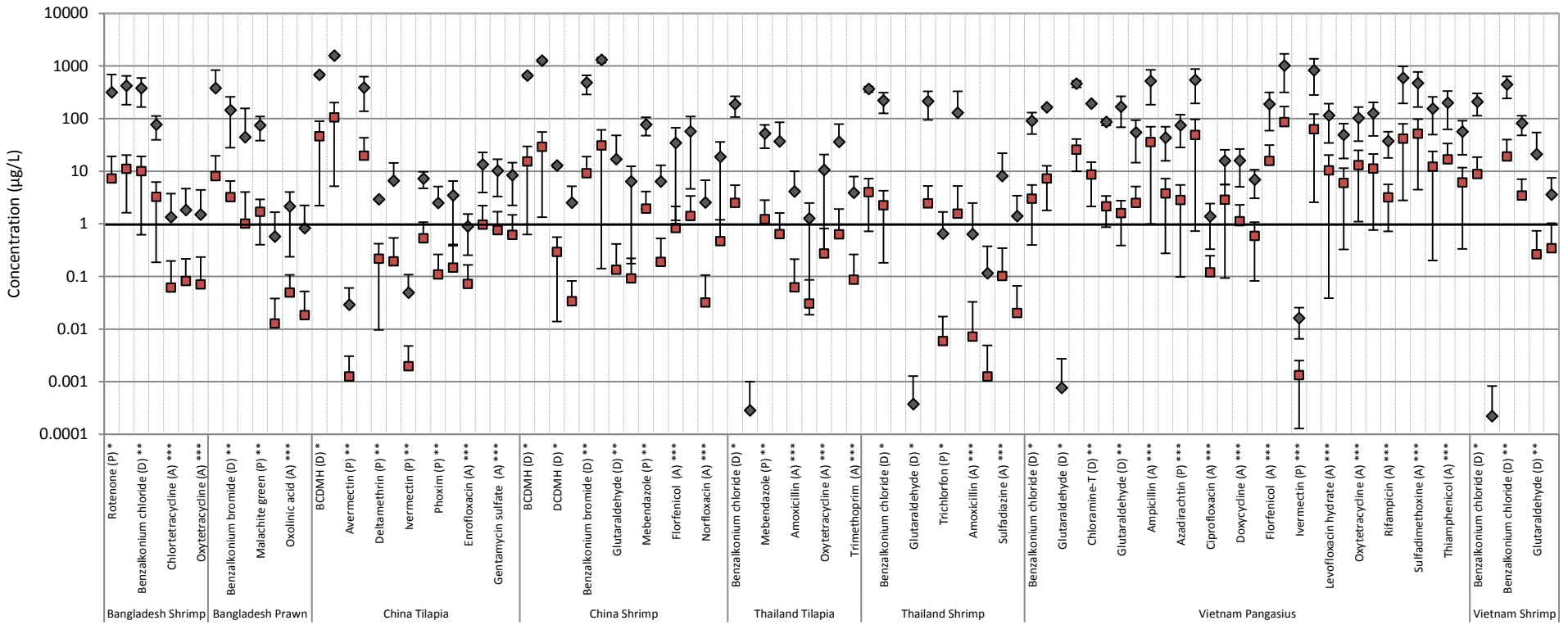


Figure 3. Calculated peak (diamonds) and time weighted average concentrations for 21 days (squares) in the aquaculture effluents (mean ± SD). The solid black line indicates the action limit above which a risk assessment has to be conducted (except for parasiticides) according to international guidelines for veterinary medicines (VICH, 2000; VICH, 2004). Values below 0.1 ng/L were not represented. The dataset used to build this graph is shown in Table S8. BCDMH: Bromochlorodimethylhydantoin; DBDMH; Dibromodimethylhydantoin ; DCDMH: Dichlorodimethylhydantoin. Left column: A = antibiotic, D = disinfectant, P = parasiticide, * = chemical applied for pond preparation, ** = chemical applied for disease management directly to water, *** = chemical applied for disease management mixed with feed.

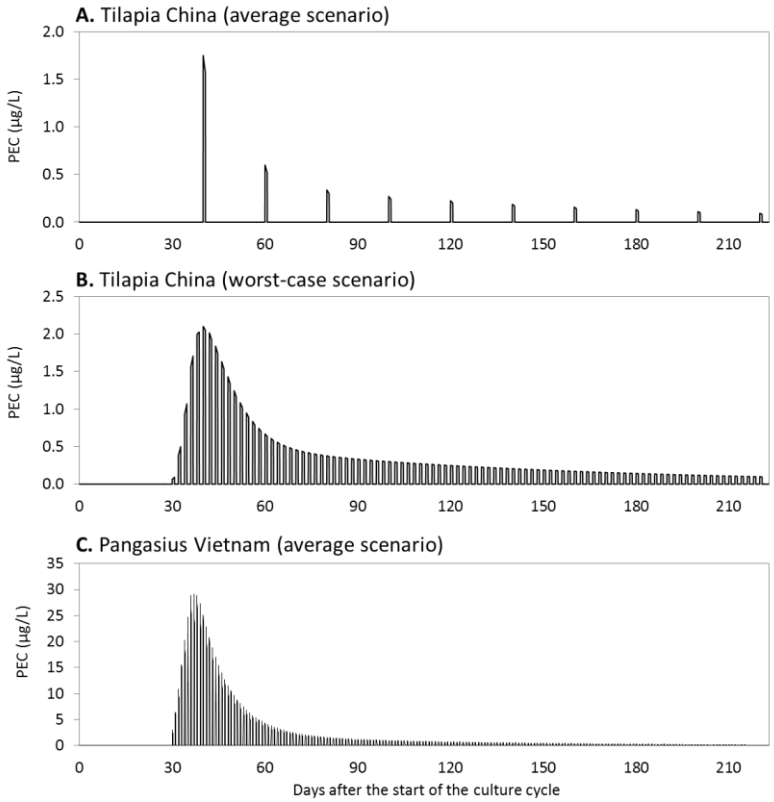


Figure 4. Example of predicted effluent concentration (PEC) dynamics for the antibiotic enrofloxacin applied to an average tilapia scenario for China (**A**), a worst-case tilapia scenario for China (**B**), and an average Pangasius scenario for Vietnam (**C**). Average scenarios were built with the mean values reported for the parameter distributions as shown in Table 1. The worst-case scenario was derived with the 10^{th} percentile of the data distribution for the percentage of water discharge per event and time interval between discharge events, and the 90^{th} percentile of the data distribution for cultured species density at the start of the culture cycle. In all scenarios enrofloxacin was applied at a dose of 20 mg/kg body weight for 7 days, and the treatment begun 30 days after the start of the culture cycle.

Results of the scenario comparisons made with the runs performed with the average (artificial) compound indicated that compounds applied directly to water are more available for effluent-mediated release into the environment (Fig. 5A), as they normally undergo less biotransformation and less biosolid deposition into the pond sediment. The highest percentage of applied drug that is expected to be released into the environment corresponds with the scenarios with higher water exchange rates: the Vietnamese Pangasius scenario, followed by the Bangladeshi shrimp scenario, and the Chinese shrimp scenario (Fig. 5A). For drugs applied mixed with feed, the calculated peak concentrations in the effluents of the Vietnamese Pangasius scenario, and the Chinese shrimp scenario were found to be one order of magnitude higher than the concentrations calculated for the other scenarios. Calculated TWACs for the Vietnamese Pangasius scenario notably exceeded the calculated values for the other scenarios in one to three orders of magnitude (Fig. 5B). The sensitivity analysis performed for drugs applied mixed with feed showed that the day of the start of the chemical treatment and the cultured species density are strongly and positively correlated to the peak drug concentrations in aquaculture effluents (TMVs: 56% and 31% respectively) (Fig. S3). TWACs were also markedly affected by the starting day of the application and the cultured species density (with TMVs values of 36-42% and 19-23%, respectively). However, the duration of the effluent discharge and the interval between effluent discharge events also played an important role, the latter being true only for long-term exposure patterns (i.e., TWACs for 21 and 28 days) (Fig. S3).

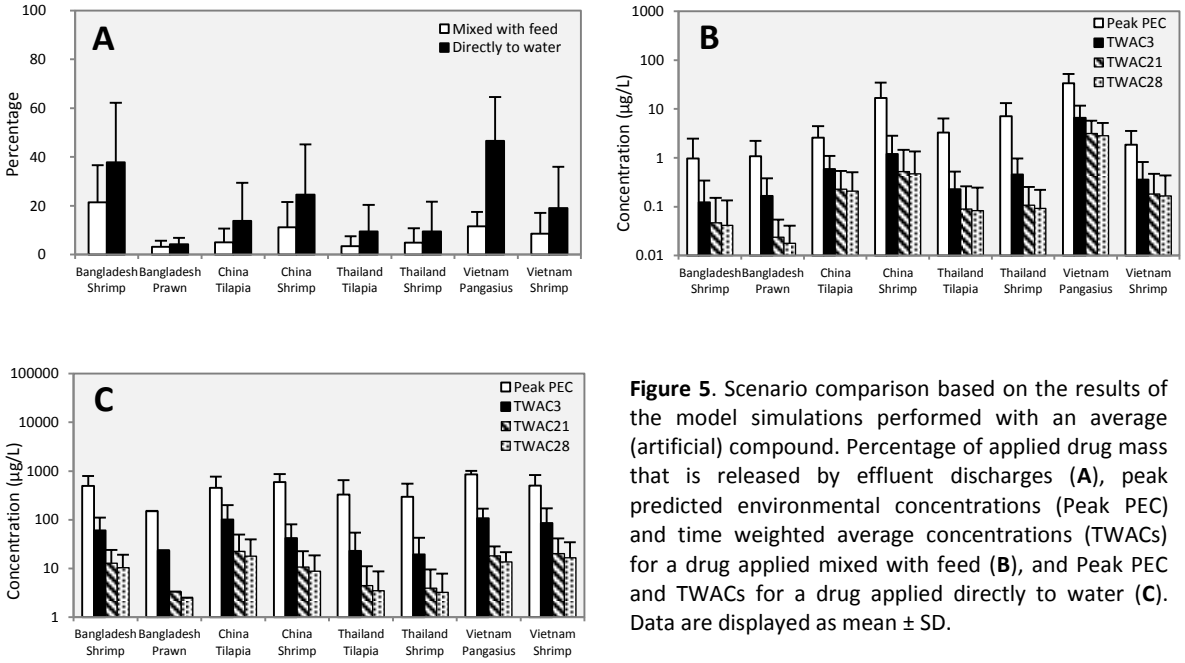


Figure 5. Scenario comparison based on the results of the model simulations performed with an average (artificial) compound. Percentage of applied drug mass that is released by effluent discharges (**A**), peak predicted environmental concentrations (Peak PEC) and time weighted average concentrations (TWACs) for a drug applied mixed with feed (**B**), and Peak PEC and TWACs for a drug applied directly to water (**C**). Data are displayed as mean ± SD.

For compounds applied directly to water, the highest peak drug concentrations were estimated for the Pangasius catfish scenario, followed by the Chinese shrimp scenario (Fig. 5C). For this mode of drug application, smaller differences in concentrations were found between the evaluated scenarios. The sensitivity analysis indicated that the most important parameter influencing the peak exposure concentrations for compounds applied directly to water is the interval between effluent discharges (TMV: 34%). Chronic exposure, however, is mostly influenced by a combination of the interval and the duration of the effluent discharge events (with TMVs values of 27-36% and 34-36%, respectively). The sensitivity analysis indicated negative correlations between the fraction of organic matter in the pond sediment and the fraction of applied drug mass that is released into the environment (TMVs of 11-19%), and the fraction of sediment's organic matter and the magnitude of the environmental exposure concentrations (TMVs of 6-11%)(Fig. S3). Therefore, this study confirms that, besides the water exchange regime, the organic matter rich sludge and sediments of earthen ponds act as a sink of chemicals and play a fundamental role in reducing the environmental release of veterinary medicines, in comparison to other aquaculture production systems such as net pens or cages. This explains the differences between the calculated percentage of the applied antibiotic mass released into surrounding ecosystems in this study (25%) and the percentages that have been speculated by other researchers, which are about 75% (Lalumera et al., 2004). Thus, the potential environmental pollution of aquaculture sludge, which has often been investigated regarding the contribution on heavy metal and nutrient loads into aquatic systems, requires further evaluation including the analysis of veterinary medicine residues. Particularly in intensive shrimp or Pangasius production, in which the disposal of high loads of sludge constitute an environmental problem (Anh et al., 2010).

3.2. Ecological effects and risk assessment

From all the evaluated treatments, 61% resulted in a median RQ higher than 1 for at least one of the evaluated ecological endpoints (Table S9). This was the case for 42% of the evaluated antibiotic treatments, 72% of the disinfectant treatments, and 90% of the parasitic treatments. Overall, antibiotics resulted in higher potential risks for primary producers. Disinfectants showed a high potential acute toxicity for all three evaluated taxonomic groups, with median RQs higher than 1 for the 41%, 63%, and 44% of the evaluated treatments for primary producers, invertebrates and fish, respectively. Parasiticide treatments posed the highest risks for

invertebrates, with 71% of the evaluated treatments resulting in median acute RQs higher than 1. The highest ecological risks (median probability of RQ>100) were calculated for the parasiticides deltamethrin and ivermectin, the disinfectants trichloroisocyanuric acid and dibromodimethylhydantoin, and the antibiotics amoxicillin, levofloxacin, and sulfadiazine (Table S7 and Fig. S2). A summary of the top-five chemical treatments posing the highest ecological risk for each of the evaluated scenarios is shown in Table 2. These chemicals have a high priority for performing further ecotoxicological work, refined modelling risk assessments and/or field monitoring assessments.

Table 2. Risk ranking of chemical treatments based on the probability (P) that the calculated Risk Quotients (RQs) exceed 100 (clear risk) and 1 (potential risk). Only the top 5 chemical treatments are shown for each aquaculture scenario, with the exception of the Vietnamese shrimp scenario, for which only 3 treatments resulted in a potential risk. When both acute and chronic RQs indicated clear risks, only the data corresponding to the worst-case exposure type is displayed.

Scenario	Treatment [a]	Sensitive Endpoints (exposure type) [b]	P(RQ>1)	P(RQ>100)	PNEC [c]
Bangladesh Shrimp	1. Rotenone (P) *	Fish (A) / Primary producers (A) / Invertebrates (A)	93/82/78	23/0.9/0.1	B/D/B
	2. Benzalkonium chloride (D) **	Primary producers (C) / Invertebrates (A) / Fish (A)	96/95/72	3.3/0/0	B/B/B
	3. Malachite green (P) **	Invertebrates (A) / Primary producers (A)	100/98	0/0	B/D
	4. Benzalkonium bromide (D) **	Invertebrates (A) / Fish (A) / Primary producers (A)	63/61/44	0/0/0	D/D/D
	5. Chlortetracycline (A) ***	Primary producers (A)	59	0	C
Bangladesh Prawn	1. Rotenone (P) *	Fish (A) / Primary producers (A) / Invertebrates (A)	92/82/78	28/2.4/0.1	B/D/B
	2. Benzalkonium chloride (D) **	Primary producers (A)	32	0.8	B
	3. Malachite green (P) **	Invertebrates (A) / Primary producers (A)	100/99	0/0	B/D
	4. Oxolinic acid (A) ***	Primary producers (A)	46	0	C
	5. Chlortetracycline (A) ***	Primary producers (A)	29	0	C
China Tilapia	1. Trichloroisocyanuric acid (D) *	Invertebrates (A) / Fish (A)	100/100	100/100	C/C
	2. Deltamethrin (P) **	Invertebrates (A)	100	100	B
	3. Ivermectin (P) **	Invertebrates (A)	100	86	C
	4. Bromochlorodimethylhydantoin (D) *	Invertebrates (C) / Fish (C)	100/100	0.5/0.4	C/C
	5. Phoxim (P) **	Invertebrates (C)	82	0.3	B
China Shrimp	1. Dibromodimethylhydantoin (D) * [and] **	Invertebrates (A) / Fish (A)	100/100	100/100	C/C
	2. Ivermectin (P) **	Invertebrates (A)	100	100	C
	3. Avermectin (P) **	Invertebrates (C)	95	23	C
	4. Flumequine (A) ***	Primary producers (A)	99	6	C
	5. Bromochlorodimethylhydantoin (D) *	Invertebrates (A) / Fish (A)	100/100	0/0	C/C
Thailand Tilapia	1. Amoxicillin (A) ***	Primary producers (A)	73	22	B
	2. Sulfadiazine (A) ***	Primary producers (A)	88	5.7	C
	3. Benzalkonium chloride (D) *	Primary producers (A) / Invertebrates (A)	100/100	0/0	B/B
	4. Mebendazole (P) **	Primary producers (A) / Invertebrates (A) / Fish (A)	100/89/10	0/0/0	D/D/D
	5. Trifluralin (P) **	Fish (A) / Invertebrates (A) / Primary producers (A)	100/99/13	0/0/0	B/B/B
Thailand Shrimp	1. Amoxicillin (A) ***	Primary producers (A)	24	1.7	B
	2. Benzalkonium chloride (D) *	Primary producers (A) / Invertebrates (A)	100/100	0/0	B/B
	3. Benzalkonium bromide (D) *	Invertebrates (A) / Fish (A)	99/94	0/0	D/D
	4. Saponin (P) *	Primary producers (A) / Fish (A)	98/84	0/0	D/C
	5. Sulfadiazine (A) ***	Primary producers (A)	44	0	C
Vietnam Pangasius	1. Levofloxacin hydrate (A) ***	Primary producers (A)	100	99	C
	2. Amoxicillin (A) ***	Primary producers (C)	100	98	B
	3. Sulfadiazine (A) ***	Primary producers (A)	100	92	C
	4. Ivermectin (P) ***	Invertebrates (A)	100	85	C
	5. Sulfamethoxazole (A) ***	Primary producers (C)	100	29	B
Vietnam Shrimp	1. Benzalkonium chloride (D) * [and] **	Primary producers (C) / Invertebrates (A) / Fish (A)	100/100/82	16/0/0	B/B/B
	2. Formaldehyde (D) **	Primary producers (A)	85	0	C
	3. Glutaraldehyde (D) **	Primary producers (A) / Invertebrates (A)	29/25	0/0	C/C

[a] A = antibiotic, D = disinfectant, P = parasiticide, * = chemical applied for pond preparation, ** = chemical applied for disease management directly to water, *** = chemical applied for disease management mixed with feed.
[b] Exposure type: A = acute; C = chronic.
[c] Predicted No Effect Concentrations (PNECs) derived from: A = model ecosystem experiment; B = species sensitivity distribution; C = single-species laboratory toxicity test; D = quantitative structure-activity relationship.

For none of the top-ranked compounds there is published data that evidence ecological effects after application in aquaculture ponds, hampering any comparison of the theoretical risks calculated in this study with field data, and indicating a need for further biological monitoring work. The majority of the PNECs used in the risk assessment were derived from the application of standard assessment factors to single-species laboratory toxicity data, with the exception of parasiticides, for which enough data was in most of the cases available to derive PNECs based on invertebrate and fish SSDs (Table S2; Fig. 6). Only ivermectin was evaluated using microcosms (Boonstra et al., 2011), while the antimicrobials were clearly underrepresented in the evaluated ecotoxicity dataset (Table S2; Fig. 6). This study indicates that more studies are needed on the toxicity of antimicrobial compounds to non-target aquatic organisms, and particular attention should be paid to test benthic species, since might be more exposed to compounds such as antibiotics that tend to accumulate in sediments down-stream aquaculture farms (e.g. Le and Munekage, 2004), and for which data is currently unavailable.

Regarding the environmental release and ecotoxicological effects of the applied veterinary medicinal treatments, the Pangasius catfish scenario in Vietnam was clearly identified as the scenario resulting in the highest ecological risks, followed by the shrimp and the tilapia production scenarios in China (Table 3). In the Pangasius scenario, highest risks were calculated for primary producers due to the contamination with several antimicrobial compounds. In the shrimp and tilapia scenarios of China, however, invertebrate and fish communities were identified as the most sensitive groups due to the environmental contamination with residues of highly toxic disinfection treatments, such as trichloroisocyanuric acid and hydantoin compounds, and parasiticides, such as deltamethrin and ivermectin.

Table 3. Scenario ranking, from highest to lowest estimated ecological risk, based on the sum of the Chemical Risk Indexes (CRIs) calculated for each endpoint in the evaluated aquaculture scenarios.

Rank	Scenario	CRI acute primary producers	CRI chronic primary producers	CRI acute invertebrates	CRI chronic invertebrates	CRI acute fish	CRI chronic fish	Σ CRIs
1	Vietnam Pangasius	9.2	5.6	3.4	3.0	2.0	0.5	24
2	China Shrimp	0.8	0.1	1.1	0.8	0.9	0.7	4.4
3	China Tilapia	0.4	0.0	0.9	0.6	0.4	0.2	2.5
4	Vietnam Shrimp	0.5	0.5	0.5	0.4	0.3	0.1	2.1
5	Thailand Tilapia	0.6	0.4	0.5	0.1	0.2	0.0	1.8
6	Thailand Shrimp	0.4	0.3	0.6	0.1	0.4	0.0	1.8
7	Bangladesh Shrimp	0.5	0.1	0.4	0.1	0.4	0.2	1.8
8	Bangladesh Prawn	0.0	0.0	0.0	0.0	0.0	0.0	0.1

The risk assessment scenarios and modelling approach used in this study provide a robust starting point for addressing further priorities and refining exposure and effect assessments. The resulting risk-based order of ranking of chemicals can be used to guide local researchers, environmental authorities, and certification schemes in order to promote and further develop regulations concerning the new registration, and post-registration monitoring of aquaculture medicines, as well as to support the development of improved aquaculture management practices. The ecological risk assessment presented here is based on the procedures proposed in the risk evaluation of veterinary medicinal products in developed countries (VICH, 2000 and 2004), which aims at protecting the structure of aquatic ecosystems (i.e., primary producers, primary consumers and secondary consumers). However, the majority of the veterinary medicines used in Asian aquaculture show specific (e.g. fungicides, antibiotics) or broad activity (e.g. chlorine-releasing compounds) against microorganisms. Recent studies show that microorganism (biofilm) communities, and important ecosystem-related processes such as carbon utilization, photosynthesis, or nutrient metabolism, might be affected by antibiotic concentrations that are below or in the order of magnitude of those that we calculated in this study (Yergeau et al., 2012; Yan et al., 2013). Preserving water quality is of crucial importance in aquaculture production

landscapes as farmers often rely on input waters that have received effluents from up-stream farms. Thus, risk assessments for aquaculture medicines should also aim to protect functions provided by aquatic ecosystems. Specific guidelines and a harmonized framework is currently not available for testing and including ecosystem functional endpoints as part of environmental risk assessments (Peters et al., 2013). However, we expect them to gain scientific relevance and to be integrated as protection goals in future risk assessments for aquaculture medicinal products.

In most of the cases, the laboratory toxicity data used in this study have been derived by using standard protocols that represent optimal conditions for the tested organisms. However, the environmental conditions in ecosystems surrounding aquaculture farms in Asia are often very different. Aquaculture pond effluents are characterized by high nutrient concentrations, high suspended (bio)solid concentrations, and often higher temperatures than running waters (Boyd, 2003). Hence, aquatic ecosystems in the surroundings of aquaculture farms often present a high level of eutrophication (e.g. Herbeck et al., 2013). The relative contribution of pulses of single compounds or mixtures of veterinary medicines to the toxicity of the total aquaculture effluent is yet poorly understood (Tello et al., 2010). Also, the influence of higher nutrient concentrations, suspended solids, temperature and pH on the toxicity of veterinary medicines has not been extensively investigated, which is needed since laboratory toxicity experiments have demonstrated that different pH, temperature, and UV-B conditions might influence the toxicity of antibiotics to *Daphnia magna* (Kim et al., 2010). Therefore, in order to better distinguish the ecological effects of multiple stressors in aquatic communities, further experiments must focus on assessing the effects of pulsed exposure patterns representing aquaculture effluent discharges on model ecosystems with high level of eutrophication representing tropical ecosystems impacted by aquaculture effluents in Asia.

In this study we used a single-compound approach to characterize risks of veterinary medicinal treatments. However, chemical residues might co-occur not only inside aquaculture ponds but also in aquaculture farm effluents, as often products contain more than one active ingredient, or several products might be applied in different moments of the production cycle to different batches. For instance, products containing more than one ingredient such as sulfonamide antibiotics potentiated with trimethoprim or ormetoprim, and the combination of apramycin and levofloxacin in the Pangasius catfish production have been reported by Rico et al. (2013a). Christensen et al. (2006) and Habenbuch and Pinckney (2012) have demonstrated that antibiotic mixtures used in aquaculture might result in additive, and even synergistic effects, on micro-algae and activated sludge microorganisms. Therefore, follow-up larger scale modelling and monitoring studies should be done in order to identify relevant chemical mixtures in aquatic ecosystems surrounding aquaculture farms for which further ecotoxicological research is needed.

Finally, the environmental and public health concerns associated to the selective pressure on bacteria by antibiotic pollution and the development of antibiotic resistance is, nowadays, one of the main challenges faced by the aquaculture industry worldwide (Tuševljak et al., 2013). High levels of antimicrobial resistance genes have been detected in aquatic ecosystems surrounding aquaculture shrimp farms of Asia (Tendencia and De la Peña, 2001; Le et al., 2005). Yet there is limited evidence as to whether monitored resistant bacteria in the environment developed as a result of the exposure to aquaculture antibiotic residues or were transported by effluents from aquaculture facilities. The knowledge and scientific tools available for the prospective assessment of resistance development, environmental transport, and ultimately ecological and public health consequences are very limited. For this reason, this endpoint was not considered in this study, and neither has been included in current international risk assessment guidelines (EMEA, 2008). Tello et al. (2012) demonstrated that the soil action limit used in the first-tier of the environmental risk assessment of antibiotics (100 µg/kg), and measured environmental concentrations, may exert a selective pressure on bacteria in the environment. This was shown by mechanistically linking exposure concentrations with bacterial SSDs based on clinically derived

minimum inhibitory concentrations. One of the future challenges for the Asian aquaculture sector is to derive prospective methods to assess the impact of antibiotic use practices on resistance development in the cultured organisms and within aquaculture facilities (e.g. water and sediment matrices), and to prioritize compounds and bacterial taxa regarding their resistance susceptibility. In this way, linking chemical fate modelling approaches as presented in this study with the modelling approach described by Tello et al. (2012) can provide promising advancements.

4. Conclusions

This study provides the first modelling exercise and quantitative risk assessment performed for veterinary medicines applied in Asian aquaculture scenarios. The risk-based ranking of compounds and scenarios indicated that aquaculture parasiticides pose a higher environmental risk than disinfectants and antibiotics, and Pangasius catfish production in Vietnam is posing the largest environmental hazard compared to the other evaluated scenarios. Research priorities for refined risk assessments include the study of antimicrobial toxicity on microorganisms and associated ecosystem functions, the toxicity evaluation of aquaculture medicines under multi-stress conditions considering chemical mixtures, and the development of antimicrobial resistance endpoints for the risk evaluation of aquaculture medicines.

Supporting Information

The Supporting Information of this chapter can be downloaded from:
http://dx.doi.org/10.1016/j.scitotenv.2013.08.063.

Chapter 6

Use, fate and ecological risks of antibiotics applied in tilapia cage farming in Thailand

Andreu Rico, Rhaul Oliveira, Sakchai McDonough, Arrienne Matser, Jidapa Khatikarn, Kriengkrai Satapornvanit, António J.A. Nogueira, Amadeu M.V.M. Soares, Inês Domingues, Paul J. van den Brink

Abstract

The use, environmental fate and ecological risks of antibiotics applied in tilapia cage farming were investigated in the Tha Chin and Mun rivers in Thailand. Information on antibiotic use was collected through interviewing 29 farmers, and the concentrations of the most commonly used antibiotics, oxytetracycline (OTC) and enrofloxacin (ENR), were monitored in river water and sediment samples. Moreover, we assessed the toxicity of OTC and ENR on tropical freshwater invertebrates and performed a risk assessment for aquatic ecosystems. All interviewed tilapia farmers reported to routinely use antibiotics. Peak water concentrations for OTC and ENR were 49 and 1.6 µg/L, respectively. Antibiotics were most frequently detected in sediments with concentrations up to 6,908 µg/kg d.w. for OTC, and 2,339 µg/kg d.w. for ENR. The results of this study indicate insignificant short-term risks for primary producers and invertebrates, but show that the studied aquaculture farms constitute an important source of antibiotic pollution.

This chapter has been accepted for publication in *Environmental Pollution*.

1. Introduction

Aquaculture production has intensified at a rapid pace across Asian countries in order to supply the increasing demand of aquatic products at a national level and in importing regions such as European or North-America (FAO, 2012a). As long as aquaculture practices have intensified and the quality of water supplies in aquaculture-clustered areas has deteriorated, the Asian aquaculture industry has been overwhelmed with a wide range of parasitic and bacterial diseases affecting the cultured species (Bondad-Reantaso et al., 2005). In order to prevent or treat such disease outbreaks, farmers often rely on a wide array of veterinary medicinal products such as antibiotics and parasiticides, which are mainly applied during periods of high stress in the cultured species (Rico et al., 2012a; Rico et al., 2013a). Residual concentrations of antibiotics used in aquaculture production have been measured in aquatic ecosystems down-stream of aquaculture production areas of Asia (Managaki et al., 2007; Zou et al., 2011; Zhou et al., 2011; Takasu et al., 2011; Shimizu et al., 2013), and due to the importance and the geographical spread of this economic activity throughout this continent, aquaculture production has been considered as one of the main pathways of veterinary medicines into the environment (Managaki et al., 2007).

Thailand is ranked sixth in aquaculture production globally, with tilapias (*Tilapia* spp.) being the most important cultured fish species group (FAO, 2012b). About 30% of Thai tilapias are produced at high densities in floating cages placed on rivers or irrigation canals (Belton et al., 2009a). Tilapias cultured under such open culturing systems are highly vulnerable to stress produced by water quality fluctuations and can easily be infected by naturally occurring parasites. Particularly, infestations with *Streptococcus* spp. and other bacteria (e.g. *Aeromonas* spp*., Pseudomonas* spp*.,* and *Vibro* spp.) have been reported to be the main causes of mortality in caged tilapia farming (Belton et al., 2009b). In order to prevent mass tilapia mortalities, farmers often apply antibiotics mixed with the fish diet. Large amounts of antibiotics applied in marine cage-based aquaculture production have been reported to end-up in the surrounding ecosystems through leaching or sedimentation of medicated feeds, or via excretion from the cultured species (Coyne et al., 1994; Capone et al., 1996). Similar situations are expected to occur in freshwater aquaculture, however, studies that report the environmental fate and distribution of antibiotics in rivers impacted by freshwater cage aquaculture are currently unavailable.

The main objective of this study was to investigate the use of antibiotics in tilapia cage farms in Thailand and to assess their environmental fate and risks for tropical aquatic ecosystems. Initially, we performed interviews with tilapia-cage farmers at two Thai rivers with significant aquaculture production. Then, we monitored residues of the most commonly used antibiotics, oxytetracycline (OTC) and enrofloxacin (ENR), in water and sediment samples collected in the environment surrounding the surveyed tilapia cage farms and in a 'non-polluted' reference area, and measured antibiotic concentrations in samples collected during and after antibiotic administration in two reference farms. In order to characterize the ecosystem sensitivity to antibiotics we performed toxicity tests with tropical invertebrates and derived safe environmental concentrations for primary producers and invertebrates. Finally, ecological risks for primary producer and invertebrate communities exposed to antibiotic residues were calculated based on the obtained measured environmental concentrations. To our knowledge, this is the first study describing the use and potential ecological risks of antibiotics applied in freshwater cage aquaculture production.

2. Material and methods

2.1. Study areas and antibiotic use data collection

This study was conducted in the Tha Chin River and in the Mun River (Fig. 1; see Supporting Information for a description of the study areas). Both rivers are subject to monsoon climate, with

the rainy season lasting from May to October. The Tha Chin and the Mun rivers significantly contribute to the total cage-based production volume of tilapia in Thailand. In these rivers, mono-sex Nile (*Oreochromis niloticus*) or (hybrid) red tilapias (mainly *O. mossambicus* x *O. aureus*) are cultured in 3x3 m (1.5-2.0 m depth) floating cages composed of steel frames and polypropylene mesh. Tilapias are fed with commercial pelleted feeds for a period of 4 months, until they reach a weight of 600-1000 g. Farms are formed by several tilapia cages (4-100) placed in parallel to the banks of the river, and normally operate in batches throughout the whole year.

Information on antibiotic use was collected by structured interviews conducted with 29 tilapia farmers (15 in the Tha Chin River and 14 in the Mun River) between November, 2010, and April, 2011. Information collected included names of antibiotic ingredients, dosages, and modes and frequencies of application. Additional information on farmer perceptions on water quality and disease occurrence was also collected.

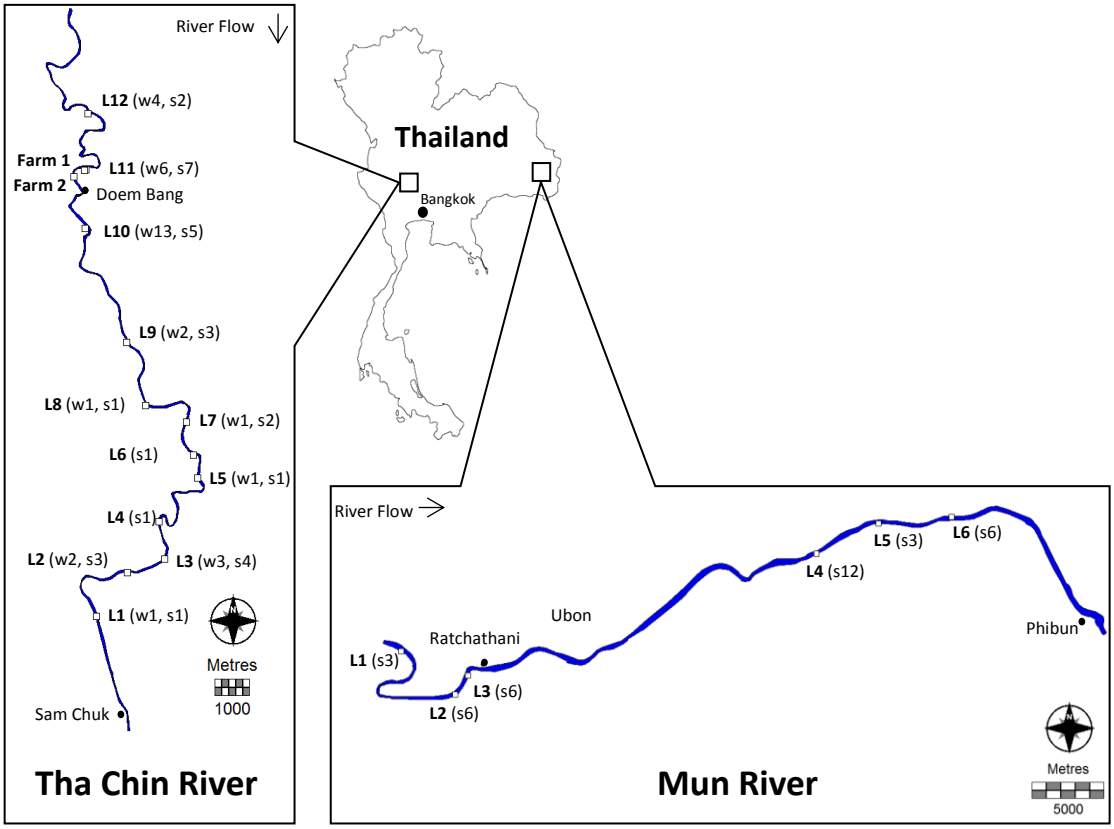


Figure 1. Sampling locations (L) in the Tha Chin River and in the Mun River (Thailand), and number of water (w) and sediment (s) samples collected in each location. In the Tha Chin River: L1,2,3 and 4 were sampled in the dry season; L5,6,8 and 9 were sampled in the wet season; and L7,10,11 and 12 were sampled in both seasons. The location L3 in the Mun River was considered as the reference site.

2.2. Sample collection

Water samples were collected in the Tha Chin River during the dry season (March; *n*=24) and in the wet season (June; *n*=10) (Fig. 1). Sediment samples were collected during the dry season in the Tha Chin River (March; *n*=19) and in the Mun River (January-February; *n*=30), and during the wet season in the Tha Chin River (June; *n*=12) (Fig. 1). In addition, six reference sediment samples were collected from a location in the Mun River isolated from the aquaculture farms. This area was assumed not to be impacted by anthropogenic activities and was considered as the 'non-polluted' reference site (Fig. 1). For a description of the antibiotic sample collection methods see the Supporting Information.

In order to assess the fate and dissipation of the studied antibiotics during and after an antibiotic administration period, extra samples were collected in two tilapia farms located in the Tha Chin River. In the first farm (FARM 1), OTC was administered mixed with feed at a dose of 40 mg/kg fish body weight (b.w.). Six cages containing 600 fish per cage (approximate weight 300 g/fish) were treated. Water samples were taken in duplicate inside the cages and next to the cages at 15 min, 1h and 15h after the antibiotic administration. In the second farm (FARM 2), OTC and ENR treatments were applied to 14 tilapia cages with 600 fish per cage (approximate weight 600-700 g/fish). Both antibiotic treatments had a duration of 7 days and overlapped in time (Fig. S1). Antibiotics were applied once a day at a dose of 14 and 8 mg/kg b.w. for OTC and ENR, respectively. Sediment samples were collected on day 6, 8, 10, 13 and 18 after the first OTC application. Water and sediment samples were collected in the afternoon (15-30 min after antibiotic administration) on day 1, 3, 5, 8, 13 and 25 after the first ENR administration. Samples were collected next to the tilapia cages, and at 30 m and 60 m downstream the cages (see Fig. S1 for a synopsis).

2.3. Toxicity experiments

The acute toxicity of oxytetracycline (OTC) and enrofloxacin (ENR), was assessed on five tropical freshwater invertebrate species: a worm (*Limnodrilus hoffmeisteri)*, two molluscs (the snails *Physella acuta* and *Melanoides tuberculata*), an insect (*Micronecta* sp.), and a crustacean (i.e., the shrimp *Macrobrachium lanchesteri*). The toxicity of OTC and ENR to invertebrates was assessed by means of static laboratory toxicity tests with a duration of 48 h. Detailed characteristics of the test organisms and experimental set-up are provided in Table 1. Test operating procedures were based on the OECD standard protocol for toxicity testing with *Daphnia magna* (OECD, 2004), however the temperature and the photoperiod (12 h of natural light) were adapted to match the environmental conditions of tropical ecosystems (Table 1).

The toxicity tests were performed with the commercial products OXYBAC 50 (oxytetracycline-HCl 50%) and EnFlocin (enrofloxacin-HCl 20%), which were typically used by tilapia farmers in the study area. Since tetracycline antibiotics have been reported to attach strongly to glass (Ciarlone et al., 1990), the test vessels used for the OTC toxicity experiments were previously rinsed with a solution of Na-EDTA in methanol (0.2% v/v). In order to assess the potential effects of rinsing the glass vessels with Na-EDTA solution, controls with rinsed and non-rinsed glass vessels were used in the OTC experiments.

In order to verify the antibiotic exposure concentrations, a sample of test media (10 mL) was taken from one replicate per treatment level at 1h and 48h after the start of the experiments. The effects of the antibiotics on the evaluated endpoints were recorded 24h and 48h after the start of the experiment. The studied endpoints were immobility, for snails and worms, and mortality, for the arthropod species (Table 1). Animals were considered immobile when no movement was observed after repeated (three times) tactile stimulation with a laboratory needle. The tests were accepted only when immobility or mortality in the controls did not exceed 20%. Finally, the Effect or Lethal Concentration for the 10% and 50% of organisms (EC10/50 or LC10/50) after an exposure period of 48h, and their 95% confidence interval (CI), were calculated. Calculations were performed with the ToxRat Professional Version 2.07 program by fitting the data to a log concentration – probit regression model. The EC and LCs were calculated with the 48h-averaged measured antibiotic concentrations.

Table 1. Characteristics of the tested organisms, experimental set-up, measured temperature (T), dissolved oxygen (DO) and pH in the test media, and endpoints evaluated in the toxicity tests (mean±SD).

	Individual's characteristics			Experimental set-up				Oxytetracycline			Enrofloxacin			Endpoint
Species	Length[a] (mm)	Weight[a] (mg)	Origin[b]	Water volume (mL)	Replicates (*n*)	Number per replicate	Aeration[c]	T (°C)	DO (mg/L)	pH	T (°C)	DO (mg/L)	pH	Immobility/ Mortality
Limnodrilus hoffmeisteri	26±5.5	NM	A	250	3	10	Yes	28.8±0.7	6.1±0.2	7.4±0.6	28.1±0.7	7.2±0.2	7.6±0.6	Immobility
Macrobrachium lanchesteri	31±4.3	329±145	A	500	6	5	Yes	28.7±0.4	5.8±0.4	7.3±0.5	28.1±0.4	5.4±0.4	7.3±0.5	Mortality
Melanoides tuberculata	17±0.7	356±67	B	250	3	10	Yes	27.6±0.7	5.8±0.2	7.2±0.6	27.5±0.7	5.5±0.2	6.5±0.6	Immobility
Micronectinae sp.	2.1±0.4	NM	B	250	3	10	No	28.7±0.7	NM	6.9±0.6	29.4±0.7	NM	7.5±0.6	Mortality
Physella acuta	7.5±1.2	63±21	B	250	3	10	Yes	27.4±0.7	5.8±0.2	7.0±0.6	27.3±0.7	5.6±0.2	7.2±0.6	Immobility

NM: not measured.

[a] $n = 10$

[b] A: purchased from fish retailers; B: collected from unpolluted freshwater tanks located in the Ornamental Fish Facilities of Kasetsart University (Bangkok, Thailand)

[c] The aeration system consisted of an air pump with plastic tubes connected to glass pipettes bubbling air into the test media. Since *Micronectinae sp.* are air breathers, aeration was not installed in these tests and dissolved oxygen levels in the test media were not measured.

2.4. Antibiotic analysis

The methods used for the extraction and analysis of the antibiotics in the water and sediment samples are described in the Supporting Information.

2.5. Ecological risk assessment

Ecological risks of OTC and ENR were calculated for the water layer and for the sediment. Ecological risks for the water layer were based on the measured antibiotic concentrations. The ecological risks for sediment were calculated by converting the measured sediment concentrations into their corresponding pore water concentration ($C_{pore\ water}$) according to Equation 1 assuming equilibrium conditions.

$$C_{pore\ water}\ (\mu g/L) = \frac{C_{sediment}}{K_{oc} \cdot f_{om}} \qquad Eq.\ 1$$

where $C_{sediment}$ is the measured concentration in sediment, K_{oc} is the organic carbon partitioning coefficient, and f_{om} is the fraction of organic matter in the sediment. The K_{oc} values for OTC (26,134 L/kg) and ENR (186,342 L/kg), were obtained from Jones et al. (2005) and Nowara et al. (1997), respectively, and were selected based on experimental data from soils with similar characteristics (i.e., clay content, organic matter content) as the sediments we sampled. The f_{om} values were based on the mean measured value in each monitoring campaign (Table S1).

The ecological risk assessment was performed by following a Risk Quotient (RQs) approach and by using Species Sensitivity Distributions (SSDs). RQs were calculated by dividing the maximum measured water and pore water concentrations by Predicted No Effect Concentrations (PNECs) for primary producers and invertebrates. PNECs were derived by using toxicity data for standard test species and assessment factors as proposed in the international guidelines for the risk assessment of veterinary medicines (VICH, 2004). SSDs were separately built for primary producers and invertebrates (for rationale see Posthuma et al., 2002). The datasets used to build the SSDs were a combination of the toxicity data generated in the current study and toxicity values (EC50 and LC50) collected from the literature (see Table S2). SSDs were built by fitting the toxicity data to a log-normal distribution using the ETX 2.0 software (Van Vlaardingen et al., 2004). The fit of the toxicity datasets to the log-normal distribution was assessed by the Anderson-Darling goodness of fit test (p = 0.05). The median Hazardous Concentration for the 5 and 50% of species (HC5 and HC50, respectively) and their 95% confidence interval (CI) were calculated according to Aldenberg and Jaworska (2000). In order to compare the primary producers and invertebrate sensitivities to the studied antibiotics, the significant differences between the distributions of toxicity data were evaluated by the two-sample Kolmogorov-Smirnov test (p = 0.05) using the GenStat 15th Edition software (VSN International Ltd, Hemel Hempstead, UK). For each SSD, the Potentially Affected Fraction (PAF) of species was conservatively calculated based on the maximum measured antibiotic concentration in the water and the estimated maximum sediment pore water concentration.

3. Results

3.1. Antibiotic use

All the interviewed tilapia farmers reported to use antibiotics. Ten different antibiotic ingredients were identified (Fig. 2), belonging to 5 different classes (β-lactams, quinolones, sulfonamides, tetracyclines, diaminopyrimidines). Overall, the most commonly used antibiotics were enrofloxacin (59% of the interviewed farmers declared to use it), followed by oxytetracycline (48%), amoxicillin (28%), and sulfadiazine potentiated with trimethoprim (28%). On average, farmers reported to use two different antibiotics per farm, however, some farmers (24%) applied

between 4 and 7 different antibiotic ingredients. Antibiotic use patterns of the interviewed farmers in the Tha Chin River and in the Mun River were found to be very similar. In all cases antibiotics were reported to be applied mixed with feed (once or twice a day) for a period ranging between 3-10 days or longer until tilapia mortalities decreased. Dosages reported in product labels ranged between 10-100 mg/kg b.w. per day, depending on the active ingredient. Farmers reported to use antibiotics to treat disease outbreaks between 1 and 3 times per production cycle (usually 4 months). In addition, about 70% of the interviewed farmers reported to apply antibiotics routinely to prevent diseases associated to the stress generated during the stocking of fingerlings. Changes in river water quality were reported to be associated to fish disease, particularly dissolved oxygen drops during the dry season and heavy water runoff events occurring during the beginning of the rainy season. Farmers also reported that high loads of organic material and traces of pesticides from the surrounding agricultural fields (mainly rice crops) could be the cause of the mortalities observed after runoff events. Besides antibiotics, farmers also reported the use of parasiticides (e.g. trifluralin, praziquantel), salts (e.g. sodium chloride, potassium permanganate), probiotic enzymes and yeasts, and other feed additives such as vitamin C, proteins, and polysaccharides.

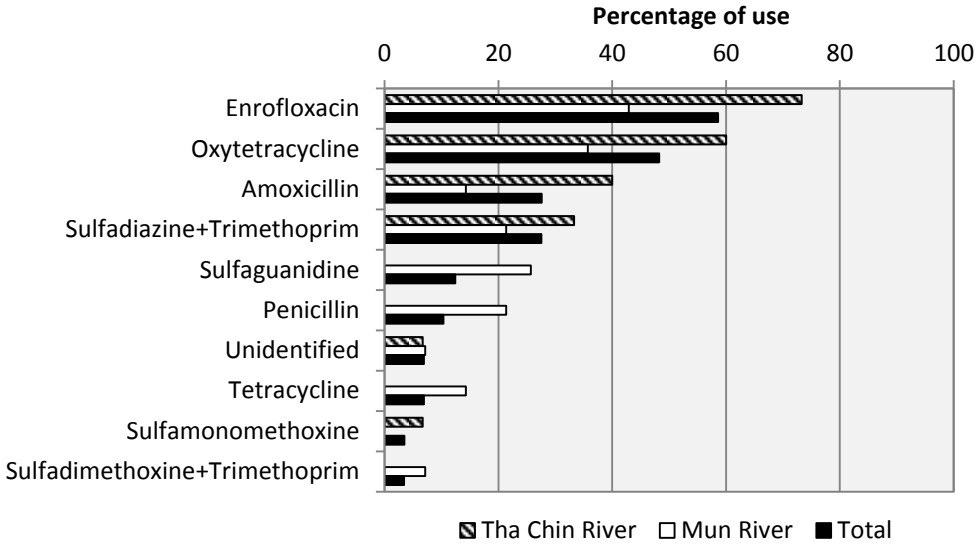


Figure 2. List of reported antibiotics and percentage of use amongst the interviewed tilapia farmers.

3.2. Antibiotic contamination

OTC was detected in 25% of the water samples collected during the dry season and in 100% of the water samples collected during the wet season in the Tha Chin River, with a maximum measured antibiotic concentration of 3.1 µg/L (Table 2). ENR was only detected in the water samples collected during the wet season, and the maximum measured concentration was 1.6 µg/L (Table 2). Both antibiotics were detected indistinctively in the majority of the sampling locations (Fig. S2). OTC and ENR were measured in all the sediment samples collected in the aquaculture-impacted areas of the Tha Chin River (dry and wet season) and Mun River. Sediment concentrations of antibiotics ranged between 4.5 and 4,062 µg/kg d.w. for OTC, and between 1.4 and 2,339 µg/kg d.w. for ENR. Overall, measured antibiotic concentrations in the Mun River were found to be higher than those measured in the Tha Chin River, and antibiotic concentrations in the wet season were found to be lower than in the dry season, especially for OTC (Fig. 3). The highest sediment concentrations in the Mun River were measured in locations 1, 2 and 4 (Fig. S2), which coincided with the river locations that held the highest density of tilapia cages. ENR was not detected in the sediments of the reference site sampled in the Mun River, and OTC was detected at low concentrations (5.4-6.0 µg/kg d.w.) in 4 out of the 6 collected samples.

Table 2. Summary of measured oxytetracycline (OTC) and enrofloxacin (ENR) concentrations in the collected water and sediment samples from the Tha Chin and Mun rivers.

		Water		Sediment	
		OTC	ENR	OTC	ENR
Tha Chin River (dry season)	Number of samples	24	24	19	19
	Detection rate	25%	n.d.	100%	100%
	Geometric mean concentration [a]	0.22	n.d.	42.4	51.2
	Maximum concentration [a]	3.05	n.d.	2119	285
Tha Chin River (wet season)	Number of samples	10	10	12	12
	Detection rate	100%	100%	100%	100%
	Geometric mean concentration [a]	0.50	0.49	12.9	45.4
	Maximum concentration [a]	1.76	1.59	94.9	67.3
Mun River (dry season)	Number of samples	NM	NM	30	30
	Detection rate	NM	NM	100%	100%
	Geometric mean concentration [a]	NM	NM	245	198
	Maximum concentration [a]	NM	NM	4062	2339
Mun River (reference site)	Number of samples	NM	NM	6	6
	Detection rate	NM	NM	67%	n.d.
	Geometric mean concentration [a]	NM	NM	5.57	n.d.
	Maximum concentration [a]	NM	NM	5.95	n.d.

[a] Concentrations are expressed in µg/L for water samples and µg/kg d.w. for sediment samples.
NM: not measured; n.d.: not detected

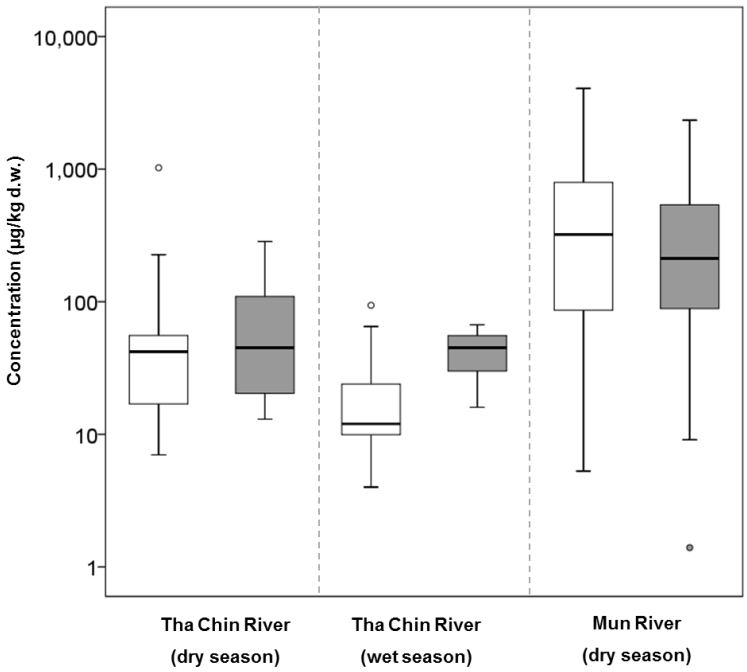


Figure 3. Measured concentrations of oxytetracycline (white boxes) and enrofloxacin (grey boxes) in the sediment samples. Each box shows the median, the 25th and 75th percentiles, the lowest datum within 1.5 times the interquartile range of the lower quartile, and the highest datum within 1.5 times the interquartile range of the upper quartile. Dots represent outliers (values between 1.5 and 3 times the range between the lower and upper interquartile).

OTC concentrations in the water samples collected 15 min after antibiotic administration in FARM 1 were 37 (26-49) µg/L inside the cages, and 6.4 (1.6-11) µg/L next to the cages (mean; minimum-maximum). Mean OTC concentrations dropped to 0.4 µg/L inside the cages and 0.1 µg/L next to the cages 1h after administration, and fell below the LOD 15h after antibiotic administration (Fig. 4A).

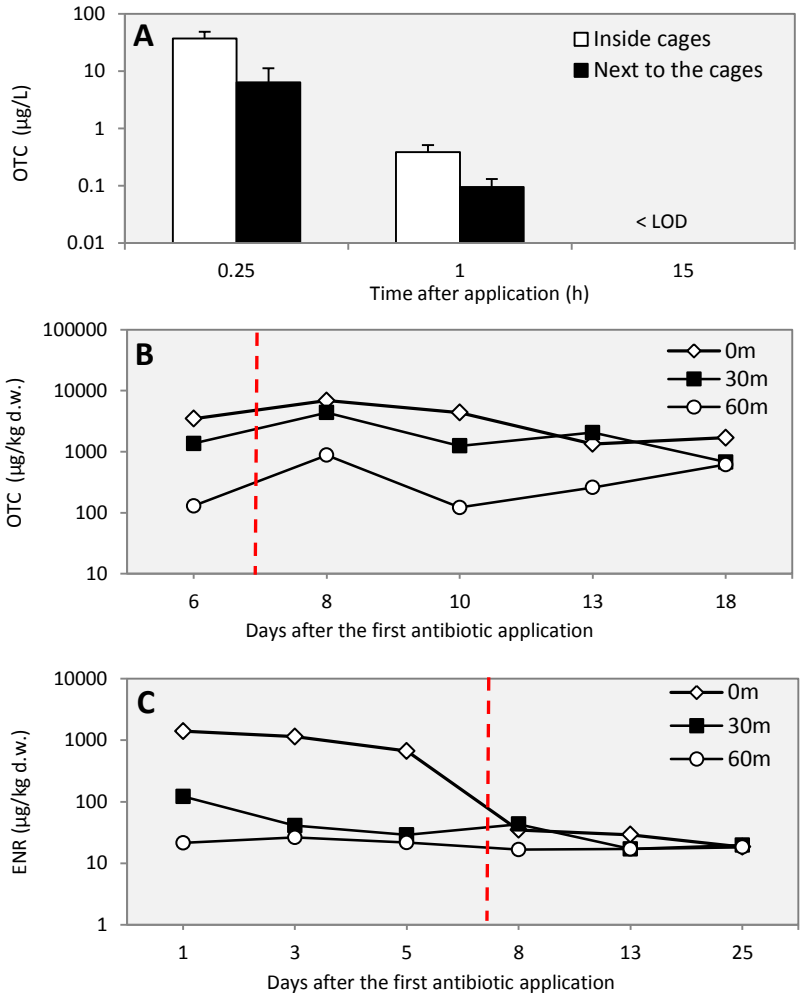


Figure 4. Measured oxytetracycline concentrations in water samples of FARM 1 (**A**), and measured oxytetracycline (**B**) and enrofloxacin concentrations (**C**) in sediment samples of FARM 2. The dashed lines indicate the end of the antibiotic administration period.

Maximum OTC concentrations in the sediments of FARM 2 (1 day after the end of the treatment) were 6,908 µg/kg d.w. in the sampling points located next to the treated cages, and 4,372 and 880 µg/kg d.w. at 30 and 60 m down-stream the cages (Fig. 4B). OTC concentrations dropped to about 20% of the peak sediment concentrations 11 days after administration, and the estimated first-order half-dissipation time in the sediment ($DT50_{sed}$) was 6.6 d. Measured ENR concentrations in the water samples collected next to the cages ranged between 0.10-0.71 µg/L during the administration period, and could not be detected in the sampling points further down the river, nor in the water samples collected after the administration period. Maximum ENR concentrations in the sediments were 1,398 µg/kg d.w. next to the cages, and 121 and 26 µg/kg d.w. at 30 and 60 m down-stream the monitored cages (Fig. 4C). Enrofloxacin concentrations in sediments next to the tilapia cages decreased sharply after the administration period, but remained stable down-stream from the sampled tilapia cages (Fig. 4C). The estimated $DT50_{sed}$ was 9.3 d (calculated for a period of 18 days after antibiotic administration).

3.3. Toxicity experiments

The results of the toxicity experiments are shown in Table 3. Average antibiotic dissipation in the test media 48-h after the start of the experiment ranged between 18 and 33% for OTC, and between 6 and 21 % for ENR. No significant differences were observed in the recorded effects between the controls that were rinsed with the Na-EDTA solution and the non-rinsed ones in the OTC experiments (data not shown). Calculated EC10 and EC50 values were in the order of mg/L, indicating that the studied antibiotics do not show a high acute toxicity to the tested invertebrate species. *L. hoffmeisteri* was found to be the most sensitive species to OTC (EC50 = 217 mg /L), whereas *M. lanchesteri* and *P. acuta* showed the highest sensitivity to ENR (LC50 = 202 and EC50 = 281 mg/L, respectively). *M. tuberculata* showed the lowest sensitivity to both antibiotics, probably due to their ability of covering their soft body with the operculum during chemical stress events. All the tested species showed higher tolerance to the tested antibiotics than *Daphnia magna*, the standard test species used as invertebrate surrogate in the risk assessment of antibiotics (Fig. 5).

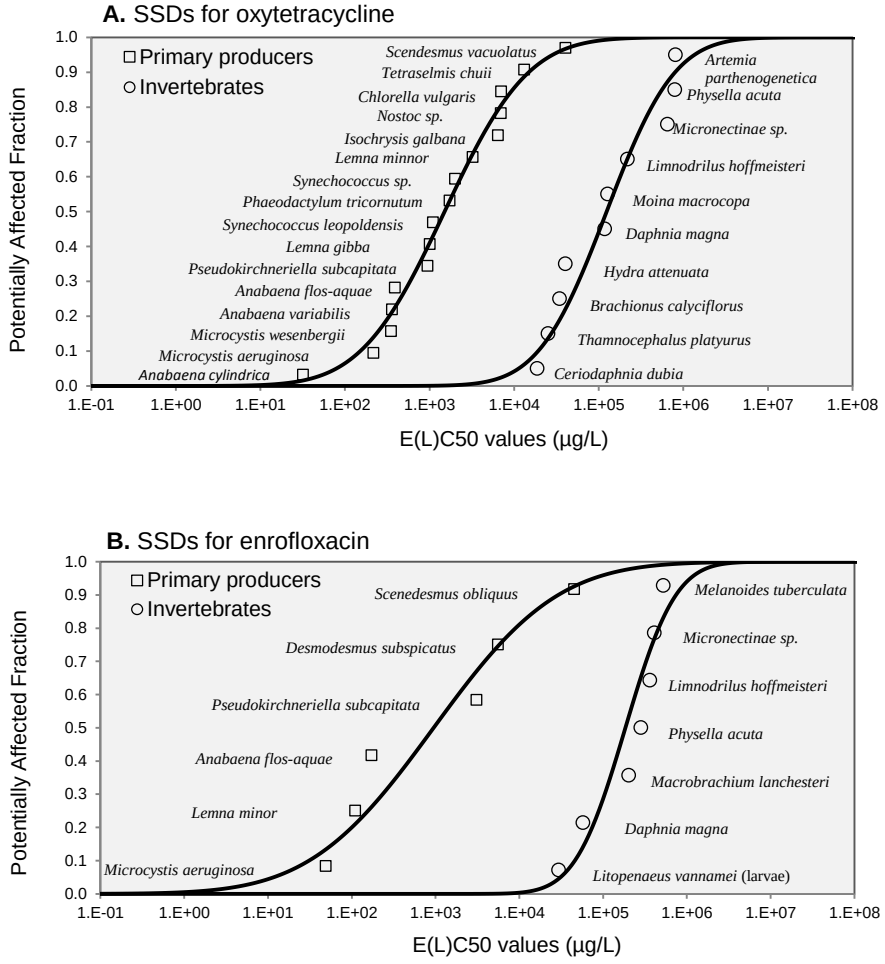


Figure 5. Species Sensitivity Distributions (SSDs) for oxytetracycline (**A**) and enrofloxacin (**B**) based on acute toxicity data. The parameters of the SSDs are shown in Table 4.

3.4. Ecological risk assessment

The risk assessment based on RQs indicated potential risks (RQ>1) for the growth of primary producer communities exposed to OTC in the water layer and river sediments, and for primary producers exposed to water concentrations of ENR. The calculated risks of both antibiotics following the RQ approach for invertebrates were found to be insignificant (Table 4).

The SSD curves for OTC and ENR are shown in Fig. 5. The Anderson-Darling goodness of fit test was accepted for the four SSD curves. The SSDs indicate that, for OTC, blue-green algae (*Anabaena* sp. and *Microcystis* sp.) are more sensitive than the other taxa represented within the primary producer community, and microinvertebrates invertebrates are generally less tolerant than macroinvertebrates. The calculated median HC5 values for OTC were 75 µg/L for primary producers and 10,405 µg/L for invertebrates. For ENR, blue-green algae (*Microcystis aeruginosa*) and macrophytes (represented by *Lemna minor*) showed a higher sensitivity compared to green algae species, and the larval stage of *Litopennaeus vanamei* was found to be more sensitive than *D. magna* and the rest of macroinvertebrate species tested in this study. The median HC5 values for ENR were 8.8 and 28,190 µg/L for primary producers and invertebrates, respectively. The results of the Kolomogorov-Smirnov test indicated that primary producers are significantly more sensitive than invertebrates for both OTC (ks=0.69, n_1=16, n_2=10, p=0.003) and ENR (ks=0.86, n_1=6, n_2=7, p=0.009). The ecological risk assessment based on the maximum antibiotic exposure concentrations and the SSDs indicated minimal risks for both investigated antibiotics on the studied aquatic communities (PAFs < 5%) (Table 4).

4. Discussion

A total of ten different antibiotics were reported to be used for disease treatment and prevention by farmers in the Tha Chin and in the Mun rivers. The frequency of antibiotic use in the interviewed farms is markedly higher than the frequencies reported in pond-based tilapia farms of Thailand (Rico et al., 2013a), probably due to the higher incidence of disease outbreaks associated to the fluctuating environmental and water quality conditions, and the high vulnerability to parasites and bacterial infestations (Belton et al., 2009b). In addition, the limited disease diagnostic capacity denoted by the interviewed farmers combined with the promotional sales by the feed distribution companies are main factors contributing to the regular antibiotic use. The batched production system and the antibiotic use patterns in each batch (i.e., characterized by long treatment durations and several applications per culture cycle) is resulting in a continuous discharge of antibiotics in the rivers in which tilapias are cultured.

The maximum OTC and ENR water concentrations measured in the Tha Chin River (49 and 1.59 µg/L, respectively) are one or two orders of magnitude higher than the concentrations monitored in other Asian aquatic environments receiving aquaculture pollution (Managaki et al., 2007; Zou et al., 2011; Yang et al., 2010; Takasu et al., 2011; Shimizu et al., 2013). The measured concentrations were as high as antibiotic concentrations monitored in pig farm effluents, hospital waste waters, sewage waters, and urban drainage systems of Asian countries (Managaki et al., 2007; Takasu et al., 2011; Shimizu et al., 2013), confirming that tilapia cage farming is an important source of antibiotic pollution. The results of this study revealed that peak antibiotic concentrations in the river water occur during antibiotic administration in the tilapia cages. This is in accordance with the study conducted by Duis et al. (1995), which demonstrated that the leaching of several antibiotics from oil-coated fish-feed pellets might range between 18-67% within 15 min after administration, and in the case of the studied tilapia cage farms this amount can be higher since only water was reported to be used as a coating agent. The water peak concentrations of OTC (49-26 µg/L) measured inside the tilapia cages of FARM 1 are in the order of magnitude of the peak concentrations measured during OTC administration in fish hatcheries (Dietze et al., 2005) and raceways (Bebak-Williams et al., 2002). However, the antibiotic dissipation from the surroundings of the administration area was found to be much quicker than in these semi-closed systems, as antibiotics are rapidly transported down-stream and diluted with the river water. This shows that water exposure is characterized by short-lasting antibiotic pulses. The higher detection rate of antibiotics in the water samples collected in the beginning of the wet season might be explained by several factors.

Table 3. Results of the oxytetracyline and enrofloxacin toxicity tests: measured exposure concentrations at the start of the experiment, dissipation of the antibiotics in the test media, effect concentrations, and slope of the fitted dose-response curve.

	Oxytetracycline					Enrofloxacin				
Species	**Exposure concentrations (mg/L)**	**Dissipation (%) after 48h (mean±SD)**	**EC10-48h in mg/L (CI 95%)**	**EC50-48h in mg/L (CI 95%)**	**Slope (L/mg)**	**Exposure concentrations (mg/L)**	**Dissipation (%) after 48h (Mean±SD)**	**EC10-48h in mg/L (CI 95%)**	**EC50-48h in mg/L (CI 95%)**	**Slope (L/mg)**
Limnodrilus hoffmeisteri	80, 136, 265, 467, 829	18±17	95.5 (NC)	217 (NC)	3.59	32, 64, 135, 293, 503	10±6	285 (238-342)	360 (317-409)	12.7
Macrobrachium lanchesteri	13, 32, 89, 167, 300	29±16	> 300 [a,b]	> 300 [a,b]	NC	147, 270, 366, 514, 634	11±8	85.2 (27.0-269) [b]	202 (128-319) [b]	3.41
Melanoides tuberculata	112, 277, 461, 868, 1052	33±13	> 958 [a]	> 958 [a]	NC	79, 113, 207, 370, 658	11±5	98 (56-173)	520 (337-801)	1.77
Micronectinae sp.	138, 330, 519, 677, 937	29±9	148 (59-369) [b]	647 (440-952) [b]	1.99	46, 91, 185, 352, 603	6±5	335 (292-384) [b]	408 (367-455) [b]	14.9
Physella acuta	142, 315, 631, 1072, 1409	29±7	528 (376-742)	791 (676-925)	7.31	58, 137, 424, 524, 749	21±7	40.1 (13.3-121)	281 (141-558)	1.51

NC: not calculated. [a] Not clear dose-response. [b] The evaluated endpoint was mortality.

Table 4. Maximum measured exposure concentrations (MECs), predicted acute no effect concentrations (PNECs), calculated parameters of the species sensitivity distributions, results of the risk assessment expressed as risk quotients (RQs) and median potentially affected fractions (PAFs) based on acute toxicity data for water and sediments.

	Exposure concentrations		Effect assessment				Risk assessment			
	MEC water in µg/L	**MEC pore water in µg/L [a]**	**PNEC in µg/L**	**HC5 in µg/L (CI 95%)**	**HC50 in µg/L (CI 95%)**	**SD of log SSD data**	**RQ water**	**RQ sediment**	**PAF (%) water (CI 95%)**	**PAF (%) sediment (CI 95%)**
Oxytetracycline										
Primary producers	49	5.08	2.18 [b]	74.5 (16.4-199)	1504 (686-3295)	0.78	22.4	2.33	3.05 (0.46-11.7)	0.09 (0.002-1.70)
Invertebrates			117 [c]	10405 (1790-28173)	123857 (53270-287982)	0.63	0.41	0.04	NC [f]	NC [f]
Enrofloxacin										
Primary producers	1.59	0.29	0.49 [d]	8.80 (0.05-91.5)	948 (105-8559)	1.16	3.24	0.60	1.25 (0.01-17.1)	0.23 (<0.001-9.81)
Invertebrates			57.5 [e]	28190 (4613-68012)	184683 (83219-409855)	0.47	0.03	0.005	NC [f]	NC [f]

[a] Pore water concentrations corresponding to a measured sediment concentrations of 6908 and 2339 µg/kg d.w. for oxytetracycline and enrofloxacin, respectively. [b] Based on the geometric mean of the EC50 values reported by Ando et al. (2007) and Holten Lützhøft et al. (1999) for *Microcystis aeruginosa*, and an assessment factor of 100. [c] Based on the geometric mean of the EC50 values reported by Park and Choi (2008), Isidori et al. (2005), and Kołodziejska et al. (2013) for *Daphnia magna,* and an assessment factor of 1000. [d] Based on the EC50 value reported by Robinson et al. (2005) for *Microcystis aeruginosa*, and an assessment factor of 100. [e] Based on the geometric mean of the EC50 values reported by Park and Choi (2008) and Kim et al. (2010) for *Daphnia magna,* and an assessment factor of 1000. [f] Not calculated. The exposure concentration falls below the lower limit used in the PAF calculations, indicating an insignificant risk.

First, the higher measured concentration of particulate organic matter during the rainy season (Table S1), together with the potential higher concentration of humic substances, are positively correlated to an increased water turbidity and, according to the study by Ge et al. (2010), are likely to contribute to a higher stability of antibiotics in the water column. Second, the increase in the water flow-rates during the wet season (Thaipichitburapa et al., 2010) are likely to contribute to a higher antibiotic desorption and re-suspension from river sediments. Finally, farmers reported higher antibiotic application rates during this season due to the observed higher fish stress, potentially caused by more frequent runoff events and the varying environmental and water quality conditions during the monsoon period.

Our analysis of the sediment samples demonstrated that antibiotics are retained in the river sediments and may reach concentrations up to several mg/kg. The OTC and ENR sediment concentrations measured in the Tha Chin and Mun rivers are within the order of magnitude of the antibiotic concentrations reported in sediments collected under marine fish cages after chemotherapy (Coyne et al., 1994; Capone et al., 1996), and are comparable or even higher than antibiotic concentrations measured in sediments of other large Asian rivers impacted by urban or agricultural pollution (Yang et al., 2010; Zhou et al., 2011; Xue et al., 2013). The results of the monitoring performed in FARM 2 show that antibiotics tend to accumulate underneath or next to the tilapia cages, suggesting that not only water-sediment sorption, but also faeces and uneaten feed sedimentation, might play a substantial role in the antibiotic's transport to the sediment. Antibiotics were found to be widely distributed along the sampled river areas and to persist for several weeks in the river sediments (Fig. 4).

Factors such as seasonal water flow variation, suspended solid concentrations in river water, river morphology, and farm density are expected to influence the distribution and occurrence of antibiotics in river sediments. For instance, higher water flow and the resulting dilution during the wet season might explain the lower OTC concentrations in the sediment, as tetracyclines have higher water solubility and lower sediment-sorption potential than fluoroquinolone antibiotics (Thiele-Bruhn, 2003). Overall, the higher magnitude of the antibiotic concentrations measured in the sediment samples from the Mun River compared to the Tha Chin River could be related to the lower relative distance of the sampling points to the tilapia cages and the higher concentration of cage farms in the sampled locations. Low OTC concentrations were detected in the sediment samples collected in the reference area sampled in the Mun River. This could be explained by the possibility that tetracycline antibiotics naturally occur in the environment, as a product of *Streptomyces* spp. bacteria (Chopra and Roberts, 2001), or because of other possible sources of contamination (e.g. transport from up-stream farms).

The RQ-based ecological risk assessment performed for OTC and ENR indicated potential risks for primary producer communities (particularly for blue-green algae), mainly in the water layer. However, the calculated risks were found to be negligible when the more realistic SSD approach was used. Knapp et al. (2005) did not find significant effects on water quality in freshwater microcosms exposed to a single-pulse of 25 µg/L of enrofloxacin. Wilson et al. (2004) found dose-response effects on phytoplankton communities exposed to a mixture of four tetracyclines for 35 days, with significant effects starting at an approximate concentration of 120 µg/L. Regarding the results of the experiments performed by Knapp et al. (2005) and Wilson et al. (2004), together with the risk assessment calculations performed in this study, the monitored antibiotic concentrations in the waters of the Tha Chin and Mun rivers are not expected to exert direct toxic effects on non-target aquatic communities. However, more experiments should be performed with long-term exposure regimes and with benthic and sediment dwelling organisms, since antibiotic exposure in sediments was demonstrated to last for long periods. These experiments should also include microorganisms and their related functional endpoints. Moreover, the nature of the antibiotic use practices and the results of the environmental monitoring show that aquatic ecosystems are exposed to antibiotic mixtures. Therefore, refined ecological risk assessments

must consider the potential consequences of combined antibiotic toxicity (and other stressors) in aquatic organisms.

Despite the low ecological risks calculated in this study, the regular antibiotic administration and the prevalence of antibiotics in river sediments is expected to exert selective pressure on the sediment bacterial communities, leading to development of (multiple) antibiotic resistance (Cabello et al., 2006). The development of multi-drug resistance in the environment and the horizontal gene transfer to human pathogens has become a serious problem in the recent years, particularly in Asia (Suzuki and Hoa, 2012). It is estimated that antimicrobial resistance is responsible for more than 30,000 deaths annually in Thailand (Pumart et al., 2012). Despite the majority of the acquired drug resistance in humans is thought to be a consequence of irrational antibiotic consumption (Sumpradit et al., 2012), the contribution of the regular antibiotic use in tilapia cage farming to the health of the tilapia farmers and riverine populations, relying on water resources and fish from antibiotic polluted rivers, remains largely uncertain. Furthermore, the development of resistant bacteria, which are usually more virulent than non-resistant strains, might pose a threat for the wild fish populations in the polluted rivers and for consumers capturing wild fish stocks exposed to antibiotic residues. Therefore, further attention must be paid by local authorities to monitor antibiotic pollution and resistant bacteria in these rivers, and to assess their impacts to the ecosystem and human's health.

5. Conclusions

This study demonstrated that the intensive use of antibiotics in tilapia cage production in Thailand constitutes an important source of contamination for freshwater ecosystems. Ecosystems are regularly exposed to antibiotic pulses and to antibiotic mixtures. Regarding the risk assessment calculations performed in this study, which is based on a single-compound approach, the measured antibiotic concentrations of OTC and ENR are not expected to result in short-term toxicity to primary producers and invertebrates. However, further research is needed in order to assess the effects of long-term exposure and mixtures of antibiotics to tropical aquatic ecosystems. Such experiments should include sediments and benthic organisms, and ecosystem functional endpoints associated to microorganism communities such as nutrient cycling and organic matter decomposition. On the other hand, the repeated antibiotic use is expected to result in the development of antibiotic-resistant bacteria, making antibiotics actually ineffective against the target pathogens. In this way, farmers are forced to increase doses and continuously change antibiotic ingredients, compromising the (environmental) sustainability of this aquaculture practice. Further attention must be paid by local farmers and Thai authorities to control the use of antibiotics in tilapia cage-farm production, and to monitor the development and potential consequences of multi-drug resistance for human's and environmental health.

Acknowledgements

The work of Rhaul Oliveira was supported by a PhD grant (SFRH/BD/62605/2009) and the work of Inês Domingues by a Post-Doc grant (SFRH/BPD/31752/2006) provided by the Portuguese Science and Technology Foundation (FCT). The authors would like to thank Frederieke Knopperts for collaborating during the interviews and field sampling, Steven Crum and Laura Buijse for their support on the antibiotic analysis, and to the farmers for their kind responses and for allowing us to take samples from their farms.

Supporting Information

Description of the study areas

The Tha Chin River (Central Thailand) is a distributary of the Chao Phraya River that drains part of the Central Plains of Thailand and mouths into the Gulf of Thailand. The investigated area of the Tha Chin River was located in the Suphan Buri Province, at a river transect of approximately 15 km situated in the Sam Chuk and Doem Bang Nang Buat districts. River width at the study area was 25-45 m, water depth 5.3-11.5 m, and water flow 0.7-1.2 m/s (Thaipichitburapa et al., 2010).

The Mun River (North-East Thailand) drains the waters of the Khorat Plateau and is one of the main tributaries of the Mekong River. The area investigated was located in the Ubon Ratchathani Province, at a river transect of approximately 40 km length and 200-250 m width, and encompassing a high density of tilapia cage farms.

Sample collection and preservation methods

A long-tail boat was used for transportation in order to collect the water samples in the Tha Chin river. Water samples were collected approximately 1 m below the surface with a water grab sampler and stored in pre-cleaned plastic bottles. The collected samples were placed in a cooler during transportation to the laboratory, and were stored in the fridge (4°C) for a maximum period of 24h until further extraction.

All sediment samples were collected on board a boat or from the cage's steel frames. In all cases, sediment samples (400-800 g) were collected from approximately the top 5 cm layer using an Ekman grab sampler (surface area: 225 cm2) and were immediately introduced into zip lock plastic bags. The plastic bags were placed in a cooler during transportation and stored in a freezer (-20°C) until further analysis.

During the sampling campaigns, basic water quality parameters were monitored in situ (temperature, dissolved oxygen, pH, and conductivity). Furthermore, extra water and sediment samples were taken in order to characterise the water (hardness, suspended solids, chlorophyll-a) and sediment (water, organic matter, texture) of the sampled areas. All water and sediment quality measurements were done according to the protocols described in APHA (1996) and are shown in Table S1.

Table S1. Water and sediment quality parameters measured in the samples collected during the monitoring campaigns in the Tha Chin and in the Mun rivers (Thailand). Data are expressed as mean ± SD.

	Tha Chin River (dry season)	Tha Chin River (wet season)	Mun River (dry season)	Mun River (reference site)
Water				
Temperature (°C)	31 ± 1.2	30 ± 0.1	26 ± 1.9	26 ± 1.4
Dissolved oxygen (mg/L)	2.7 ± 1.2	2.8 ± 0.3	5.2 ± 1.6	6.7 ± 0.2
pH	6.8 ± 0.1	6.9 ± 0.5	6.9 ± 0.3	6.9 ± 0.2
Conductivity (µS/cm)	203 ± 5.1	194 ± 2.2	203 ± 33	201 ± 18
Hardness (mg CaCO3/L)	69 ± 1.8	59 ± 3.5	NM	NM
Total suspended solids (mg/L)	16 ± 2.8	70 ± 16	27 ± 11	35 ± 1.1
Chlorophyll-a (µg/L)	1.9 ± 0.7	0.9 ± 0.2	NM	NM
Sediment				
Water content (%)	33 ± 8.3	41 ± 8.6	33 ± 13	36 ± 20
Organic matter (%)	7.5 ± 1.8	5.2 ± 1.4	4.3 ± 1.9	1.6 ± 2.2
Clay (%)	26 ± 12	62 ± 22	13 ± 6.5	3.4 ± 5.5
Sand (%)	34 ± 15	30 ± 23	37 ± 26	80 ± 32

NM: not measured.

Antibiotic extraction methods and analysis

Antibiotic extraction from the toxicity test media and the water samples collected in the Tha Chin River were done with solid phase extraction (SPE) OASIS HLB cartridges (3cc, Waters, USA), preconditioned with 5 mL of MeOH and 5 mL of distilled water. For the analysis of the experimental media, 10 mL were passed through the SPE cartridges by means of a plastic syringe. For the analysis of the monitoring samples, a water volume of approximately 1 L was passed through the SPE cartridges by using a vacuum pump (average speed: 10 mL/min). Before extraction, the water samples collected during the field monitoring were filtered through a 1.2 µm pore size glass microfiber filter (Whatman GF/C), and the pH was adjusted to 5.5-6.0 by drop adding acetic acid/acetate buffer solution, in order to improve the antibiotic retention in the SPE cartridges. After SPE the cartridges were stored in a freezer at -20°C until elution. Elution was performed with 5 mL (5x1 mL) of 0.01 M NaOH-acetonitrile (75:25, v/v) solution. The extracts were transferred into 2 mL plastic vials and stored at -20°C until analysis.

Antibiotic extraction from the sediment samples was based on the method described by Yang et al. (2010). Approximately 20 g of sediment were introduced into plastic centrifuge tubes. Subsequently, 100 µL of internal standard (tetracycline and enrofloxacin d-5) were spiked into the centrifuge tubes and kept in dark for 30 min. Twenty mL of a mix of 0.2 M citric acid buffer (pH4) and acetonitrile (50:50; v/v) were added to the centrifuge tubes and shaken manually. Subsequently, the tubes were sonicated for 15 min and shaken for 30 min (150 rpm). After shaking, the samples were centrifuged at 25 °C for 10 min at 12,000 rpm. The obtained supernatant was placed in 30 mL glass tubes and evaporated at 55°C under a gentle stream of air to remove the organic solvent. The aqueous sediment extract was diluted in 200 mL of Milli-Q water with 0.2 g of Na2-EDTA. The aqueous sediment extracts were then cleaned-up and enriched by means of SPE Oasis HLB cartridges (500 mg, 6 cc, Waters, USA), preconditioned with 10 mL of MeOH and 10 mL of Milli-Q water. After all the extract was loaded completely, the glass bottle containing the aqueous extract and the cartridge were rinsed with 10 mL of Milli-Q water, and the cartridges were vacuum dried for 1 h. Next, the antibiotics were eluted from the cartridges with 10 mL of MeOH (5x2mL) and the eluents were evaporated to dryness at 55°C. The pellet was dissolved by adding 400 µL of acetonitrile and 1600 µL of Milli-Q water, with vortex and sonication (10 min). Finally, the extracts were transferred into 2 mL plastic vials and stored at -20 °C until analysis.

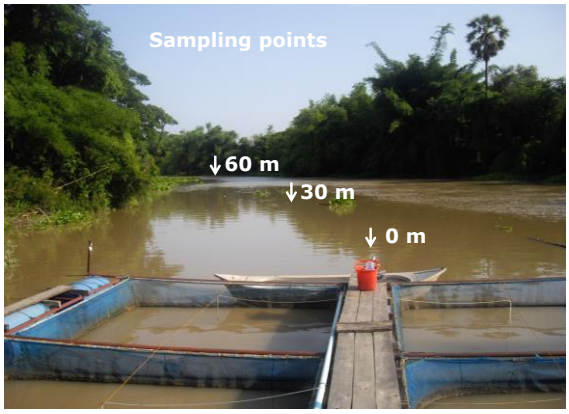
The antibiotic analysis of the water and sediment extracts was made by LC-MS/MS. Prior to injection, the extracts obtained from the toxicity test experimental media were diluted. An extract volume of 50 µL was injected into the chromatographic system by means of an Agilent 1200 series (Agilent Technologies, Germany). Separation was done on a Zorbax XDB-C18 column (4.6 x 150 mm, 5 µm), set to a temperature of 25 °C, using binary gradient elution. Mobile phase A consisted of formic acid solution in Milli-Q water (0.01% v/v) and mobile phase B consisted of formic acid solution in acetonitrile (0.01% v/v). The mobile phase lasted for 20 min and was performed at a constant flow rate of 0.7 mL/min according to the following elution gradients: held at 10% B for 10 min, then moved to 80% and held for 4 min, and then moved to 20% and held for 6 min. In order to prevent contamination of the MS, part of the eluent was sent to the waste (0-5 min and after min 16 for water samples, and 0-7 min and 12-20 min for sediment samples). The mass spectrometry analysis was conducted with a triple quadrupole mass spectrometer (Agilent Technologies 6410) equipped with an ESI+. The nebulizer pressure was set to 35 psi and the flow rate of drying gas (nitrogen) was 8 L/min. The capillary voltage was 3000 V and the dry temperature 350 °C. Sample acquisition was performed in the multiple reaction monitoring (MRM) mode. Calculated recoveries of the method used for the toxicity test media (with an antibiotic concentration of 500 mg/L) were 97±11% for OTC, and 72±6% for ENR (Mean±SD, *n*=3). The calculated recoveries for OTC and ENR following the method used for the environmental samples were 48±2% and 112±13% (*n*=4), respectively, for an antibiotic concentration of 10 µg/L. The calculated recoveries in the sediment samples collected from the reference site were 78±3%

and 99±6% for OTC and ENR, respectively. When the method recovery was below 70% the final measured antibiotic concentrations were re-calculated. The limit of detection (LOD) and limit of quantification (LOQ) in the water samples were 0.02 and 0.05 µg/L for OTC, and 0.01 and 0.02 µg/L for ENR. The LOD and LOQ in the sediment samples were 0.8 and 2.6 µg/kg d.w. for OTC, and 0.3 and 1.1 µg/kg d.w. for ENR, respectively.

Table S2. Toxicity data used to build the species sensitivity distributions. Only EC50 or LC50 values calculated for a exposure duration of 1-7 days for primary producers and 1-4 days for invertebrates were selected. The geometric mean was calculated when more than one toxicity value was available for a species.

Species name	Species group	Evaluated endpoint	Toxicity value (mg/L)	Reference
Oxytetracycline				
Anabaena cylindrica	Blue-green algae	EC50-6d (growth inhibition)	0.032	Ando et al. (2007)
Anabaena flos-aquae	Blue-green algae	EC50-6d (growth inhibition)	0.39	Ando et al. (2007)
Anabaena variabilis	Blue-green algae	EC50-6d (growth inhibition)	0.36	Ando et al. (2007)
Microcystis aeruginosa	Blue-green algae	EC50-6d (growth inhibition)	0.23	Ando et al. (2007)
Microcystis aeruginosa	Blue-green algae	EC50-72h (growth inhibition)	0.207	Holten Lützhøft et al. (1999)
Microcystis wesenbergii	Blue-green algae	EC50-6d (growth inhibition)	0.35	Ando et al. (2007)
Nostoc sp.	Blue-green algae	EC50-6d (growth inhibition)	7	Ando et al. (2007)
Synechococcus leopoldensis	Blue-green algae	EC50-6d (growth inhibition)	1.1	Ando et al. (2007)
Synechococcus sp.	Blue-green algae	EC50-6d (growth inhibition)	2	Ando et al. (2007)
Chlorella vulgaris	Green algae	EC50-72h (growth inhibition)	7.05	Eguchi et al. (2004)
Scendesmus vacuolatus	Green algae	EC50-24h (growth inhibition)	40.4	Kołodziejska et al. (2013)
Isochrysis galbana	Green algae	EC50-96h (growth inhibition)	6.43	De Orte et al. (2013)
Pseudokirchneriella subcapitata	Green algae	EC50-72h (growth inhibition)	0.342	Eguchi et al. (2004)
Pseudokirchneriella subcapitata	Green algae	EC50-72h (growth inhibition)	4.5	Holten Lützhøft et al. (1999)
Pseudokirchneriella subcapitata	Green algae	EC50-72h (growth inhibition)	0.17	Isidori et al. (2005)
Pseudokirchneriella subcapitata	Green algae	EC50-96h (growth inhibition)	3.1	Zounková et al. (2011)
Pseudokirchneriella subcapitata	Green algae	EC50-48h (growth inhibition)	1.26 [a]	Christensen et al. (2006)
Tetraselmis chuii	Green algae	EC50-72h (growth inhibition)	13.16	Ferreira et al. (2007)
Phaeodactylum tricornutum	Diatom	EC50-96h (growth inhibition)	1.73	De Orte et al. (2013)
Lemna gibba	Macrophyte	EC50-7d (wet weight)	1.01	Brain et al. (2004)
Lemna minor	Macrophyte	EC50-7d (growth inhibition)	4.92	Pro et al. (2003)
Lemna minor	Macrophyte	EC50-7d (growth inhibition)	2.1	Zounková et al. (2011)
Lemna minor	Macrophyte	EC50-7d (growth inhibition)	3.26	Kołodziejska et al. (2013)
Daphnia magna	Crustacean	EC50-48h (immobilization)	621.2	Park and Choi (2008)
Daphnia magna	Crustacean	EC50-48h (immobilization)	114	Kołodziejska et al. (2013)
Daphnia magna	Crustacean	EC50-48h (immobilization)	22.64	Isidori et al. (2005)
Moina macrocopa	Crustacean	EC50-48h (immobilization)	126.7	Park and Choi (2008)
Artemia parthenogenetica	Crustacean	LC50-48h (mortality)	806	Ferreira et al. (2007)
Ceriodaphnia dubia	Crustacean	EC50-48h (immobilization)	18.65	Isidori et al. (2005)
Thamnocephalus platyurus	Crustacean	EC50-48h (immobilization)	25	Isidori et al. (2005)
Brachionus calyciflorus	Rotifer	EC50-48h (immobilization)	34.21	Isidori et al. (2005)
Hydra attenuata	Cnidarian	EC50-96h (morphology)	40.13	Quinn et al. (2008)
Limnodrilus hoffmeisteri	Worm	EC50-48h (immobilization)	217	This study
Micronectinae sp.	Insect	EC50-48h (mortality)	647	This study
Physella acuta	Mollusc	EC50-48h (immobilization)	791	This study
Enrofloxacin				
Microcystis aeruginosa	Blue-green algae	EC50-5d (growth inhibition)	0.049	Robinson et al. (2005)
Anabaena flos-aquae	Blue-green algae	EC50-72h (growth inhibition)	0.173	Ebert et al. (2011)
Desmodesmus subspicatus	Green algae	EC50-72h (growth inhibition)	5.57	Ebert et al. (2011)
Pseudokirchneriella subcapitata	Green algae	EC50-72h (growth inhibition)	3.1	Robinson et al. (2005)
Scenedesmus obliquus	Green algae	EC50-72h (growth inhibition)	45.1	Qin et al. (2011)
Lemna minor	Macrophyte	EC50-7d (growth inhibition)	0.114	Robinson et al. (2005)
Lemna minor	Macrophyte	EC50-7d (growth inhibition)	0.107	Ebert et al. (2011)
Daphnia magna	Crustacean	EC50-48h (immobilization)	58.3 a	Kim et al. (2010)
Daphnia magna	Crustacean	EC50-48h (immobilization)	56.7	Park and Choi (2008)
Litopenaeus vannamei (larvae)	Crustacean	EC50-48h (mortality and morbidity)	29.4	Williams et al. (1992)
Macrobrachium lanchesteri	Crustacean	EC50-48h (mortality)	202	This study
Limnodrilus hoffmeisteri	Worm	EC50-48h (immobilization)	360	This study
Melanoides tuberculata	Mollusc	EC50-48h (immobilization)	520	This study
Micronectinae sp.	Insect	EC50-48h (mortality)	408	This study
Physella acuta	Mollusc	EC50-48h (immobilization)	281	This study

[a] Geometric mean of the values reported in this study.



Date	Day after start OTC treatment	OTC treatment	OTC sediment samples	Day after start ENR treatment	ENR treatment	ENR water and sediment samples
8-May	1	X		-		
9- May	2	X		-		
10- May	3	X		-		
11- May	4	X		-		
12- May	5	X		-		
13- May	6	X	X	1	X	X
14- May	7	X		2	X	
15- May	8		X	3	X	X
16- May	9			4	X	
17- May	10		X	5	X	X
18- May	11			6	X	
19- May	12			7	X	
20- May	13		X	8		X
21- May	14			9		
22- May	15			10		
23- May	16			11		
24- May	17			12		
25- May	18		X	13		X
26- May	19			14		
27- May	20			15		
28- May	21			16		
29- May	22			17		
30- May	23			18		
31- May	24			19		
1-June	25			20		
2- June	26			21		
3- June	27			22		
4- June	28			23		
5- June	29			24		
6- June	30			25		X

Figure S1. Antibiotic dose preparation and administration in FARM 2. Synopsis of the sampling points and sampling days in relation to the oxytetraycline (OTC) and enrofloxacin (ENR) treatments applied in FARM 2.

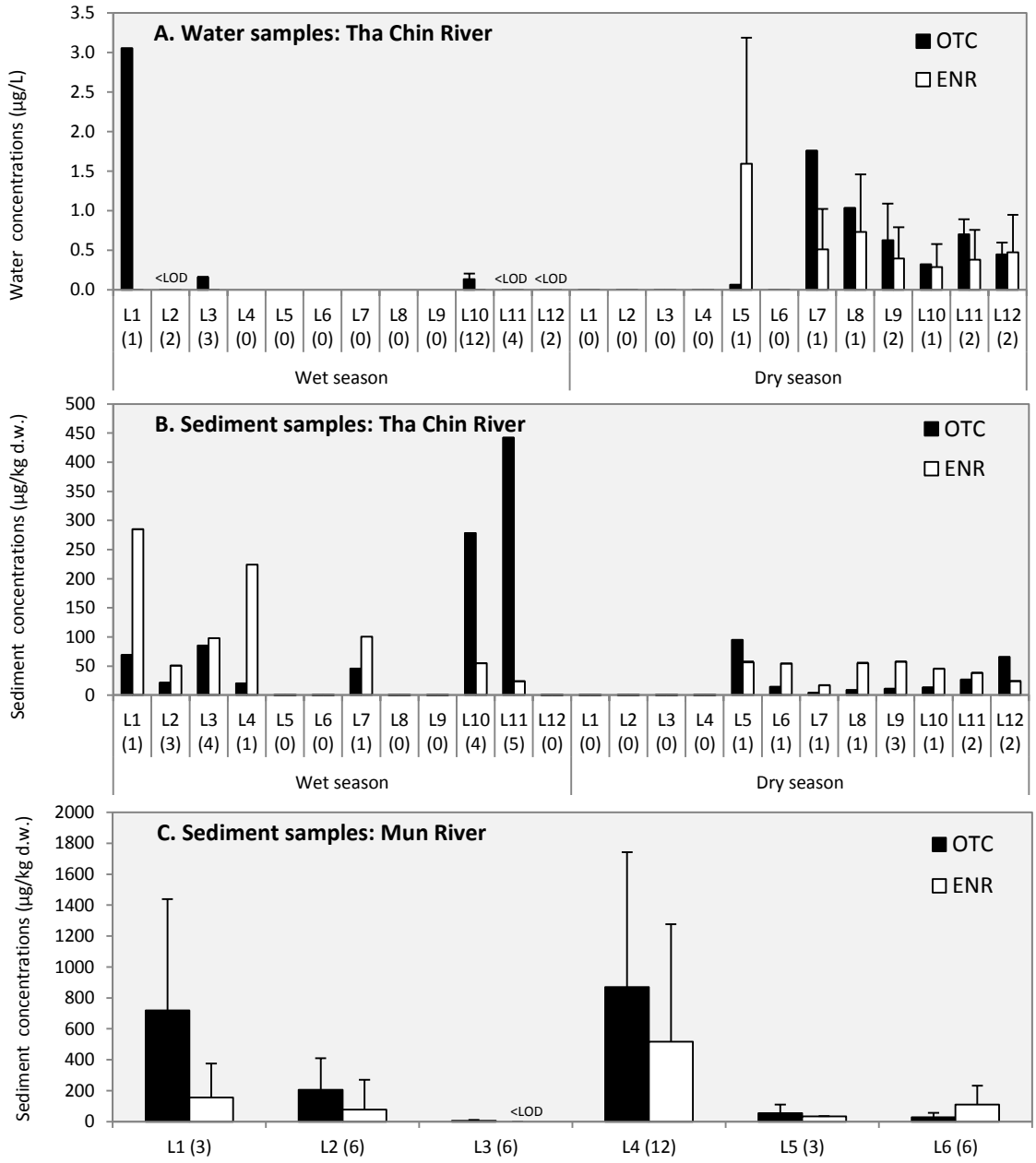


Figure S2. Measured water and sediment oxytetracycline (OTC) and enrofloxacin (ENR) concentrations in the different sampling locations of the Tha Chin and Mun rivers (mean ± SD). The number between brackets indicates the number of analysed samples in each sampling location.

Ecological risk assessment of the antibiotic enrofloxacin applied to *Pangasius* catfish farms in the Mekong delta, Vietnam

Margot Andrieu, Andreu Rico, Tran Minh Phu, Do Thi Thanh Huong, Nguyen Thanh Phuong, Paul J. van den Brink

Abstract

In this study we assessed the ecological risks posed by the use of the antibiotic enrofloxacin (ENR), and its main metabolite ciprofloxacin (CIP), in a *Pangasius* catfish farm in the Mekong Delta, Vietnam. Water and sediment samples were collected in a stream receiving effluents from a *Pangasius* catfish farm that had applied ENR. The toxicity of ENR and CIP was assessed on three tropical aquatic species: the green-algae *Chlorella* sp. (72h - growth inhibition test), the micro-invertebrate *Moina macrocopa* (48h - immobilization test), and the Nile tilapia (*Oreochromis niloticus*). The toxic effects on *O. niloticus* were evaluated by measuring the cholinesterase (ChE) and catalase (CAT) activities in the fish brain and muscles, respectively, and by considering feed exposure and water exposure separately. Ecological risks were assessed by comparing maximum exposure concentrations with predicted no effect concentrations for cyanobacteria, green algae, invertebrates and fish derived with available toxicity data. The results of this study showed that maximum antibiotic concentrations in *Pangasius* catfish farm effluents were 0.68 µg/L for ENR and 0.25 µg/L for CIP (dissolved water concentrations). Antibiotics accumulated in sediments down-stream the effluent discharge point at concentrations up to 2590 µg/kg d.w. and 592 µg/kg d.w. for ENR and CIP, respectively. The calculated EC50 values for ENR and CIP were 111,000 and 23,000 µg/L for *Chlorella* sp., and 69,000 and 71,000 µg/L for *M. macrocopa*, respectively. Significant effects on the ChE and CAT enzymatic activities of *O. niloticus* were observed at 5 g/kg feed and 400-50,000 µg/L, for both antibiotics. The results of the ecological risk assessment performed in this study indicated only minor risks for cyanobacteria communities, suggesting that residual concentrations of ENR and CIP after medication are not likely to result in severe toxic effects on exposed aquatic ecosystems. However, more studies should be performed by considering other antibiotic treatments used in *Pangasius* catfish production and the potential ecotoxicological effects of relevant antibiotic mixtures on sediment communities.

Submitted

1. Introduction

Vietnam is the third largest aquaculture producing country in the world (FAO, 2012a). The Vietnamese aquaculture production sector has sharply increased mainly due to the development and expansion of *Pangasius* catfish (*Pangasius hypophthalmus*) production in the Mekong River Delta (Phan et al., 2009), which has increased ten-fold in the last decade, reaching an annual production of 1.14 million tonnes in 2012 (FAO, 2012b). The success of this aquaculture species is explained by its ability to be reproduced in captivity, its fast growth, its capability to tolerate low water oxygen concentrations, the development of improved culture and feeding techniques, and the expansion of the export markets (Phuong and Oanh, 2010). *Pangasius* catfish are produced in earthen ponds with relatively high water depth (3.5 to 4.5 m), which are stocked with exceptionally high fish densities (about 50 fish/m^2) and rely on heavy water exchange regimes with the surrounding aquatic ecosystems - once or twice a day, from 30 to 100% replenishment (Phan et al., 2009). The intensification of *Pangasius* production practices has been accompanied by the outbreak of several bacterial and parasitic infestations, which has led to the introduction of a wide array of veterinary medicines for their prevention and treatment (Phan et al., 2009; Rico et al., 2013a). For example, the results of a recent study on the use of veterinary medicines in the *Pangasius* catfish grow-out farms of the Mekong Delta identified 17 different antibiotic ingredients (Rico et al., 2013a). Eventually, residual concentrations of veterinary medicines used in *Pangasius* production can be released into the environment by untreated effluent and sludge discharges, and have raised concerns about their potential toxic effects on aquatic ecosystems surrounding the aquaculture farms (Rico et al., 2012a; Thuy et al., 2011). Several studies have warned about the spread and the high levels of antibiotic pollution in the aquatic ecosystems of the Mekong Delta due to the input of aquaculture, livestock and urban effluents (Managaki et al., 2007; Shimizu et al., 2013). However, the contribution of the *Pangasius* catfish farms to this pollution problem and their potential ecological consequences have not been investigated so far. A recent modelling study identified the *Pangasius* farming areas of the Mekong Delta as potential hot-spots for environmental pollution due to their widespread use of veterinary medicines and their intensive discharge of untreated effluents, and stressed the need to monitor and further assess the ecological effects of selected aquaculture antibiotics on streams impacted by *Pangasius* catfish effluents (Rico and Van den Brink, 2014).

The aim of this study was to get a better understanding on the environmental fate and ecological risks posed by the use of antibiotics in *Pangasius* catfish production. For this, we evaluated the discharge of the fluoroquinolone antibiotic enrofloxacin (ENR) and its main metabolite ciprofloxacin (CIP) on freshwater ecosystems surrounding *Pangasius* catfish farms and assessed their potential toxicological effects on tropical freshwater ecosystems. ENR was chosen as model compound because of its widespread use in *Pangasius* production as well as in other important aquaculture species produced in Asia (Rico et al., 2013a). It is mainly applied to treat bacillary necrosis and the red spot disease affecting *Pangasius*, typically caused by the bacteria *Edwardsiella ictaluri* and *Aeromonas* spp., respectively (Crumlish et al., 2002; Dung et al., 2008). In this study, the concentrations of ENR and CIP were monitored in environmental samples collected in a tropical stream receiving effluents from a *Pangasius* farm during and after ENR treatment. Moreover, the toxicological effects of these antibiotics were assessed on tropical aquatic organisms representing three different trophic levels: the algae *Chlorella* sp., the invertebrate *Moina macrocopa*, and the fish *Oreochromis niloticus*. Finally, the ecological risks posed by the use of ENR in *Pangasius* catfish farms were assessed by comparing the concentrations measured in the field with the predicted no effect concentrations derived with toxicity data for aquatic organisms.

2. Material and methods

2.1. Antibiotic exposure assessment

2.1.1. Sample collection

This study was conducted in February of 2012 in a *Pangasius* catfish pond of 0.26 ha, with an average water depth of 3.3 m. At the moment of the antibiotic treatment, the pond contained approximately 10 tons of fish, with an individual size distribution of 103±9.1 g (mean±SD). ENR was administered daily mixed with feed at a dose of approximately 10 mg/kg of fish body weight for a period of 5 days, according to the dosages typically reported by *Pangasius* farmers (Rico et al., 2013a). The medicated diet was daily prepared by diluting the antibiotic in water. This mixture was then sprayed over the commercial pelleted feed (28% protein content) and further mixed manually. In the studied pond, about 22% of the pond's water was daily replaced by tidal flushing. The draining water was discharged by two pipes into a natural stream of approximately 3.7 m width, 0.7 m depth, and with an average water flow of 0.15 m/s. Water and sediment samples were collected in the two water discharge points of the pond (DP1 and 2) after the first application (day 0), after the third application (day 3) and on day 1, 3, 7, 14 and 21 after the last antibiotic application. In addition, extra water and sediment samples were collected one day after the last application in 5 sampling points (separated by 25 m) located in a longitudinal transect of the stream receiving the farm effluents (S1 to 5) (Fig. 1). Water samples were taken at 10 cm depth in previously rinsed plastic bottles. Depth integrated (5 cm) sediment samples were taken with a core sampler and immediately introduced into zip lock plastic bags. All samples were kept in a cooler during transportation to the laboratory and stored in the refrigerators until further analysis (water samples: 4°C; sediment samples: -20°C).

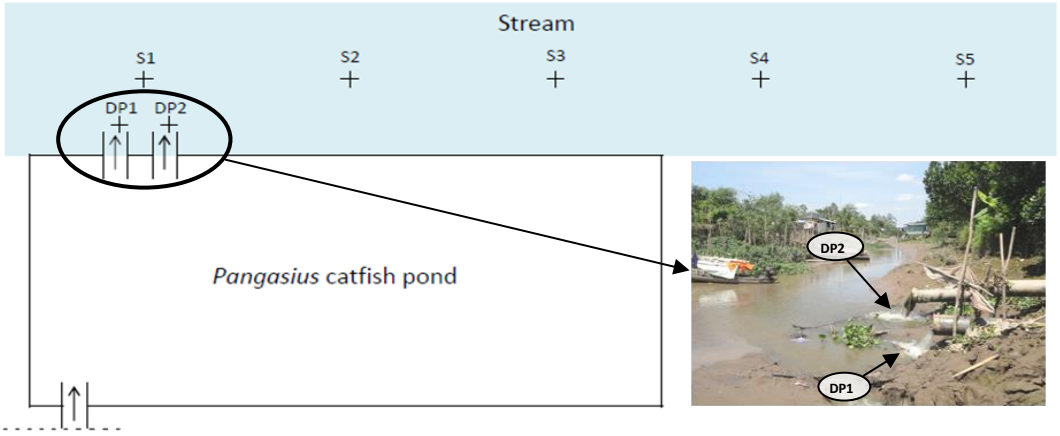


Figure 1. Scheme of the *Pangasius* catfish pond and the water stream receiving the pond effluent, with the selected water and sediment sampling points (crosses). The effluent discharge points are indicated as DP1 and 2, and the sampling points located in the water stream as S1 to 5.

2.1.2. Determination of antibiotic concentrations

The day after the sample collection, a water volume of approximately 400 mL was filtered (0.45 µm Whatman GF/C) and passed through Oasis HLB solid phase extraction (SPE) cartridges (3 cc, Waters, USA), preconditioned with 5 mL of MeOH and 5 mL of distilled water. Elution was performed by passing 5 mL of 0.01 M NaOH-acetonitrile (75:25, v/v) solution through the SPE cartridges. The extracts were transferred into 2 mL vials and stored at -20°C until further analysis. The dissolved antibiotic concentrations (C_{diss}) in the water samples were determined by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) according to the method described in Rico et al. (Submitted). The analytical method recoveries were 112±13% and 91±9.6% for ENR and CIP, respectively (mean±SD; n=4), and the limit of detection (LOD) and

quantification (LOQ) were 0.01 and 0.02 µg/L for ENR, and 0.01 and 0.04 µg/L for CIP, respectively.

The total antibiotic concentration in the water samples (C_{total}) was calculated according to Equation 1 (Adriaanse, 1996):

$$C_{total} = C_{diss}\,(1 + ss \cdot m_{om,ss} \cdot K_{oc} \cdot 0.58) \qquad \textit{Eq. 1}$$

with,
C_{total} = calculated total antibiotic concentration in water (µg/L)
C_{diss} = measured dissolved concentration (µg/L)
ss = mass concentration of suspended solids in the water sample (kg/L)
$m_{om,ss}$ = mass fraction of organic matter in suspended solids (-)
K_{oc} = sorption coefficient on organic carbon (L/kg)

The mass concentration of suspended solids (ss) and the percentage of organic matter in suspended solids ($m_{om,ss}$) were determined according to the methods described in APHA (1996), and were 149±105 mg/L and 30±7.8 %, respectively (mean±SD). The antibiotic elution and analysis from the sediment samples was performed according to the method described by Rico et al. (Submitted). The recoveries of the analytical method at a sediment concentration of 50 µg/kg d.w. were 99±6% and 84±15% for ENR and CIP, respectively (mean±SD; *n*=3), and the LOD and LOQ were 0.3 and 1.1 µg/kg d.w. for ENR, and 0.7 and 2.3 µg/kg d.w. for CIP. In order to assess the antibiotic exposure for benthic aquatic organisms, the pore water concentration ($C_{pore\ water}$) equivalent to the measured sediment concentrations ($C_{sediment\ dw}$) were determined by using Equation 2.

$$C_{pore\ water} = \frac{C_{sediment\ dw}}{K_{oc} \cdot f_{oc}} \qquad \textit{Eq. 2}$$

with,
$C_{pore\ water}$ = calculated pore water concentration (µg/L)
$C_{sediment\ dw}$ = measured sediment concentration (µg/kg d.w.)
K_{oc} = organic carbon normalized sorption coefficient (L/kg)
f_{oc} = fraction of organic carbon in sediment

The fraction of organic carbon in the sediment was determined according to APHA (1996) and was 2.7±0.4% (mean±SD). The K_{oc} values for ENR and CIP used in Equation 1 and 2 were based on the study by Gagliano and McNamara, (1996) (289,568 L/kg for ENR and 48,341 L/kg for CIP), and were selected from experimental values calculated for soils with similar texture characteristics to the sediments we sampled (sand: 30±6.7%; silt: 49±14%; clay: 22±11%; *n*=6; mean±SD).

2.2. Toxicity tests

2.2.1. Test compounds

Formulated products of ENR (i.e., 20% active ingredient; liquid) and CIP (i.e., 50% active ingredient; powder) were purchased from a chemical outlet specialized in veterinary medicinal products for aquaculture production in Can Tho city (Vietnam).

2.2.2. Toxicity tests with *Chlorella* sp.

Toxicity tests with ENR and CIP on *Chlorella* sp. were conducted according to the OECD 201 standard procedure (OECD, 2006). The experiment was performed in triplicate (*n*=3) with cultures of exponentially growing *Chlorella* sp. (0.6 x 10^6 cells/mL) exposed for 72h in 250 mL Erlenmeyer flasks to a series of six antibiotic concentrations (0; 21,000; 42,000; 75,000; 150,000; 300,000; 600,000 µg/L for ENR, and 0; 3,150; 6,300; 12,500; 25,000; 50,000; 100,000 µg/L for CIP). The

culture media (100 mL) consisted of sterilized tap water, 200 µL of concentrated growth medium, prepared according to Walne (1970), and 3 drops of vitamins. Algae were grown in a temperature controlled room (temperature: 26±1°C) with constant illumination intensity (provided by neon light). The flasks were shaken manually 3 times per day. The temperature and pH of the culture media were monitored 1h, 36h, and 72h after the start of the experiment using a portable pH meter (SevenGo, Mettler Toledo). One mL of the culture media (one per treatment level) was taken for antibiotic analysis right after spiking and at the end of the experimental period. Algae were sampled 1h, 36h and 72h after the start of the experiment from each test unit and were fixated with formaldehyde (4%; v/v). The number of algae cells per mL was counted using a counting chamber (Bürker CE-Marienfeld Germany; Tiefe depth: 0.1 mm, largest squares size: 1 mm^2, smallest squares size: 2.5 μm^2) and a powerful microscope (OLYMPUS CX 21; magnification: x400). Finally, the average specific growth rate and the percentage of growth inhibition were calculated as a function of time for each treatment level (including the controls) according to the methods described in the OECD guideline 201 (OECD, 2006).

2.2.3. Toxicity tests with *Moina macrocopa*

M. macrocopa were obtained from an outdoor shallow pond in Can Tho University (Vietnam) and kept in a tank with water originating from the pond in which they were collected. Toxicity tests with ENR and CIP on *M. macrocopa* were conducted according to the OECD 202 standard procedure (OECD, 2004). This protocol was adapted to the higher temperatures and light regime occurring in tropical ecosystems. *M. macrocopa* neonates (< 24h old) were exposed to a series of six antibiotic concentrations (0; 40,000; 80,000; 160,000; 320,000; 640,000; 1280,000 µg/L for ENR, and 0; 20,000; 40,000; 80,000; 160,000; 320,000; 640,000 µg/L for CIP) for 48h with five replicates per treatment level. The tests were performed in 5 mL glass cuvettes containing 3 mL of exposure media, prepared by diluting the stock solutions of antibiotics on filtrated pond water, and with three *M. macrocopa* individuals per test unit. All cuvettes were placed on a platter in the laboratory (temperature: 31±1°C; 12-12h light/dark regime). Four extra cuvettes per treatment level were installed in order to collect samples for antibiotic analysis and water quality measurements. One mL water samples were taken for antibiotic analysis (one per treatment level) after spiking and at 24h and 48h after the start of the experiment. On the same sampling times, the temperature and pH of the exposure media were monitored using a portable pH meter (SevenGo, Mettler Toledo) and the number of immobilized *M. macrocopa* individuals in each cuvette was recorded. The *M. macrocopa* individuals were considered to be immobile when no movement was observed in the cuvette after 15 seconds of gentle agitation.

2.2.4. Toxicity tests with *Oreochromis niloticus*

Juvenile *Oreochromis niloticus* of 11±2 g (Mean±SD) were obtained from a hatchery and reared in a big composite tank (2 m^3) with aeration for one month in order to acclimatize to the food (commercial pelleted feed: 30% protein, 5% fat) and the water used in the experiments. The effects of ENR and CIP on the muscle catalase (CAT) and brain cholinesterase (ChE) activities of *O. niloticus* were studied by performing separate toxicity tests with two different modes of antibiotic administration: antibiotic added mixed with feed (oral administration) and added directly to water (bath treatment). Three days before the start of the experiments, 24 fishes were introduced in 60 L composite tanks with oxygenated tap water. All experiments lasted for 14 days, with 5 days of antibiotic treatment period and 9 days of post-exposure period (i.e., recovery period). During the exposure and recovery periods, 20 L of test media were daily replaced in order to prevent excessive water quality deterioration. Food was administered two times per day (at 8 am and 5 pm) at a feeding rate of 3% and 5% of fish body weight during the treatment and recovery period, respectively. On day 1, 3, 5, 7, 10 and 14 after the start of the experiment, temperature and pH were recorded in each tank using a waterproof digital pH meter (HI 98127, Hanna Instruments, USA) and water samples (1 mL) were taken for antibiotic analysis (one sample

per treatment level). On day 3, 5 and 14, after the start of the experiment, three fish per tank were killed by a cold shock in ice and directly dissected to extract their brains and part of their muscles. The samples were then stored at -80°C until further analysis.

The oral administration experiments were performed in triplicate (n = 3) for a series of antibiotic dosages of 0, 1, 2.5, 5, 10 g ENR/kg of feed (i.e., representing 0, 30, 75, 150, 300 mg ENR/kg of fish body weight), and 0, 0.50, 1.12, 2.5, 5.0 g CIP/kg of feed (i.e., representing 0, 15, 33.6, 75, 150 mg CIP/kg of fish body weight). Prior to the start of these experiments, small bags of antibiotic-treated and antibiotic-free feed were prepared. For this, stock solutions of ENR and CIP were sprayed over the food pellets and further mixed with a spoon in order to reach the desired antibiotic concentrations. Subsequently, all the feed was placed into a freezer (-20 °C) to avoid antibiotic degradation until the moment of administration. The bath treatment experiments were also performed in triplicate (n=3). During the exposure period, stock solutions of ENR and CIP were daily prepared and added to the fish culture media in order to generate the following water concentrations: 0; 100; 800; 10,000; 100,000 µg ENR/L, and 0; 50; 400; 5,000; 50,000 µg CIP/L.

The *O. niloticus* brain and muscle samples collected from each experimental unit were mixed and homogenized with phosphate buffer (KH_2PO_4/K_2HPO_4 50 mM; pH = 7.5) using a sample homogenizer (T18 basic Ultra Turrax, USA). Subsequently, they were centrifuged at 10,000 rpm for 10 min at 4°C (Hettich Mikro 22R, Germany). The supernatants were collected in Eppendorf tubes and stored at -80 °C. The total protein content was measured in each sample according to the method described by Lowry et al. (1951) in order to normalize the CAT and ChE levels.

CAT activity in fish muscles was assessed according to the method described by Baudhuin et al. (1964). Briefly, 25 µL of homogenate muscle sample was mixed in an Eppendorf cuvette with 25 µL of 2% Triton X-100 and 1,250 µL of substrate solution composed by bovine serum albumin, hydrogen peroxide and imidazol. After incubation at 0°C for 6 min, 750 µL of titanous sulfate were added. Between the next 5 to 10 min, absorbance was read at 420 nm using a Varian – Cary 50 UV/ Visible spectrophotometer. One unit of CAT activity was defined as the amount of enzyme causing the destruction of 90% of the substrate in 1 min in a volume of 50 mL. Catalase activity was expressed in Baudhuin Units (BU)/min/mg protein.

ChE activity in fish brains was determined according to Ellman et al. (1961). Briefly, 50 µL of homogenate brain sample was mixed with 50 µL of acetylthiocholine (ATC) and 900 µL of beta-dystrobreving (DTNB) in an Eppendorf cuvette. After 10 min of incubation at ambient temperature, absorbance was measured at 412 nm (Varian – Cary 50 UV / Visible spectrophotometer). ChE was expressed as mols of hydrolysed ATC/min/mg protein.

2.2.5. Chemical analysis

Antibiotic concentrations in the exposure media of the toxicity experiments were measured with a Shimadzu SCL-10A high performance liquid chromatography (HPLC) system equipped with a Shimadzu RF-10AxL fluorescence detector (FLD). The FLD excitation wavelength was 280 nm and the emission wavelength 420 nm. The chromatographic separation was performed with isocratic elution on a Gemini C18 110A (150 mm × 3.0 mm, 5 micron) analytical column (Phenomenex, Torrance, CA, USA). A C18 guard cartridge (4 mm × 2 mm, Phenomenex) was used prior to the analytical one. The mobile phase for LC–MS/MS analyses consisted of acetonitrile and phosphate buffer (50 mM, pH 3.5). Prior to the analysis, all samples were diluted taking the theoretical antibiotic concentration to approximately 50 µg/L. Subsequently, samples were placed into a 13 mm syringe and pushed through a 0.2 µm filter and injected into the HPLC (injection volume: 20 µL). The separation of the compounds was performed at ambient temperature and with a constant flow rate of 0.3 mL/min. The recovery of the analytical method was 98 ± 3.5 % for ENR, and 100 ± 2.6 % for CIP (mean±SD; n=3). The efficiency tests were performed using ENR (ENR >

98%) and CIP (CIP > 98%), purchased from Sigma Aldrich (St Louis, MO, USA). LOQs for ENR and CIP were 1 µg/L. The quantification was based on an external standard curve using experimental blank water as the matrix, with ENR and CIP concentrations ranging from 1 to 100 µg/L.

2.2.6. Data analysis

The concentrations resulting in 10% and 50% (EC10 and EC50) inhibition of algae growth (for *Chlorella* sp.) and immobilization (for *M. macrocopa*) at the end of the experimental period and their 95% confidence intervals (CIs) were calculated by probit analysis using linear maximum likelihood regression with the ToxRat Professional Version 2.01 software (Toxrat, 2003). All calculations were performed using the measured antibiotic concentrations.

For the experiments conducted with *O. niloticus*, the differences between the enzymatic activities measured in the controls and those measured for the different antibiotic treatment levels were assessed for each sampling date using a one-way analysis of variance (ANOVA) test followed by a Dunnett's post-hoc test. In addition, the Kolmogorov-Smirnov test and the Levene's test were performed in order to verify the normality and the variance homogeneity assumptions of the tested data. For the datasets for which these two criteria were not met, the ANOVA and Dunnett's test were substituted by the Kruskal-Wallis test followed by a Mann-Whitney U test. These statistical tests were performed using the SPSS statistical package (ver. 19.0, SPSS Company, Chicago, IL, USA). Differences between controls and antibiotic treatments were considered to be statistically significant when $p < 0.05$.

2.3. Ecological risk assessment

The toxicological risks for cyanobacteria, green algae, invertebrates and fish in the aquatic ecosystems surrounding *Pangasius* catfish farms were assessed by following a risk quotient (RQ) approach. RQs were calculated by dividing the highest ENR and CIP measured exposure concentrations by the predicted no effect concentrations (PNECs) for each taxonomic group. The aquatic exposure RQs for algae and cyanobacteria were calculated based on the C_{diss}, as they will be more exposed to the freely dissolved antibiotics in the water. For invertebrates and fish, the aquatic exposure RQs were conservatively calculated based on the C_{total}, given their capacity to filter and/or feed on suspended organic particles. The sediment exposure RQs were calculated based on the $C_{pore\ water}$. The PNECs were derived by using the toxicity values calculated in this study as well as other toxicity values collected from the literature. PNECs were calculated by dividing the lowest acute EC50 value available for each taxonomic group by an assessment factor (AF) of 100 for algae, and 1000 for invertebrates and fish according to the international risk assessment guidance document for veterinary medicines (VICH, 2004).

3. Results

3.1. Aquatic exposure assessment

Measured antibiotic concentrations in the water samples (dissolved fraction) collected in the pond effluents (DP 1 and 2) ranged between 0.05 and 0.68 µg/L for ENR, and between <LOD and 0.25 µg/L for CIP (Table 1). According to our calculations, the total antibiotic concentrations in the pond effluent ranged between 0.24 and 3.15 µg/L for ENR, and between <LOD and 0.39 µg/L for CIP. Measured sediment concentrations for ENR and CIP in the effluent discharge points were found to be insignificant (<LOD).

Table 1. Enrofloxacin (ENR) and ciprofloxacin (CIP) concentrations measured in the water samples collected on both discharge points (DP) of the studied *Pangasius* catfish pond. The total antibiotic concentrations (C_{total}) were calculated using Equation 1.

	ENR						CIP					
	C_{diss} (µg/L)			C_{total} (µg/L)			C_{diss} (µg/L)			C_{total} (µg/L)		
	Mean ± SD	Min	Max	Mean ± SD	Min	Max	Mean ± SD	Min	Max	Mean ± SD	Min	Max
DP 1	0.23 ± 0.12	0.08	0.37	1.22 ± 1.10	0.33	3.15	0.08 ± 0.10	<LOD	0.25	0.12 ± 0.15	<LOD	0.39
DP 2	0.27 ± 0.23	0.05	0.68	1.00 ± 1.03	0.24	3.00	0.06 ± 0.08	<LOD	0.20	0.08 ± 0.12	<LOD	0.31

Measured ENR and CIP concentrations in the water samples collected down-stream the effluent discharge point (S1 to 5) fell below the detection limit. Sediment concentrations showed a gradual increase in the samples monitored down-stream the effluent discharge points, with the maximum concentrations being 2590 µg/kg d.w. for ENR, and 592 µg/kg d.w. for CIP, in S5 (Fig. 2). The corresponding maximum pore water concentrations were 0.33 µg/L for ENR and 0.45 µg/L for CIP.

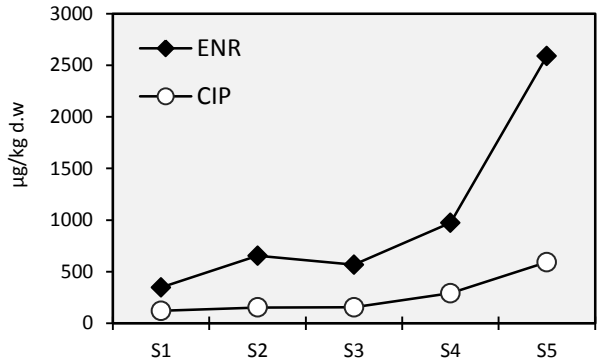


Figure 2. Measured enrofloxacin (ENR) and ciprofloxacin (CIP) concentrations in the sediment samples collected down-stream the effluent discharge point (S1 to 5) one day after the last antibiotic administration.

3.2. Toxicity tests

3.2.1. Toxicity tests with *Chlorella* sp.

In both experiments conducted with *Chlorella* sp., the cultured algae population grew exponentially in the control treatment, and the growth was totally inhibited by the highest antibiotic concentration (Fig. 3). The measured antibiotics concentrations in the test media showed an average dissipation of 22% for ENR and 29% for CIP at the end of the experimental period. The results of the toxicity experiments showed that *Chlorella* sp. is more sensitive to CIP (EC50-72h = 23,400 µg/L, which corresponds to 70.6 x 10^{-6} mol/L) than to ENR (EC50-72h = 111,000 µg/L, which corresponds to 309 x 10^{-6} mol/L) (Table 2).

Table 2. Results of the toxicity tests with enrofloxacin (ENR) and ciprofloxacin (CIP) on *Chlorella* sp. (i.e., growth inhibition after 72h) and on *Moina macrocopa* (i.e., immobilization after 48h), and water quality parameters measured during the toxicity experiments.

Species	Antibiotic	Exposure duration	EC10 (µg/L) (95% CI)	EC50 (µg/L) (95% CI)	Temperature (°C) (Mean±SD)	pH (Mean±SD)
Chlorella sp.	ENR	72h	3,000 (4-14,000)	111,000 (40,000-281,000)	26 ± 1.8	9.1 ± 1.1
	CIP	72h	5,200 (600-10,000)	23,000 (13,000-40,000)	25 ± 1.5	8.7 ± 1.2
Moina macrocopa	ENR	48h	41,000 (30,400-55,000)	69,000 (57,000-84,000)	32 ± 1.0	8.5 ± 0.2
	CIP	48h	50,000 (36,000-69,000)	71,000 (61,000-83,000)	33 ± 2.4	8.4 ± 0.3

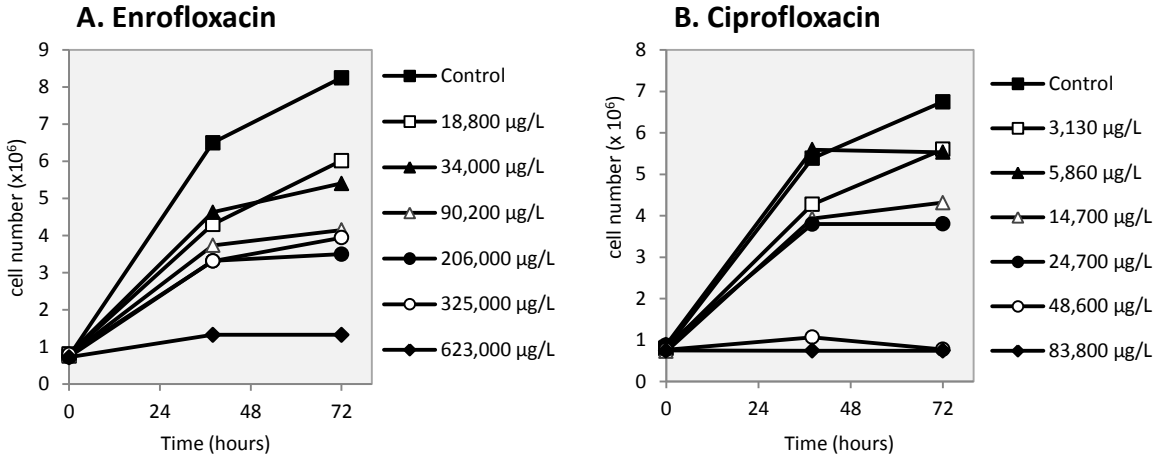


Figure 3. Average *Chlorella sp.* cell numbers measured in the toxicity experiments performed with (**A**) enrofloxacin and (**B**) ciprofloxacin.

3.2.2. Toxicity tests with *Moina macrocopa*

For both antibiotics, a dose-response effect on the immobility of *M. macrocopa* was observed. ENR and CIP had similar toxicity to *M. macrocopa* (ENR: EC50-48h= 69,100 µg/L, which corresponds to $192x10^{-6}$ mol/L, and CIP: EC50-48h= 71,200 µg/L, which corresponds to $214x10^{-6}$ mol/L) (Table 2). In these tests, CIP showed a fast dissipation in the *M. macrocopa* culture media (average 48h dissipation: 42%), whereas ENR was found to be rather stable (average 48h dissipation: 2%).

3.2.3. Toxicity tests with *Oreochromis niloticus*

CAT activity measured in the toxicity experiments performed with ENR and CIP administered orally to *O. niloticus* are displayed in Fig. 4A and 4B, respectively. For all CIP tested concentrations, CAT activity was not significantly different from the controls during the entire experimental period ($p > 0.05$). A clear trend was observed towards a decrease in CAT activity on the last day of the exposure period (day 5) in the tests performed with ENR, showing a significant effect for the lowest and the highest tested concentrations (Fig. 4A). ChE activities in the fish brain samples measured in the in-feed antibiotic administration toxicity experiment with ENR and CIP are displayed in Fig. 4C and 4D, respectively. A significant increase of the ChE activity was observed on day 3 and 5 for the fish that were exposed to 5 and 10 g ENR/kg of feed and 5 g CIP/kg of feed, however, the ChE activity returned to basal enzymatic levels on day 14. In general, the temperature and pH values measured in this experiment remained rather constant during the whole experimental period (ENR: T=28 ±1.7°C, pH=8.1±0.3, and CIP: T=28 ±1.2°C, pH=7.9±0.1).

The antibiotic concentrations in the experimental media of the toxicity experiments performed with ENR and CIP administrated in bath treatment increased gradually during the exposure period, up to 132 ± 20% (mean ± SD) of the nominal concentration for ENR and up to 182 ± 86% (mean ± SD) of the nominal concentration for CIP on day 5. Then, the concentration of antibiotics in the experimental media decreased slowly to below LOD on day 14. CAT and ChE activities measured in the *O. niloticus* samples taken in the toxicity experiments performed with ENR and CIP administered in bath treatment are shown in Fig. 5. Fishes exposed to 10,000 µg/L of ENR showed an impaired swimming behaviour with no or very slow movements during the whole exposure period. These effects on fish mobility were not noticeable 24h after the exposure period. CAT activities measured in fish exposed to ENR were not significantly different from the controls during the entire experimental period (Fig. 5A). However, CAT activity in fish muscle decreased significantly in the individuals exposed to 50,000 µg CIP/L on day 14 (Fig. 5B). Conversely, ChE activity in brain samples appeared to increase with increasing ENR and CIP water

concentrations, showing a clearer dose-response effect in the experiment conducted with CIP (Fig. 5C and 5D). In the experiment conducted with ENR, a significant increase in the brain ChE activity was observed for the concentration of 10,000 and 800 µg of ENR/L on day 3 and 14, respectively (Fig. 5C), and on day 5 for the three highest tested concentrations in the experiment conducted with CIP (Fig. 5D). In general, the measured temperature and pH values remained rather constant during the whole experimental period (ENR: T=27±0.5°C, pH=8.1±0.4 and CIP: T=28±1.0°C, pH=7.8±0.2).

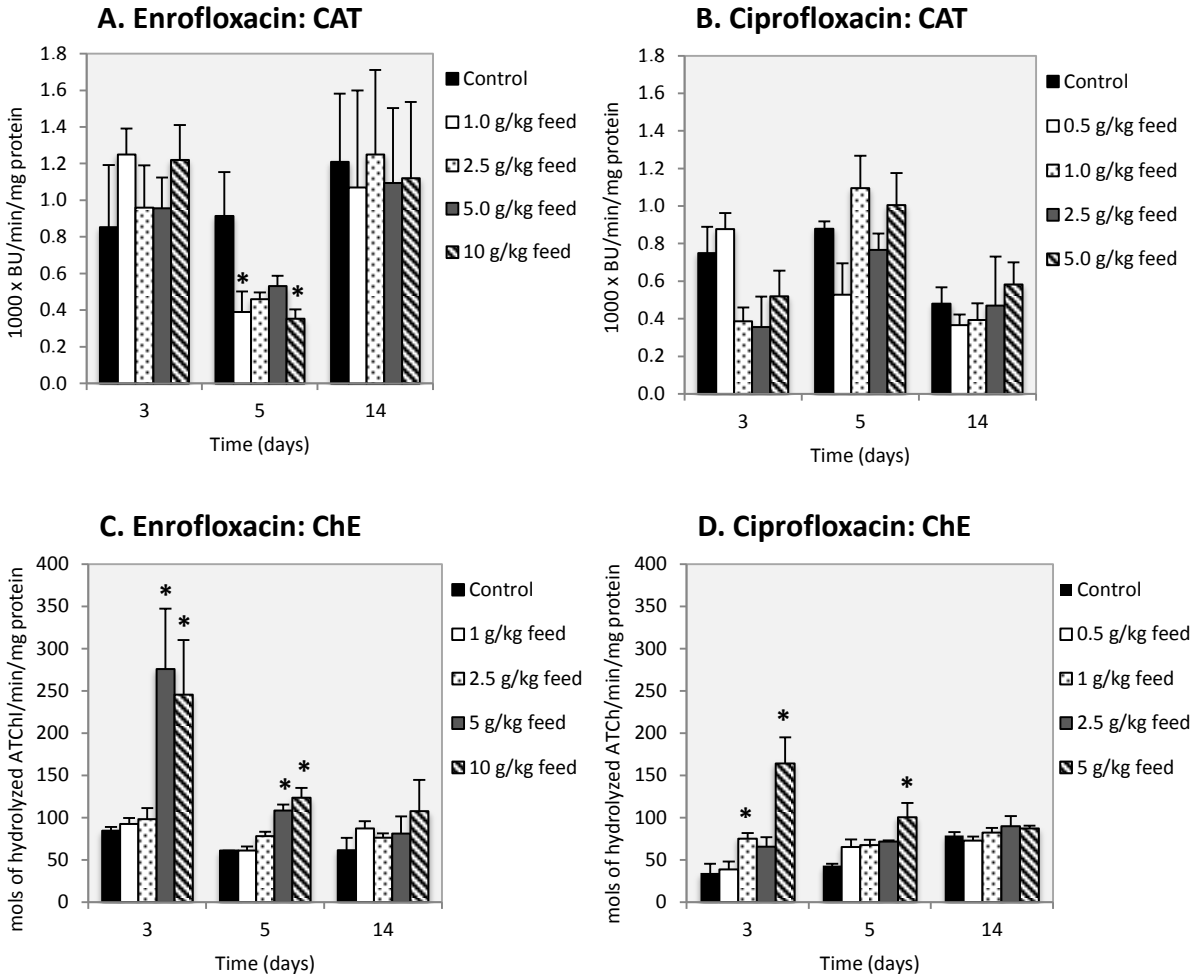


Figure 4. Results of the catalase (CAT) and cholinesterase (ChE) activities (Mean ± SD) measured in the toxicity experiments performed with enrofloxacin (**A,C**) and ciprofloxacin (**B,D**) administered via medicated feeds to *O. niloticus.*

3.3. Ecological risk assessment

The results of the risk assessment showed potential risks for cyanobacteria exposed to antibiotic concentrations in the water layer, with calculated RQs of 1.4 and 5.0 for ENR and CIP, respectively (Table 3). However, the results of the risk assessment performed for green algae, invertebrates and fish, indicated insignificant risks (RQs<1; Table 3).

4. Discussion

4.1. Antibiotic fate and exposure

To our knowledge, this is the first study that monitored antibiotic concentrations in freshwater aquaculture pond effluents during and after antibiotic medication. Based on the measured antibiotic concentrations in the pond effluents and the water exchange rates in the monitored

pond, we estimated that about 18% of the applied ENR mass is discharged into the surrounding aquatic ecosystems (4.4% during the administration period and 13.7% during the 20 successive days), and 5.3% of the applied ENR applied mass is discharged in form of CIP. The estimated amount of ENR discharged into the environment corresponds fairly well with the modelling calculations performed by Rose and Pedersen, (2005), who predicted that about 10-15% of the administered mass of the antibiotic oxytetracycline (OTC) is released from fish hatcheries to the receiving water during treatment and in the first 5 days thereafter. Rico and Van den Brink (2014) estimated that 7.6±3.6% (mean±SD) of the ENR mass applied in *Pangasius* catfish ponds of the Mekong Delta is released unaltered into the environment. However, differences between this study and ours could be related to the fact that the fish density in the our monitored pond (1.1 kg/m^3) notably exceeded the density distribution at the start of the culture cycle used in the modelling calculations performed by Rico and Van den Brink (2014; 0.2 kg/m^3).

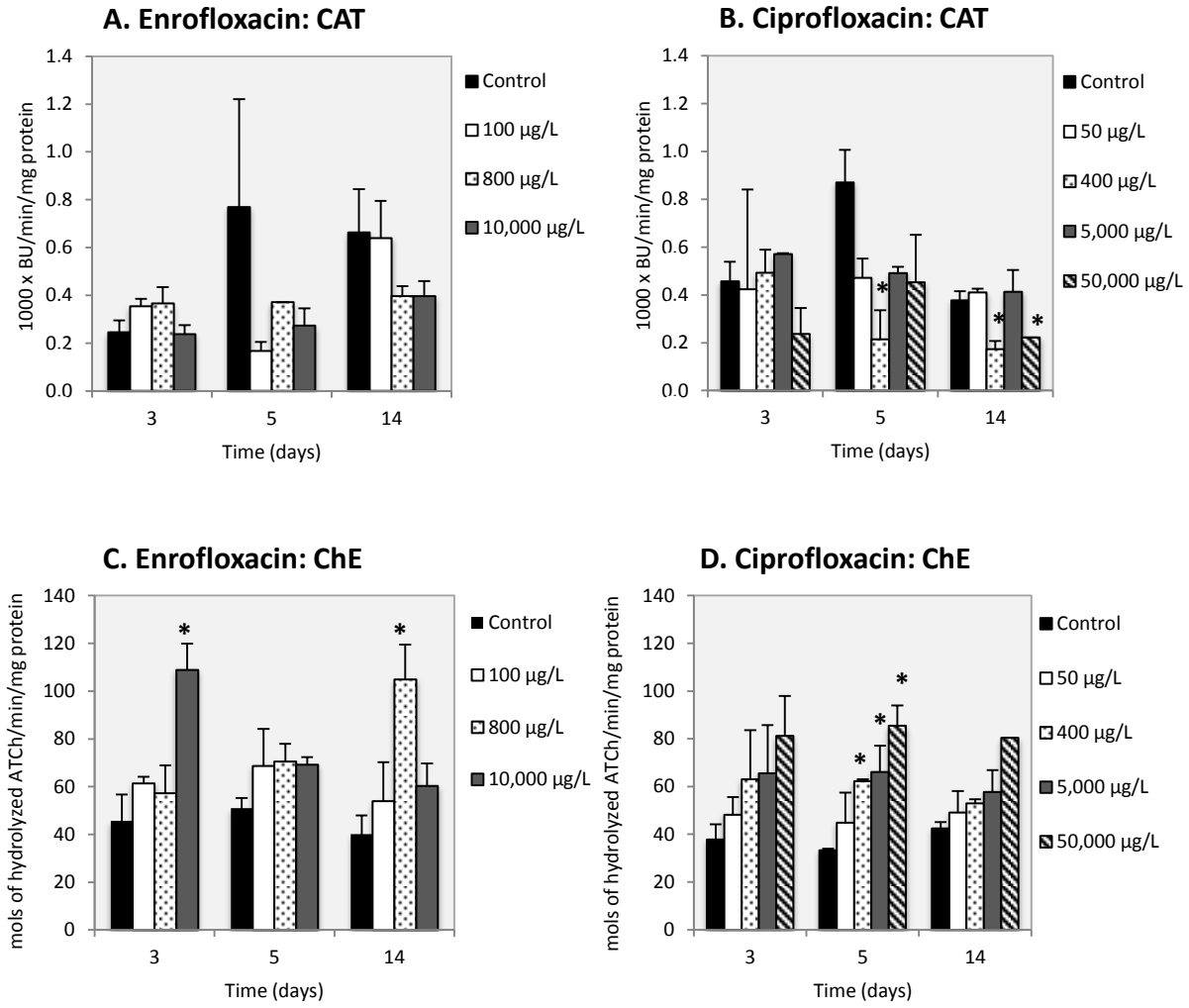


Figure 5. Results of the catalase (CAT) and cholinesterase (ChE) activities (Mean ± SD) measured in the toxicity experiments performed with enrofloxacin (**A,C**) and ciprofloxacin (**B,D**) administered in bath treatments to *O. niloticus.* The results of the highest ENR concentration (100,000 µg/L) are not displayed since all fish died before the third day after the start of the experiment. The standard deviation for the highest CIP concentration could not be calculated since all fish died in 2 out of the 3 replicates after day 5.

Table 3. Calculated risk quotients (RQ) for enrofloxacin (ENR) and ciprofloxacin (CIP) based on the highest measured exposure concentrations in the water (C_{diss} for cyanobacteria and algae, C_{total} for invertebrates and fish) and sediment samples ($C_{pore\ water}$) and on the predicted no effect concentration (PNEC) determined using the lowest available short-term EC50 values.

	Exposure Concentrations						Effect Assessment						Risk Quotients			
	ENR			CIP			ENR			CIP			ENR		CIP	
	Water		Sediment	Water		Sediment										
Taxonomic group	C_{total} (µg/L)	C_{diss} (µg/L)	$C_{pore\ water}$ (µg/L)	C_{total} (µg/L)	C_{diss} (µg/L)	$C_{pore\ water}$ (µg/L)	EC50 (µg/L)	AF	PNEC (µg/L)	EC50 (µg/L)	AF	PNEC (µg/L)	Water	Sediment	Water	Sediment
Cyanobacteria							49.0 [a]	100	0.49	5.00 [b]	100	0.05	1.39	0.67	5.00	9.000
Algae							3,100 [a]	100	31.0	2,970 [b]	100	29.7	0.02	0.01	0.02	0.011
	3.15	0.68	0.33	0.39	0.25	0.45										
Invertebrate							53,300 [c]	1000	53.3	1,200 [c]	1000	1.20	0.06	0.01	0.32	0.375
Fish							79,500 [d]	1000	79.5	>60,000 [e]	1000	>60	0.04	<0.01	<0.01	<0.007

[a] Value for *Microcystis aeruginosa* (Robinson et al., 2005).
[b] Value for *Pseudokirchneriella subcapita* (Halling-Sørensen et al., 2000).
[c] Value for *Daphnia magna* (Kim et al., 2010).
[d] LC50 Value for *Lepomis macrochirus* (Gagliano and Mc Namara, 1996).
[e] Value for *Gambusia holbrooki* (Martins et al., 2012).

Monitored ENR concentrations in the *Pangasius* catfish farm effluents were found to be in the range of the antibiotic concentrations measured in livestock farm effluents, sewage treatment plant effluents and drainage systems of urban areas in the Mekong Delta (Managaki et al., 2007; Shimizu et al., 2013) and elsewhere (Sukul and Spiteller, 2007; Wei et al., 2012). ENR and CIP concentrations in the river sediments were found to be in the range of the antibiotic concentrations measured by Rico et al. (Submitted) in tropical river sediments of Thailand impacted by aquaculture pollution, and are comparable to other monitoring studies performed in other Asian rivers impacted by urban or agricultural pollution (e.g. Xue et al., 2013; Zhou et al., 2011). Our study showed that ENR and residual concentrations of CIP are released for periods up to several weeks after treatment, indicating that surrounding ecosystems are exposed to low-concentration antibiotic pulses for relatively long periods, with worst-case water exposure situations occurring next to the effluent discharge point. In addition, our study demonstrated that antibiotic residues tend to accumulate in the sediments down-stream the effluent discharge point, where the water flow speed is reduced. This suggests that settling of organic matter particles are likely routes of transport and deposition of antibiotics into natural sediments, as proposed by modelling studies (e.g. Rose and Pedersen, 2005). Further research must be dedicated to evaluate the efficiency of waste-water treatment options such as the construction of decantation ponds or wetlands for the removal of antibiotic residues from *Pangasius* effluents, and should also evaluate the contribution of the eventual discharges of sludge from *Pangasius* ponds to the environmental contamination with antibiotics.

4.2. Antibiotic toxicity on aquatic organisms

4.2.1. Antibiotic toxicity for *Chlorella* sp.

The EC50-72h value for CIP calculated from our study results is very similar to the value calculated by Nie et al. (2007) for *Chlorella vulgaris* (EC50-96h = 20,600 µg/L). Marked sensitivity differences between microalgae species to antibiotics have been reported (e.g. Qin et al., 2011; Robinson et al., 2005). The toxicity values for *Chlorella* sp. calculated in this study are one or two orders of magnitude higher than those reported for cyanobacteria, and slightly higher than those reported for other green algae species (*Pseudokirchneriella subcapitata*) used in ecotoxicological evaluations (Halling-Sørensen et al., 2000; Robinson et al., 2005). Therefore, this study supports the use of toxicity data for cyanobacteria to protect the structure of primary producer communities in antibiotic risk assessments. The higher tolerance of *Chlorella* sp. to antibiotics compared to other green algae species might be explained by the fact that their cell walls contain glucosamine polymers such as chitin and chitosan (Kapaun and Reisser, 1995; Eckard, 2010), which could act as an extra permeability barrier, thus limiting antibiotic uptake (Bernard and Latgea, 2001).

4.2.2. Antibiotic toxicity on *Moina macrocopa*

Due to their high abundance and distribution in tropical aquatic ecosystems as well as their high sensitivity to toxicants, *Moina* sp. have been considered as good candidates for representing invertebrate communities and replacing testing with *Daphnia magna* in ecotoxicological assessments performed in tropical and subtropical regions (e.g. Daam et al., 2008; Park and Choi, 2008). The calculated toxicity values for *M. macrocopa* in this study are in the order of the available toxicity values of ENR (EC50-48h = 56,700 µg/L; Park and Choi, 2008) and CIP (EC50-48h = 65,300 µg/L; Martins et al., 2012) for *Daphnia magna*, suggesting no major sensitivity differences between the temperate and tropical cladocerans to the studied antibiotics. The EC50-48h determined for ENR in the present study is more than two times lower than the one reported by Park and Choi (2008) for *M. macrocopa* (EC50-48h > 200,000 µg/L; T=25±1°C). This could be

related to the higher temperature set in our tests (>5°C difference), which could have enhanced chemical uptake (see Kim et al*.,* 2010).

4.2.3. Antibiotic toxicity on *Oreochromis niloticus*

Several studies have demonstrated that CAT and ChE activities are reliable biomarkers of antibiotic exposure in aquatic organisms (Table 4). Similarly to the results of the present study, Oliveira et al. (2013) measured a significant inhibition of CAT in zebra fishes exposed to OTC and amoxicillin via water. Wang et al. (2009) showed that CAT activity increases in the gills when fish are treated with ENR medicated feed. Moreover, this enzymatic increase is higher when fish density increases (Wang et al., 2009). Our study was the first showing a trend towards an increase of ChE activity in brains of fishes exposed to fluoroquinolone antibiotics. This increase might be explained by the fact that fluoroquinolones can act as negative allosteric modulators and block or change the conformation of the orthosteric or allosteric binding sites of Acetylcholine (ACh) receptors, as reported in humans (Gregory, 2007). Therefore, all ACh released in the synaptic cleft would only bind to ChE such that its activity would increase. Moreover, fluoroquinolones could act as cholinergic agonists or modify the gene coding for choline acetyl-transferase, leading to an increase of ACh production (Rawi et al., 2011), which would be further hydrolysed by ChE resulting in a significant increase of the enzymatic activity.

In general, bath treatment application resulted in higher toxic effects than the oral administration method when considering the same applied dose (antibiotic weight per kg of fish). Milan et al. (2006) found that pharmaceuticals caused QT prolongation and cardiac disorders in zebra fishes, in a similar way that pharmaceutical overdoses affect humans. Therefore, according to the symptoms observed in our fishes exposed to the highest ENR and CIP water concentrations (i.e., muscle spasms and impaired movements until death), the (transient) failure of the cardiac system seems to be the most plausible cause of death and an important toxicity mechanism for these antibiotics in fishes.

In conclusion, the results of our biomarker experiments suggest that measured ChE activity in brain samples seems to be an appropriate biomarker for fluoroquinolone antibiotics and should be used in combination with CAT and Gluthation S-Transferase (GST) (Oliveira et al., 2013) in order to describe sub-lethal effects and physiological impairments in fishes treated with antibiotics at high dosages. However, the effective exposure concentrations calculated in this study (10,000 µg/L for ENR and 400 µg/L for CIP), and in other studies (Table 4), are one order of magnitude higher than the antibiotic concentrations measured in aquatic ecosystems polluted with aquaculture effluents. This suggests that the use of biomarkers for monitoring ecotoxicological effects of antibiotic pollution on wild fish populations has severe limitations, and should be restricted to the evaluation of the stress caused by antibiotics at the therapeutic doses used in aquaculture facilities. Furthermore, the symptoms and biomarker responses observed in the studied fishes seem to correspond fairly well with the available literature for humans, suggesting that the receptors and toxicity mechanisms of antibiotics might be somehow similar for different vertebrate species.

Table 4. Overview of the available literature assessing the effects of antibiotics on the Cholinesterase (ChE) and Catalase (CAT) enzymatic activities of aquatic organisms.

Compound	Species name (common name)	Biomarker/Organs	Exposure method	Tested concentrations	Exposure duration (days)	Biomarker response	Reference
Enrofloxacin	*Pangasianodon hypophthalmus* (Pangasius catfish)	CAT/ gills, brain, liver, muscles	Oral exposure	1 g/kg feed	7	Increase of CAT in the gills during the exposure period	Wang et al. (2009)
Enrofloxacin	*Penaeus monodon* (Black tiger shrimp)	CAT/muscles, hepatopancreas, gills	Oral exposure	4 g/kg feed	7	Increase of CAT in the gills in intensive systems	Tu et al. (2008)
Enrofloxacin	*Penaeus monodon* (Black tiger shrimp)	ChE/muscles and gills	Oral exposure	4 g/kg feed	7	No significant effects	Tu et al. (2009a)
Enrofloxacin	*Pangasianodon hypophthalmus* (Pangasius catfish)	ChE/gills, brain, liver, muscles	Oral exposure	1 g/kg feed	7	No significant effects	Wang et al. (2009)
Oxytetracycline	*Danio rerio* (Zebra fish)	CAT/ gills, brain, liver, muscles	Water exposure	0; 1,000; 10,000; 25,000; 50,000; 100,000 µg/L	4	Decrease of CAT in all tested organs, especially in brain	Oliveira et al. (2013)
Amoxicillin	*Danio rerio* (Zebra fish)	CAT/ gills, brain, liver, muscles	Water exposure	0; 1,000; 10,000; 25,000; 50,000; 100,000 µg/L	4	Decrease of CAT in brain and gills for almost all tested concentrations	

4.3. Ecological risk assessment

The results of the risk assessment performed in this study indicate that the environmental release of ENR and CIP residues after ENR medication in *Pangasius* catfish farms is posing minimal risks for algae, invertebrate and non-target fish communities. Only cyanobacteria and other bacterial taxa might be affected by antibiotic pollution. A microcosm study investigating the effects of enrofloxacin on tropical freshwater communities could not identify significant effects of this antibiotic on cyanobacteria species (Rico et al., 2014). The authors of this study, however, argued that the low dominance of cyanobacteria and the higher pH of the monitored waters could have limited the observation of effects. More research should be performed to better understand the toxicity of antibiotics on cyanobacteria communities and to assess potential side-effects on ecosystem structure, especially under tropical conditions. Due to a lack of available data on the toxicity of antibiotics to benthic organisms, the risk assessment for sediment communities was performed based on estimated pore water concentrations and toxicity data for pelagic species. Since our study indicated that antibiotics tend to accumulate in sediments down-stream effluent discharge points, further assessments should be carried out by testing potential toxic effects of contaminated sediments on sediment dwelling organisms. In our study, we focused on one single antibiotic treatment, however *Pangasius* farms are often clustered around water sources and share water drainage systems. Therefore, more research should be dedicated to monitor the ocurrence of several antibiotic residues in aquatic ecosystems surrounding *Pangasius* farms and to assess the ecological effects of antibiotic mixtures from different groups and antimicrobial modes of action. This information is of crucial importance for assessing the risks of antibiotics to tropical freshwater ecosystems and the sustainability of current antibiotic use practices in Vietnamese *Pangasius* production and in other intensive aquaculture species in Asia.

5. Conclusions

The results of our study indicate that the discharge of untreated effluents from *Pangasius* catfish farms should be considered as an important pathway of antibiotic pollution into the aquatic environment. The administration of ENR for treating bacterial diseases in *Pangasius* catfish farms is not likely to result in major risks for non-target aquatic organisms inhabiting water bodies receiving farm effluents. However, further investigations must be dedicated to assess potential consequences for microbial communities and associated ecological functions (Rico et al., 2014), as well as to evaluate the contribution of antibiotic residues to the development of antibiotic resistant bacteria in the environment. After the completion of this study, ENR and CIP were banned for use in Vietnamese aquaculture (VMARD, 2012) due to the significant number of international market rejections related to food safety alerts (Love et al., 2011), and it is therefore expected that their sells and use have recently seen a significant decline. However, given the large number of antibiotics that are currently used in *Pangasius* catfish production in the Mekong Delta region of Vietnam and the lack of regulations controlling their environmental discharge, further monitoring of aquaculture antibiotics in aquatic ecosystems and cost-effective methods for reducing their environmental discharge are urgently required.

Acknowledgements

The authors would like to thank the students of the College of Aquaculture and Fisheries from Can Tho University for their collaboration during sample collection, and to Nguyen Tran Phuong Thao, Nguyen Le Nhat Khoa and Nguyen Thi Kim Ha for their collaboration on the toxicity tests.

Effects of the antibiotic enrofloxacin on the ecology of tropical eutrophic freshwater microcosms

Andreu Rico, Mauricio R. Dimitrov, René P.A. Van Wijngaarden, Kriengkrai Satapornvanit, Hauke Smidt, Paul J. van den Brink

Abstract

The main objective of the present study was to assess the ecological impacts of the fluoroquinolone antibiotic enrofloxacin on the structure and functioning of tropical freshwater ecosystems. Enrofloxacin was applied at a concentration of 1, 10, 100 and 1000 µg/L for 7 consecutive days in 600-L outdoor microcosms in Thailand. The ecosystem-level effects of enrofloxacin were monitored on five structural (macroinvertebrates, zooplankton, phytoplankton, periphyton and bacteria) and two functional (organic matter decomposition and nitrogen cycling) endpoint groups for four weeks after the last antibiotic application. Enrofloxacin was found to dissipate relatively fast from the water column (half-dissipation time: 11.7 h), and about 11% of the applied dose was transformed into its main by-product ciprofloxacin after 24 h. Consistent treatment-related effects on the invertebrate and primary producer communities and on organic matter decomposition could not be demonstrated. Enrofloxacin significantly affected the structure of leaf-associated bacterial communities at the highest treatment level, and reduced the abundance of ammonia-oxidizing bacteria and ammonia-oxidizing archaea in the sediments, with calculated NOECs of 10 and <1 µg/L, respectively. The ammonia concentration in the microcosm water significantly increased in the highest treatment level, and nitrate production was decreased, indicating a potential impairment of the nitrification function at concentrations above 100 µg/L. The results of this study suggest that environmentally relevant concentrations of enrofloxacin are not likely to result in direct or indirect toxic effects on the invertebrate and primary producer communities, nor on important microbially mediated functions such as nitrification.

This chapter has been published in *Aquatic Toxicology* 2014, 147: 92-104.

1. Introduction

Antibiotics used in human and veterinary medicine can enter aquatic ecosystems directly, through the discharge of waste water treatment plant effluents or aquaculture residues, or indirectly, by leaching and runoff of agricultural soils amended with manure from livestock facilities (Ternes et al., 2004; Sarmah et al., 2006; Rico et al., Submitted). Over the last few years, a considerable amount of work has been done on assessing the occurrence and environmental fate of antibiotics in the aquatic environment, indicating that measured water concentrations are, in most cases, relatively low (i.e. from 0.001 µg/L to about 10 µg/L) (Kümerer, 2009). Acute and chronic laboratory studies suggest that antibiotics are not expected to result in direct toxic effects on fish and aquatic invertebrates at environmentally relevant concentrations (Robinson et al., 2005; Park and Choi, 2008). However, several experiments indicated that cyanobacteria and non-phototrophic microbial communities could be affected by antibiotic pollution at concentrations that are orders of magnitude lower than the threshold concentrations derived from toxicity data for standard test species (Maul et al., 2006; Ebert et al., 2011; Yergeau et al., 2012; Wunder et al., 2013). Possibly, effects of antibiotics on cyanobacteria could affect the community structure of primary producers, which might propagate to primary and secondary consumers (Rico et al., 2014). Furthermore, the disruption of important ecosystem processes such as organic matter mineralization (Maul et al., 2006), nitrification (Klaver and Mattews, 1999), and/or degradation of organic pollutants (Näslund et al., 2008) could result in changes in water quality and might induce additional stress to aquatic organisms. To date, our knowledge on the effects of antibiotics on ecological interactions is still very limited and, therefore, further research needs to be undertaken to assess the potential side effects of antibiotics on ecological functions and on the structure of aquatic communities in multitrophic systems.

Model ecosystem studies (i.e., microcosms and mesocosms) have been used in the risk assessment of pesticides and veterinary medicines since they provide more ecological realism as compared to laboratory bioassays and allow the identification of potential interactions between aquatic communities and ecosystem functions (Van den Brink et al., 2005). The number of studies evaluating the fate and effects of antibiotics on aquatic model ecosystems is very limited, and all of them have been performed under temperate climatic conditions (e.g. Wilson et al., 2004; Knapp et al., 2005; Maul et al., 2006). Recent monitoring studies have detected antibiotic residues in several rivers impacted by urban and intensive animal production in (sub-)tropical regions of Asia (Yang et al., 2011; Shimizu et al., 2013; Rico et al., Submitted), suggesting that the study of the potential ecotoxicological effects of antibiotics in the tropical zone requires further attention.

The main objectives of the present study were (1) to get a better understanding on the potential direct and indirect toxic effects of antibiotic pollution on tropical aquatic ecosystems, (2) to identify sensitive structural and functional endpoints for the risk assessment of antibiotics, and (3) to assess whether the use of threshold concentrations derived from laboratory toxicity data would result in a sufficient level of protection for tropical aquatic ecosystems. For this, we assessed the effects of the fluoroquinolone antibiotic enrofloxacin on five structural (macroinvertebrates, zooplankton, phytoplankton, periphyton and bacteria) and two functional (organic matter decomposition and nitrogen cycling) endpoint groups in outdoor freshwater microcosms in tropical Thailand. Enrofloxacin was chosen as test compound because of its broad use in livestock and aquaculture production in tropical countries (e.g. Lampang et al., 2007; Rico et al., 2013a), and because of the availability of data on its environmental fate and aquatic toxicity (Knapp et al., 2005; Robinson et al., 2005; Park and Choi, 2008; Ebert et al., 2011; Rico et al., Submitted). In our study, enrofloxacin was applied in daily pulses for a period of 7 days to eutrophic microcosms, simulating exposure patterns in tropical ecosystems receiving aquaculture effluents that contain enrofloxacin residues (Rico and Van den Brink, 2014). Enrofloxacin shows antibacterial activity against a broad spectrum of (gram-positive and gram-negative) bacteria and is believed to act by inhibiting bacterial DNA gyrase or topoisomerase IV, thus preventing bacterial DNA synthesis and reproduction (Hooper, 1999). Under environmental conditions, enrofloxacin is

rapidly de-ethylated to form ciprofloxacin (Knapp et al., 2005), which is an antibiotic that has been listed as critically important for its use in human medicine (WHO, 2012). The occurrence of antibiotics such as enrofloxacin and ciprofloxacin in the environment has raised concerns about their selective pressure on clinically relevant bacteria and the development of antibiotic resistance (Suzuki and Hoa, 2012), and therefore the assessment of their degradation and transformation under tropical conditions adds crucial information to perform refined exposure assessments.

2. Material and methods

2.1. Experimental design

The present experiment was performed in ten outdoor microcosms at the Faculty of Fisheries of Kasetsart University (KU, Bangkok, Thailand; see Fig. 1). Each microcosm consisted of a PVC tank (top diameter: 122 cm; bottom diameter: 101 cm; total depth: 80 cm; water depth: 63 cm; water volume: 600 L) initially filled with approximately 3 cm of silica-based fine gravel (1-2 mm diameter) extracted from natural rivers in the north of Thailand, and tap water pre-stored for one week to allow dissipation of possible chlorine residues. An aeration system was installed in each microcosm in order to provide mixing of the water during the experimental period. The experiment was performed during March and April 2012 (dry season). The weather conditions during the experimental period were: air temperature 32 (24-40) °C (mean, minimum-maximum), relative humidity 63 (50-75) %, and daily precipitation 1.7 (0-37) mm (rained on 19% of days) (Don Muang Weather Station, Bangkok, Thailand). The microcosms were stocked with plankton and macroinvertebrates collected from freshwater outdoor tanks located at the Ornamental Fish Facilities of KU, from a water reservoir at KU, from the water canal located at the Asian Institute of Technology (AIT, Bangkok, Thailand) described in Daam and Van den Brink (2011), and from outdoor freshwater tanks located at the hatchery of the AIT. These sampling sites were selected because they were uncontaminated sources that showed a relatively high biodiversity of phytoplankton and invertebrates native to Thailand. The stock of the macroinvertebrates was performed by distributing the same number of animals into each microcosm, and the stock of plankton by introducing equal volumes of concentrated plankton sample into each microcosm. The planktonic and macroinvertebrate communities were allowed to establish for a period of 4 weeks prior to the application of the test substance. During this period, water was exchanged between microcosms biweekly in order to homogenise the structure of the communities between the systems. Nitrogen (1.4 mg/L as urea) and phosphorus (0.18 mg/L as triple super phosphate) were added biweekly to the systems according to the recommendations provided by Daam and Van den Brink (2011) during the entire experimental period. The resulting experimental systems were plankton dominated and showed a high eutrophication level, mimicking uncontaminated aquatic systems receiving nutrient-rich effluents from aquaculture or livestock production areas which may be contaminated by antibiotic residues.

2.2. Application of the test substance

Enrofloxacin was applied to the microcosms in daily pulses (at around 4 pm) at a nominal concentration of 1, 10, 100 and 1000 µg a.i./L during a period of seven days (starting on April 3rd 2012). The selected dosing scheme tried to simulate exposure regimes in aquatic ecosystems resulting from antibiotic treatments used in aquaculture or livestock production. The enrofloxacin application was performed in eight microcosms in duplicate replicated treatments, while the remaining two microcosms were used as controls. Enrofloxacin stock solutions (667 mg/L) were prepared daily with enrofloxacin powder purchased from Sigma Aldrich (purity ≥ 98%, Lot Number: 0001369030). In order to dissolve the enrofloxacin crystals, the weighted amount of the compound was introduced with distilled water in a volumetric flask and sonicated for 30 minutes at 45 °C. Subsequently, 200 µL of ammonia solution (25% v/v ammonia) were introduced in the volumetric flasks. The solutions were shaken gently by hand and then sonicated for another 15-30

minutes under the same temperature conditions until the compound was completely dissolved. Dosing solutions of 0.60, 6.03, 60.3, and 603 mg/L were created by diluting aliquots of the stock solutions in 1 L of distilled water. Finally, the prepared dose solutions were poured over the water surface of the microcosms and mixed by stirring with a wooden stick.

Figure 1. Experimental set-up and detail of the freshwater microcosms used in this study.

2.3. Sampling and analytical verification

The concentration of enrofloxacin and ciprofloxacin (main by-product of enrofloxacin) were determined in water samples collected approximately 30 min after the first application, 24h after the first application (prior to the second application), approximately 30 min after the last application (i.e., seventh application), 2 days after the last application, and 7 days after the last application. Depth-integrated water samples (500 mL) were collected with a Perspex tube and stored in the fridge (4°C) for a maximum period of 24h until analysis.

On the day of the analysis, internal standard (norfloxcin-D5) was added to 1 mL sub-samples of the cosm water samples in order to reach a concentration of 5 µg/L. Subsequently, the sub-samples were filtered through a nylon membrane with 0.22 µm pore size and transferred into glass vials. Enrofloxacin and ciprofloxacin were analysed by High-Performance Liquid Chromatography (HPLC) using a Waters 2695 Alliance HPLC Separation Module. The chromatographic separation was performed by means of a Shiseido Capcell Pak C18 column (150 x 2 mm; 3 µm) at 30°C. The mobile phase was formed by (A) 50 mM ammonium acetate (pH = 3.0) and (B) acetonitrile, and the flow rate was set to 0.2 mL/min. The mobile phase composition for the separation method lasted for 15 min with the following elution gradients: 90% A, to 70% A in min 3, to 40% A in min 5, to 10% A in min 6, held for 4 min, to 90% A in min 10 and held for 5 min. Sample injection volumes were 20 µL. The detection was performed by MS/MS using a Quattro Ultima (Micromass, UK, Ltd.) triple stage quadrupole mass spectrometer with the following conditions: ionization mode ESI^+, capillary voltage of 3.0 kv, cone voltage of 50 v, source temperature of 120 °C, desolvation temperature of 350 °C, and nitrogen gas flow of 50 L/h in the cone and 600 L/h in the desolvation. The detection limit for both antibiotics in the water samples was 0.1 µg/L. The calculated recoveries of the analytical method (at a concentration of 10 µg/L) were 89 ± 2% for enrofloxacin, and 104 ± 3% (mean ± SD; *n* = 3) for ciprofloxacin. The measured concentrations in the cosm water samples were corrected for the method recovery.

2.4. Water quality

Dissolved oxygen (DO), pH, electrical conductivity (EC) and temperature (T) were monitored on day 7 and 1 before the antibiotic treatment, 1 hour after the first antibiotic application, and on day 2, 7, 9, 14, 21 and 28 after the first antibiotic application. Measurements were made in the morning (at 8 am) and at the end of the afternoon (around 6 pm) at an approximate water depth of 10 cm. DO, pH and T were measured with a HQ40d multimeter and EC with an EC-meter (Eijkelkamp 18.28).

Alkalinity levels and the concentration of ammonia, nitrite, nitrate and total phosphorus were measured in microcosm water samples collected on the same days as the other water quality parameters, except for day 9 after the first antibiotic application. A depth-integrated water sample (1 L) was collected with a Perspex tube and stored at 4°C until analysis. Analysis of the alkalinity and nutrient concentrations was performed according to the methods described in APHA (2005).

2.5. Phytoplankton and zooplankton

Phytoplankton and zooplankton samples were taken on day 7 and 1 day before the start of the antibiotic treatment, and on day 2, 7, 9, 14, 21 and 28 after the first antibiotic application. Depth-integrated water samples of 5 L were collected using a Perspex tube and were passed through a plankton net with a mesh size of 20 µm for phytoplankton, and 55 µm for zooplankton. The 5 L water samples were concentrated to an approximate volume of 100 mL. Subsequently, the concentrated samples were fixated with Lugol's iodine solution and stored at 4 °C until further identification.

Sub-samples (200 µL) of the concentrated phytoplankton samples were analysed with an inverted microscope (400x). Phytoplankton taxonomy was determined to the lowest practical level, and the species or genus densities were calculated as the number of individuals per litre of microcosm water. In addition, the chlorophyll-a content of the phytoplankton was used as a proxy for the phytoplankton biomass in the microcosm water. For the analysis of the chlorophyll-a, 150 mL of the microcosm water was filtered through a Whatman GF/C glass-fibre filter (mesh size: 1.2 µm). Chlorophyll-a samples were extracted according to the acetone extraction procedure described in APHA (2005).

Cladocerans, ostracods and copepods were counted in the concentrated zooplankton sample using a binocular microscope with a magnification of 15-25x. Furthermore, a sub-sample (1-2 mL) of the zooplankton sample was taken for the identification of rotifers and copepod nauplii using an inverted microscope (magnification 100x). Rotifers and cladocerans were identified to the lowest practical taxonomic level. Copepods were identified to suborder (i.e., calanoids or cyclopoids), and a distinction was made between nauplii stages and the more mature stages. Ostracods were not further identified. The number of individuals of each species was re-calculated to numbers per litre of microcosm water. The phytoplankton and zooplankton species identification was made by using several taxonomic classification keys for tropical aquatic organisms (e.g. Wongrat, 2000; Fernando et al., 2002).

2.6. Periphyton

The effects of the treatment on the periphyton community was assessed by measuring the chlorophyll-a content of the periphyton biomass on artificial substrates. Three series of 5 microscopic glass slides (7.5 x 2.5 cm) were introduced at a water depth of 30 cm in each microcosm 7 days before the first antibiotic application. On day 7, 14 and 28 after the first antibiotic application, a glass slide series was retrieved and the attached periphyton was collected by scraping them (in 0.5 L of water) until slides were visually clean. The chloropyll-a in the water containing the scraped periphyton was measured according to APHA (2005). Finally, the mass of chlorophyll-a per square centimetre of glass slide was calculated by dividing the total chlorophyll-a content of the water sample by the area of the glass slide that was scraped.

2.7. Macroinvertebrates

The diversity and abundance of macroinvertebrate organisms were monitored by using pebble stone baskets that served as artificial substrates. Two pebble baskets (20x20x10cm) were placed on the sediment's surface of each microcosm three weeks before the antibiotic treatment. Macroinvertebrates were sampled 1 day before the start of the antibiotic treatment, and on day 2, 9, 14, 21 and 28 after the first antibiotic application. The artificial substrates were sampled

alternately. On each sampling day, one of the substrates was gently lifted from the sediment and directly enveloped by a net (51x38 cm; mesh size: 0.5 mm). The substrates were gently shaken inside of the net to collect the invertebrates inhabiting the substrates. Moreover, the net was passed through the water column next to the tank's wall covering approximately one quarter of the walls' surface in order to catch swimming macroinvertebrates. The collected invertebrates were introduced in a white plastic tray, where they were identified and counted alive. Finally, the counted invertebrates were released back into their original microcosm.

2.8. Organic matter decomposition

In order to study the effects of the antibiotic treatment on microbial organic matter decomposition, three litter bags containing approximately 2 g of *Musa* (banana) leaves were introduced in each microcosm one day before the first antibiotic application. First, the banana leaves were leached in tap water for two days and dried in the oven at 70°C for 48 h. A known weight (approximately 2 g) of the dried banana leaves was introduced into nylon bags (mesh size: 0.5 mm). The litter bags were suspended at an approximate water depth of 30 cm in the microcosms. One litter bag was retrieved from each microcosm on day 7, 14 and 28 after the start of the treatment. The decomposed material was dried at 70°C for 48 h and weighted. The percentage of organic matter decomposition was calculated by comparing the initial dry weight of the banana leaves (before introduction into the microcosms) and the final dry weight after the incubation period in the microcosms.

2.9. Microorganisms

Changes in bacterial community structure present on leaf material and sediment were monitored after antibiotic application. *Musa* leaves were dried at 70°C for 48h and introduced into nylon bags (mesh size: 0.5 mm). Two nylon bags were hung at 30 cm depth in each microcosm seven days before the first antibiotic application. The nylon bags were retrieved from the microcosms on day 7 and 14 after the first antibiotic application. The nylon bags were opened and leaves were carefully transferred into plastic bags. Integrated sediment samples (3 cm) were collected from each microcosm on day 7, 14 and 21 after the first antibiotic application, and were introduced into plastic bags. Plastic bags containing the leaf and sediment material were frozen at -20°C until further analysis.

Three leaf discs (1 cm diameter) were taken from every leaf sample collected, and a sub-sample of 2 g was collected from the sediment samples for further analysis. Leaf discs and sediment sub-samples were subjected to total DNA extraction, using the FastDNA® Spin kit for Soil (MP Biomedicals, Santa Ana, CA) according to manufacturer's instructions (Mincer et al., 2005). The quality and quantity of the isolated DNA were checked by using a Nanodrop ND-100 spectrophotometer (Thermo Scientific, San Jose, CA, USA). Before using the DNA samples in further experiments an equal dilution was made for all samples. The 16S rRNA gene was partially amplified (V1 to V2 region) by polymerase chain reaction (PCR). PCR products were analysed by denaturing gradient gel electrophoresis (DGGE) according to Lin et al., (2012). Briefly, DGGE was performed on polyacrylamide gels with a denaturant gradient from 30 to 60% (100% denaturing acrylamide was defined as 7 M urea and 40% (v/v) formamide) using a DCode Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA) (Muyzer et al., 1993). Aliquots of the PCR products were loaded on the gel and electrophoresis was carried out with 1 x Tris-acetate-EDTA buffer (60°C, 85 V) for 16h. The resulting gels were silver-stained according to Sanguinetti et al. (1994) and scanned. Finally, the Bionumerics software version 4.61 (Applied Maths, Belgium) (Tzeneva et al., 2009) was used for DGGE band detection and band intensity quantification. The results of this analysis were used to assess total operational taxonomic units (OTUs), as proxy for bacterial richness, and the relative intensity of the present bands, as a proxy for relative abundance (RA) of different OTUs (Massana and Jürgens, 2003).

Quantitative PCR (qPCR) was used to determine the abundance of total bacteria (16S rRNA gene), bacterial and archaeal ammonia oxidizers (*amoA* gene) and nitrogen-fixing bacteria (*nifH* gene) in the leaf and sediment samples. All qPCR reactions were performed in a 384-well plate (Bio-Rad) using a CFX384 Real-Time PCR Detection System (Bio-Rad). All samples were analysed in triplicate, and reactions were carried out in a total volume of 10 μL**.** Single qPCR reactions were prepared using 5 μL of iQ SYBR Green super mix (Bio-Rad), 0.4 μL of forward and reverse primers (10 μM), 0.1 μL of BSA (20 mg/mL), 0.1 μL of VisiBlue™ qPCR mix colorant (TATAA Biocentre) and 4 μL of DNA (1.25 μg/mL). Primer combinations and cycle conditions are described on Table 1. At the end of each qPCR run, a melting curve analysis was performed from 60 to 99 °C with an increase of 0.5 °C every 10 seconds. Purity of the qPCR products was checked by the observation of a single peak on the melting curve, while correct size amplification was confirmed on a 1% (w/v) agarose gel. For each qPCR reaction a standard curve comprising 10 serial 10-fold dilutions of the target gene was created. Standards were obtained by amplifying the target genes from the following sources: *Escherichia coli* (16S rRNA gene), *Nitrososphaera viennensis* (archaeal *amoA* gene), *Nitrosospira multiformis* (bacterial *amoA* gene) and *Pseudomonas stutzeri* (bacterial *nifH* gene).

Table 1. Primers and cycle conditions used in the quantitative PCR reactions.

Target gene	Primers	Cycle conditions	References
16S rRNA	BACT1369F PROK1492R	95 °C – 3min; 40 cycles of 95 °C – 30 sec, 56 °C – 45 sec, 72 °C 60 sec.	Suzuki et al. (2000)
Archaeal *amoA*	Arch-amoAF Arch-amoAR	95 °C – 3min; 40 cycles of 95 °C – 30 sec, 56 °C – 45 sec, 72 °C 60 sec.	Francis et al. (2005)
Bacterial *amoA*	amoA-1F amoA-2R	95 °C – 3min; 40 cycles of 95 °C – 30 sec, 55 °C – 45 sec, 72 °C 60 sec.	Rotthauwe et al. (1997)
nifH	nifHF nifHR	95 °C – 3min; 40 cycles of 95 °C – 30 sec, 63 °C – 45 sec, 72 °C 60 sec.	Rösch et al. (2002)

2.10. Data analysis

No observed effect concentrations (NOECs) were calculated for all water quality parameters, chlorophyll-a content of the phytoplankton and periphyton community, organic matter decomposition data, and for all taxa of phytoplankton, zooplankton and macroinvertebrates. Effects were considered to be consistent when they showed statistically significant deviations pointing in the same direction for at least two consecutive sampling days or occurred on a single sampling day during or immediately after the treatment period. The NOEC calculations were performed by using the Williams test (Williams, 1972), which assumes a monotonic increasing effect with increasing exposure dose. The Williams tests were performed with the Community Analysis computer program, version 4.3.05 (Hommen et al., 1994), using a significance level of 0.05. Prior to the analysis, the species abundance data and the OTU's RA dataset were ln(*Ax*+1) transformed, where x stands for the abundance value and *Ax* makes 2 by taking the lowest abundance value higher than zero for *x*. This was done in order to down-weigh high abundance values and approximate a normal distribution of the data (for rationale see Van den Brink et al., 2000).

The phytoplankton, zooplankton and macroinvertebrate datasets were analysed by the Principal Response Curve (PRC) method (Van den Brink and Ter Braak, 1999) using the CANOCO Software package, version 5 (Ter Braak and Šmilauer, 2012). The PRC method is a specific type of redundancy analysis (RDA) that is able to explain the variation in species composition between replicate microcosms from the exposure to a stressor by including the treatment regime as explanatory variable, and the interaction between the treatment regime and the sampling times as covariables. The overall significance of the antibiotic treatment regime on the variation in species composition ($p \leq 0.05$) was tested by performing 499 Monte Carlo permutations (Van den Brink and Ter Braak, 1999). The significance of the antibiotic treatment regime per sampling date was calculated by performing single RDA permutation tests for the dataset of each sampling date

separately using Ln-transformed treatment concentrations as explanatory variable. Finally, the NOEC values at community level were calculated for each individual sampling date by applying Williams test to the sample scores of the first principal component of each sampling date (for rationale see Van den Brink et al., 1996).

The use of the PRC method for the analysis of microbial data requires perfect alignment of the DGGE profiles obtained from different samples, which is a laborious and difficult task, and potentially introduces an extra source of variability to the dataset (Lin et al., 2012). For this reason, the statistical significance of the antibiotic treatment on the OTU and OTU's RA datasets derived from the bacterial DGGE profiles were analysed by RDAs performed for each sampling date separately using the Ln-transformed treatment concentrations as explanatory variables (Monte Carlo permutation test: 499 permutations; $p \leq 0.05$). In addition, Principal Component Analysis (PCA) bi-plots were constructed in order to graphically show the within treatment variations. The PCA and RDA analyses were performed using the CANOCO Software package version 5 (Ter Braak and Šmilauer, 2012). Bacterial community NOECs were calculated for each sampling date following the same procedure as described above. The NOECs for the total bacterial abundance, abundance of bacterial and archaeal *amoA* gene, and abundance of the *nifH* gene were calculated with the Williams test ($p \leq 0.05$; Williams, 1972).

3. Results

3.1. Exposure concentrations

Measured enrofloxacin concentrations after the first application were, on average, 102% of the intended concentrations (range: 88-121%) (Fig. 2). Based on the enrofloxacin concentrations measured 24 h after the first application and the equations described in Hoang et al. (2012), a first-order half dissipation time (DT50) of 11.7 ± 1.35 h and a dissipation rate constant of 1.44 ± 0.17 d^{-1} (mean ± standard deviation) were calculated. The concentrations of enrofloxacin measured two days after the last application were below the detection limit, except for the treatment with 100 and 1000 µg/L, which were 1.8 and 292 µg/L, respectively. One week after the last application, all measured enrofloxacin concentrations fell below the detection limit, except for the highest treatment level (1000 µg/L), which had a concentration of 23 µg/L (Fig. 2). The calculated 7-day average concentrations of enrofloxacin in the treated microcosms were approximately 0.7, 7, 69 and 686 µg/L, for the lowest to the highest treatment level, respectively. Enrofloxacin was rapidly transformed into ciprofloxacin. Measured ciprofloxacin concentrations 24h after the first enrofloxacin application were, on average, 11% of the applied dose . Seven days after the last enrofloxacin application, ciprofloxacin was detected only in the 100 and 1000 µg/L treatments at concentrations of 1.1 and 40 µg/L, respectively.

3.2. Water quality parameters

The daily average water temperature in the microcosms ranged between 30 and 35°C during the experimental period. The water temperature gradually increased after the treatment period reaching a maximum water temperature of 38°C on day 28 after the first antibiotic application (Fig. 3A, B). Average DO concentrations in the control microcosms ranged between 4.1 in the morning, to concentrations above the oxygen saturation level in the afternoon(average morning value: 5.5 mg/L; average afternoon value: 14 mg/L). On day 21 after the first antibiotic application, morning DO concentrations dropped to critical levels (below 2 mg/L) in some microcosms. The average daily oxygen production in the control microcosms (i.e., difference between morning and afternoon concentration) was 8.4 mg/L, denoting a very high primary productivity. A trend was observed towards lower DO concentrations and lower daily oxygen production in the highest treatment level (1000 µg/L), however, significant differences with the control treatment were only calculated for the oxygen production values after the second enrofloxacin pulse (Table 2; Fig. 3C, D). The pH in the microcosms ranged between 8.0 and 10.7.

Although a decrease in the pH was observed in the highest treatment level (1000 µg/L) during the treatment period, deviations to the controls were lower than 0.8 pH units and differences were not statistically significant (Table 2; Fig. 3E). The measured EC and alkalinity levels during the whole experimental period were 259 (213-349) µs/cm and 90 (57-140) mg $CaCO_3$/L (mean, minimum-maximum), respectively. No treatment-related effects could be demonstrated for these two parameters during the experimental period (Table 2; Fig. 3F).

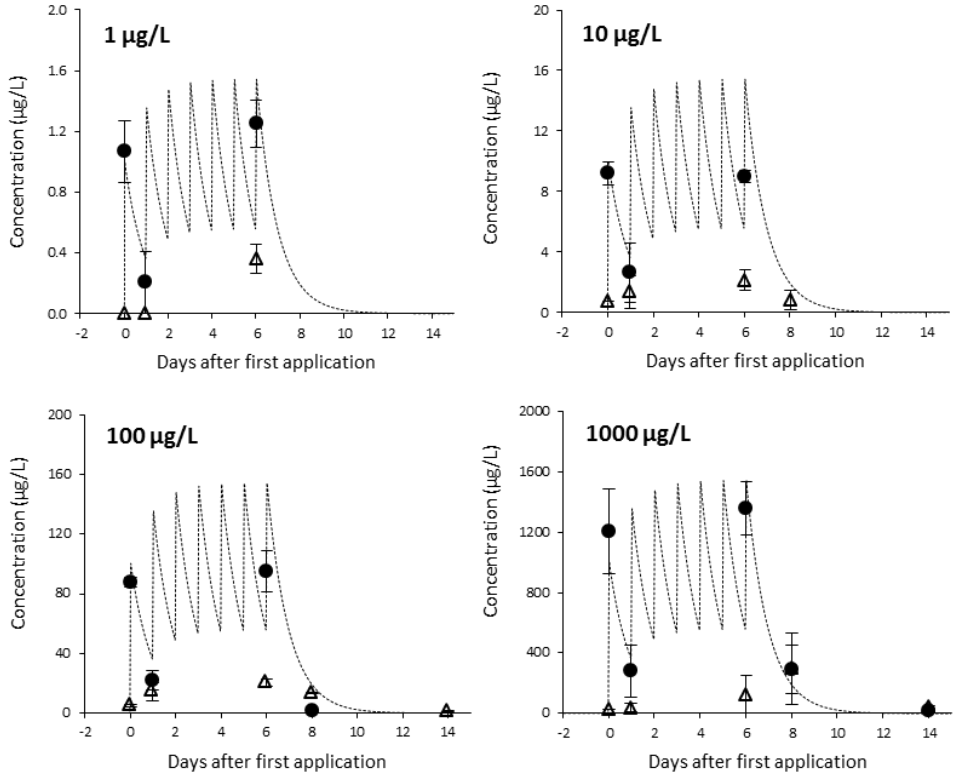


Figure 2. Measured water concentrations of enrofloxacin (dots) and ciprofloxacin (triangles) in the different treatments (mean ± standard deviation). The figure only displays those measured antibiotic concentrations that exceeded the detection limit of the analytical method (0.1 µg/L). The dashed line represents the theoretical enrofloxacin concentration in the microcosm water calculated with the first-order dissipation rate constant derived from the present study (k = 1.44 d^{-1}).

Ammonia concentrations showed a significant increase at the highest treatment level (1000 µg/L) during the treatment period, and one week after the treatment period (Table 2, Fig. 4A). The average ammonia concentrations on day 2, 7 and 14 after the first antibiotic application were 2.6, 3.2, and 1.0 mg/L in the highest treatment level (1000 µg/L), and 1.3, 0.7 and 0.4 in the controls, respectively. Nitrite concentrations were considerably higher in the control treatment samples than in the rest of the treatments during the pre-treatment and treatment period (Fig. 4B), and were found to decrease in the highest treatment level on day 2, 7, and 14 after the first antibiotic application, although the data did not show significant differences. Nitrate concentrations during the pre-treatment and treatment periods showed a high variability, with considerably higher values in the control (0.4-0.5 mg/L) and in the lowest antibiotic treatment (0.7-0.9 mg/L), compared to the other treatments (Fig. 4C). This variability could be visually associated to different periphyton or phytoplankton dominating states in the microcosms. Microcosms with high quantities of filamentous algae adhered to the walls of the tanks generally showed lower dissolved nitrate concentrations. A trend was observed towards lower nitrate concentrations in the highest treatment level during the antibiotic application period although, due to the high variability observed in the other treatment levels, significant differences could not be demonstrated (Table 2; Fig. 4C). Total phosphorus concentrations ranged between 0.07-0.69 mg/L during the whole experimental period (average: 0.24 mg/L), and did not show any treatment-related significant variation (Table 2).

Table 2. No observed effect concentrations (NOECs; Williams test, $p \leq 0.05$) in µg/L (expressed in terms of nominal single-dose enrofloxacin concentration) for water quality parameters measured on each sampling date. The shaded area indicates the treatment period.

	Days after first application									
Endpoint	**-7**	**-1**	**0**	**1**	**2**	**7**	**9**	**14**	**21**	**28**
DO a.m.	>	>	>	>	>	>	>	>	>	>
DO p.m.	>	>	>	>	>	>	>	>	>	>
DO production	>	>	>	>	10 (↓)	>	>	>	10 (↓)	>
pH a.m.	>	>	>	>	>	>	>	>	>	>
pH p.m.	>	>	>	>	>	>	>	>	>	>
EC a.m.	>	>	>	>	>	>	>	>	>	>
EC p.m.	>	>	>	>	>	>	>	>	>	>
Alkalinity	>	>	NM	NM	>	>	NM	>	>	>
Ammonia	>	>	NM	NM	100 (↑)	100 (↑)	NM	100 (↑)	>	>
Nitrite	< 1 (↓)	>	NM	NM	>	>	NM	>	>	>
Nitrate	>	>	NM	NM	>	>	NM	>	>	1 (↓)
Total phosphorus	>	>	NM	NM	>	>	NM	>	>	>
Chlorophyll-a	>	>	NM	NM	>	>	NM	>	>	>

↑ = increase, ↓ = decrease, > = no significant effect (NOEC > 1000 µg/L), NM = not measured

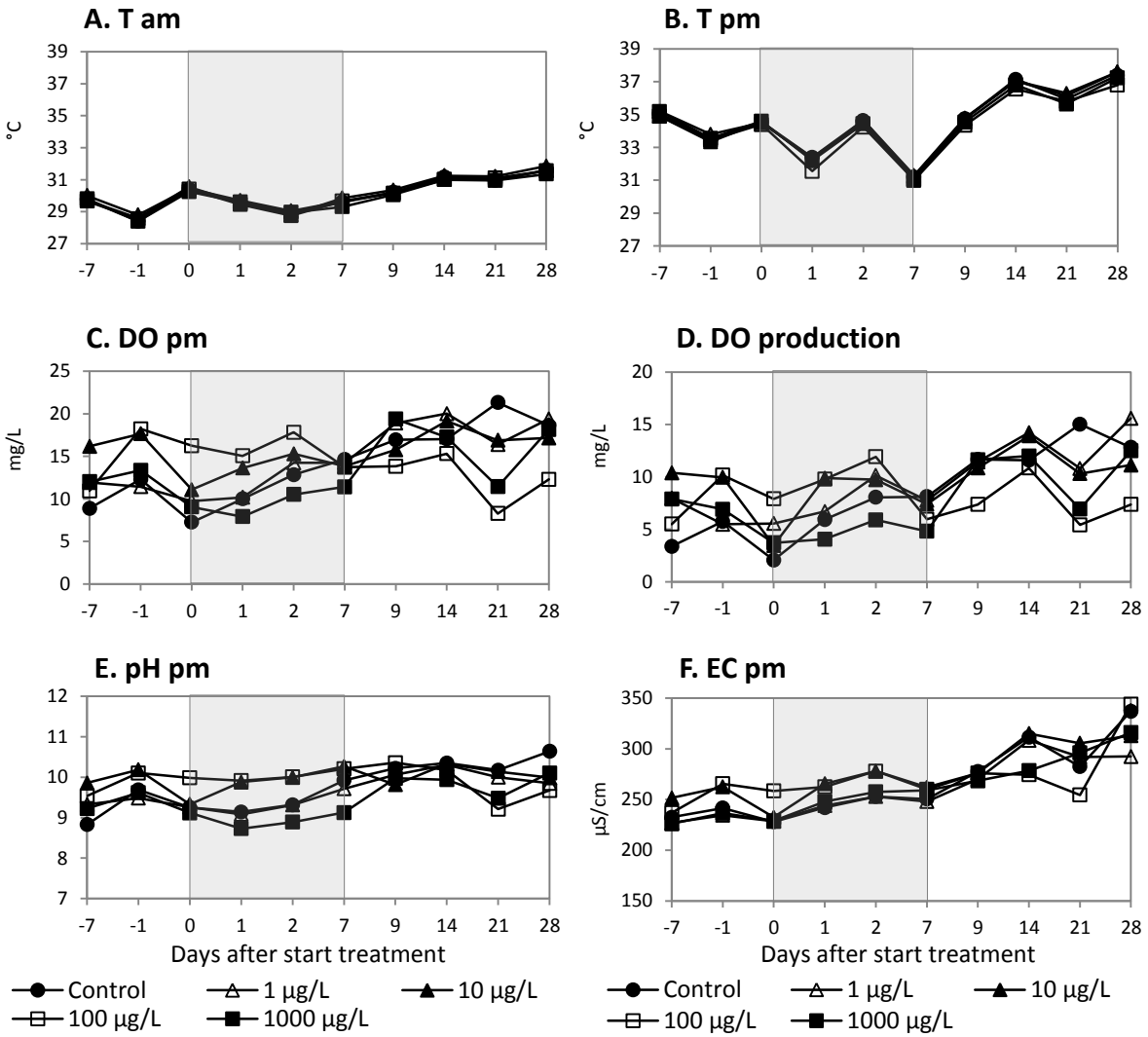


Figure 3. Water quality parameter dynamics measured during the experimental period. The figures show temperature (T) measured early in the morning (8 am) (**A**) and late in the afternoon (6 pm) (**B**), afternoon dissolved oxygen (DO) measurements (**C**) and dissolved oxygen production (difference between morning and afternoon levels) (**D**), and afternoon pH (**E**), and electric conductivity (EC) measurements (**F**). Afternoon dissolved oxygen values exceeded the oxygen saturation levels (about 7 mg/L at 35°C) due to the high primary production in the microcosms. The shaded area indicates the treatment period.

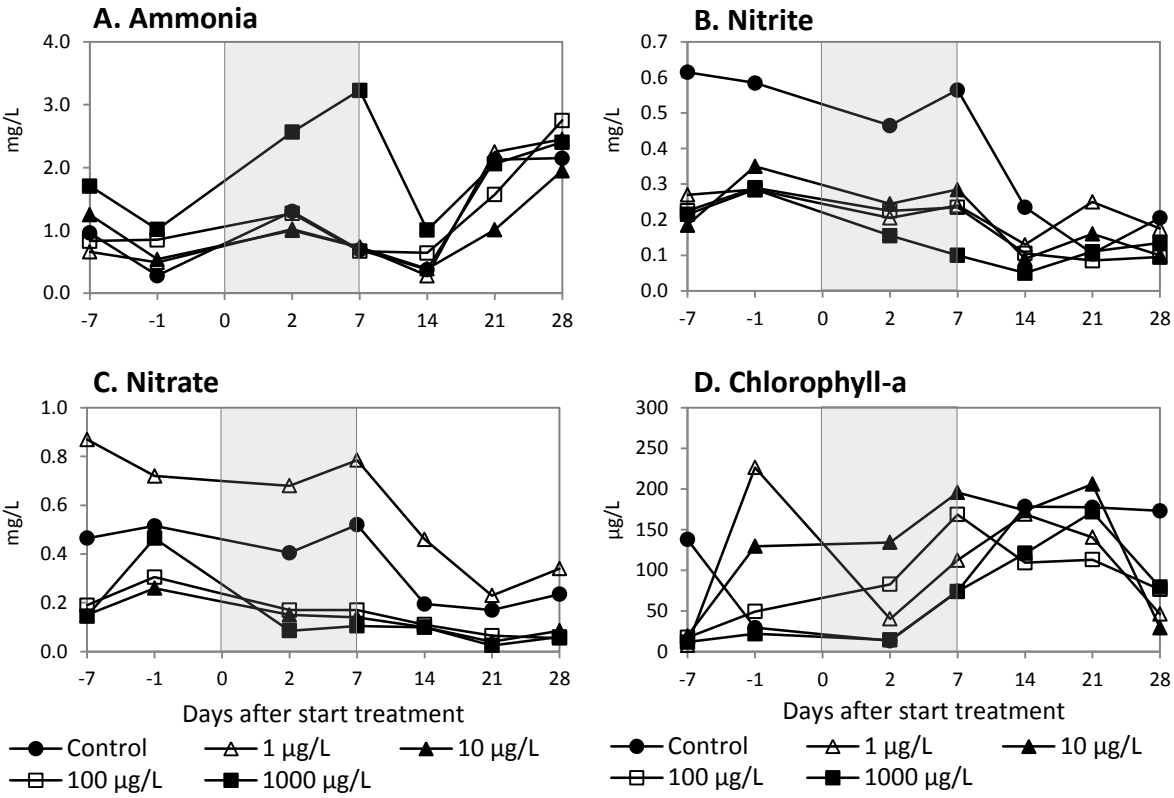


Figure 4. Ammonia (**A**), nitrite (**B**), nitrate (**C**), and chlorophyll-a (**D**) dynamics measured during the experimental period. The shaded area indicates the treatment period.

3.3. Phytoplankton community

Thirty nine phytoplankton taxa were identified in the current study, belonging to 5 different taxonomic groups Chlorophyceae (20 taxa), Cyanobacteria (8), Bacillariophyceae (5), Desmidiaceae (3), Dinophyceae (2), and Euglenophyceae (1). The phytoplankton community was dominated by a limited number of taxa, and many occurred at low densities (< 1 individual/mL) and/or were only observed on a limited number of sampling days (Table S1). The most abundant phytoplankton taxa in decreasing order were: *Chlorella sp.* (Chlorophyceae) and *Coelastrum sp.* (Chlorophyceae). The total phytoplankton abundance in the controls considerably decreased after the pre-treatment period, however the relative abundance of species remained relatively constant (Fig. S1).

The total taxa richness observed on day 14 was slightly higher in all treated microcosms compared to the controls (Table 3; Fig. S2A). These differences, however, occurred in one isolated sampling day and were very small (i.e., from 11 taxa in controls to 16 taxa in the 1000 µg/L treatment level) and, hence, a clear dose-response effect relationship could not be identified. The water concentration of chlorophyll-a in the microcosms were relatively high, indicating a high primary productivity in the systems, and increased during the treatment period. However, significant effects of the antibiotic could not be demonstrated (Table 2; Fig. 4D). The results of the PRC analysis did not show significant effects of the enrofloxacin treatment on the composition of the phytoplankton community (p = 0.53). Consistent statistically significant treatment-related effects were calculated for only 1 out of the 39 phytoplankton taxa. A *Scenedesmus* species showed a higher abundance at the three highest treatment levels compared to the controls (Table 3; Fig. S2B,C).

3.4. Periphyton biomass

The periphytonic chlorophyll-a density in the control microcosms ranged between 2 and 7 µg/dm^2 of glass slide (Fig. S5B). On average, chlorophyll-a contents increased on day 14 after the first

antibiotic application of the four treatments. However, the results of the univariate analysis did not show a significant effect of the antibiotic treatment on the chlorophyll-a content at any of the sampling dates.

3.5. Zooplankton community

The sampled zooplankton community consisted of 20 Rotifera taxa, 6 Cladocera taxa, 2 Copepoda taxa and 1 Ostracoda taxon. The most abundant taxa belonged to Rotifera (i.e., *Brachionus angularis, Filinia longiseta, Brachionus caudatus, Hexathra sp., Ploesoma sp., Brachionus calyciflorus, Polyarthra vulgaris, and Trichocerca sp.*) and Copepoda (i.e., nauplii stages and cyclopoids) (Table S2). The control microcosms were dominated by cyclopoid copepods, the rotifers *Ploesoma sp.*, *P. vulgaris, B. angularis,* and the cladoceran *Ceriodaphnia reticulata.* During the experimental period, the relative abundance of Copepoda, Cladocera, and *Ploesoma sp.*, decreased. The numbers of the *Brachionus* rotifers increased sharply during the last two weeks of the experimental period, probably due to the increased water temperatures, and resulted in a notable increase of the total zooplankton abundance (Fig. S1).

The results of the PRC analysis did not show significant effects of the enrofloxacin treatment on the zooplankton community (p = 0.62). Significant univariate responses were calculated for 8 taxa, but only one species (*C. reticulata*) showed a consistent response (Table 3). *C. reticulata* abundance was significantly lower in the treated microcosms than in the controls after the start of the treatment. However such differences were already appreciable in the pre-treatment period (Fig. S3D).

3.6. Macroinvertebrate community

During the experimental period, 17 different macroinvertebrate taxa were identified, the majority of which belonged to Insecta (11 taxa), followed by Mollusca (5) and Annelida (1) (Table S3). The most abundant genera in decreasing order were Chironomidae, *Micronecta* sp., and Notonectidae. The relative abundance of these three taxa in the control microcosms remained relatively constant during the experimental period. The total macroinvertebrate abundance was generally low in the pre-treatment period and in the last two weeks of the experimental period (Fig. S1).

The total macroinvertebrate taxa in all the treatment levels slightly decreased during the experimental period. The results of the PRC analysis did not show a significant effect of the enrofloxacin treatment on the macroinvertebrate community (p = 0.30). The results of the univariate analysis indicated a significant increase in the abundance of two snail species (*Melanoides tuberculata* and *Physella acuta*) in the highest enrofloxacin treatment level (1000 µg/L) (Table 3). However, these significant effects were observed on isolated sampling dates and the abundance of these species in the microcosm samples was very low (Fig. S4B,C).

3.7. Organic matter decomposition

The decomposition of the *Musa* leaves in the control microcosms were 24%, 43% and 76%, after an incubation period of 1, 2 and 4 weeks, respectively (Fig. S5A). The results of the univariate analysis did not show treatment-related effects on the percentage of decomposition in any of the sampling dates. It must be noted, however, that in some instances macroinvertebrates (e.g. Chironomidae) were found to be feeding on the leaves, which could have influenced the leaf breakdown.

Table 3. No observed effect concentrations (NOECs; Williams test, $p \leq 0.05$) expressed in terms of nominal single-dose of enrofloxacin concentration (µg/L) for the phytoplankton, zooplankton, macroinvertebrate and microorganism endpoints evaluated. Only individual taxa that showed a treatment-related effect on at least one sampling date are included. The shaded area indicates the treatment period.

Endpoint	Day after first application								Note
	-7	-1	2	7	9	14	21	28	
Phytoplankton									
Community	>	>	>	>	>	>	>	>	
Total taxa richness	>	>	>	>	>	<1(↑)	>	>	Fig. S2A
Chlorophyta	>	>	>	>	>	>	>	>	
Scenedesmus sp. II	NP	>	>	>	10(↑)	<1(↑)	<1(↑)	>	Low density [a], Fig. S2B
Cyanophyta	>	>	>	>	>	>	>	>	
Desmidiaceae	>	>	>	>	>	>	>	>	
Diatomeae	>	>	>	>	>	>	>	>	
Diatom sp. IV	>	>	>	>	>	>	10(↑)	>	Low density [a], Fig. S2C
Dinoflagellata	>	>	NP	NP	NP	NP	NP	NP	
Euglenophyceae	>	NP	NP	NP	NP	NP	NP	NP	
Zooplankton									
Community	>	>	>	>	>	>	>	>	
Total taxa richness	>	>	>	>	>	>	>	>	Fig. S3A
Cladocera	>	>	>	>	>	>	1(↑)	>	Fig. S3B
Alonella sp.	>	>	>	100(↑)	>	>	>	>	Low density [b], Fig. S3C
Ceriodaphnia reticulata	>	>	<1(↓)	<1(↓)	100(↓)	>	>	>	Low density [b], Fig. S3D
Diaphanosoma senegal	>	>	>	>	100(↑)	>	>	>	Low density [b], Fig. S3E
Copepoda	>	>	>	>	>	>	>	<1(↑)	Fig. S3F
Nauplii	>	>	>	>	>	>	>	<1(↑)	Fig. S3G
Ostracoda	>	>	>	>	>	>	100(↑)	>	Fig. S3H
Rotifera	10(↑)	>	>	>	>	>	>	>	Fig. S3I
Brachionus angularis	>	>	>	>	>	>	>	100(↓)	Fig. S3J
Brachionus caudatus	>	>	>	>	>	100(↑)	>	>	Fig. S3K
Brachionus forficula	NP	>	NP	NP	>	>	>	<1(↓)	Low density [b], Fig. S3L
Filinia longiseta	NP	NP	NP	NP	>	>	100(↓)	>	Fig. S3M
Hexarthra sp.	>	>	>	>	>	100(↑)	10(↑)	>	Fig. S3O
Macroinvertebrates									
Community	NM	>	>	NM	>	>	>	>	
Total taxa richness	NM	<1(↑)	>	NM	>	>	>	>	Fig. S4A
Insecta	NM	>	>	NM	>	>	>	>	
Mollusca	NM	>	>	NM	>	>	>	>	
Melanoides tuberculata	NM	>	100(↑)	NM	>	NP	NP	NP	Low density [c], Fig. S4B
Physella acuta	NM	10(↑)	>	NM	>	100(↑)	>	>	Low density [c], Fig. S4C
Annelida	NM	NP	NP	NM	>	NP	NP	NP	
Microorganisms									
Leaf samples									
Bacterial OTUs	NM	NM	NM	100	NM	100	NM	NM	
Bacterial RA OTUs	NM	NM	NM	100	NM	100	NM	NM	Fig. 5A
Total bacteria	NM	NM	NM	100(↓)	NM	>	NM	NM	Fig. S6A
Bacterial *amoA* gene	NM	NM	NM	>	NM	>	NM	NM	Fig. S6C
Archaeal *amoA* gene	NM	NM	NM	>	NM	>	NM	NM	Fig. S6D
nifH gene	NM	NM	NM	100(↓)	NM	>	NM	NM	Fig. S6E
Sediment samples									
Bacterial OTUs	NM	NM	NM	>	NM	>	>	NM	
Bacterial RA OTUs	NM	NM	NM	>	NM	>*	>	NM	Fig. 5B
Total bacteria	NM	NM	NM	>	NM	>	>	NM	Fig. S6B
Bacterial *amoA* gene	NM	NM	NM	10(↓)	NM	10(↓)	>	NM	Fig. 5A
Archaeal *amoA* gene	NM	NM	NM	<1(↓)	NM	<1(↓)	<1(↓)	NM	Fig. 5B
nifH gene	NM	NM	NM	>	NM	100(↓)	>	NM	Fig. S6F

> = no significant effect (NOEC > 1000 µg/L), NM = not measured, NP = not present (taxa not present in the analysed samples).

[a] The number of individuals per sample was, on average, lower than 1 individual/mL when the statistically significant effect was observed.

[b] The number of individuals per sample was, on average, lower than 10 individuals/L when the statistically significant effect was observed.

[c] The number of individuals was, on average, lower than 5 per sample when the statistically significant difference was observed.

* Significant effects (Monte Carlo permutation test $p = 0.05$), but calculated NOEC was higher than 1000 µg/L.

3.8. Microorganism community

The RDA analysis indicated significant effects of the antibiotic treatment on the bacterial OTUs and the RA of OTUs in the *Musa* leaf samples at the end of the treatment period (day 7) and one week after the antibiotic treatment (day 14), with calculated NOECs of 100 µg/L for both datasets and both sampling dates (Table 3; Fig. 5A). The total bacteria and the *nifH* gene abundance in the leaf samples of the highest treatment level decreased on day 7 after the first antibiotic application (Fig. S6A,E), however, the abundance of the bacterial and archaeal *amoA* gene did not show significant treatment-related effects (Fig. S6C,D; Table 3).

The RDA analysis on the bacterial OTUs in the sediment samples did not show any treatment-related effects. The sediment bacterial OTUs' RA dataset only showed significant antibiotic-related effects for the samples collected one week after the antibiotic treatment using the Monte Carlo permutation test, but the calculated NOEC was higher than 1000 µg/L (Table 3; Fig. 5B). The total abundance of bacteria in the sediment samples did not show significant treatment related effects (Fig. S6B). A significant decrease was observed in the *amoA* gene abundance during and after the antibiotic treatment, with calculated NOECs of 10 µg/L and below 1 µg/L for the sediment bacteria and archaea communities, respectively (Table 3; Fig. 6A,B). A significant decrease of the bacterial *nifH* gene abundance was only observed at the highest treatment level on the sediment samples collected one week after the antibiotic treatment (Fig. S6F; Table 3).

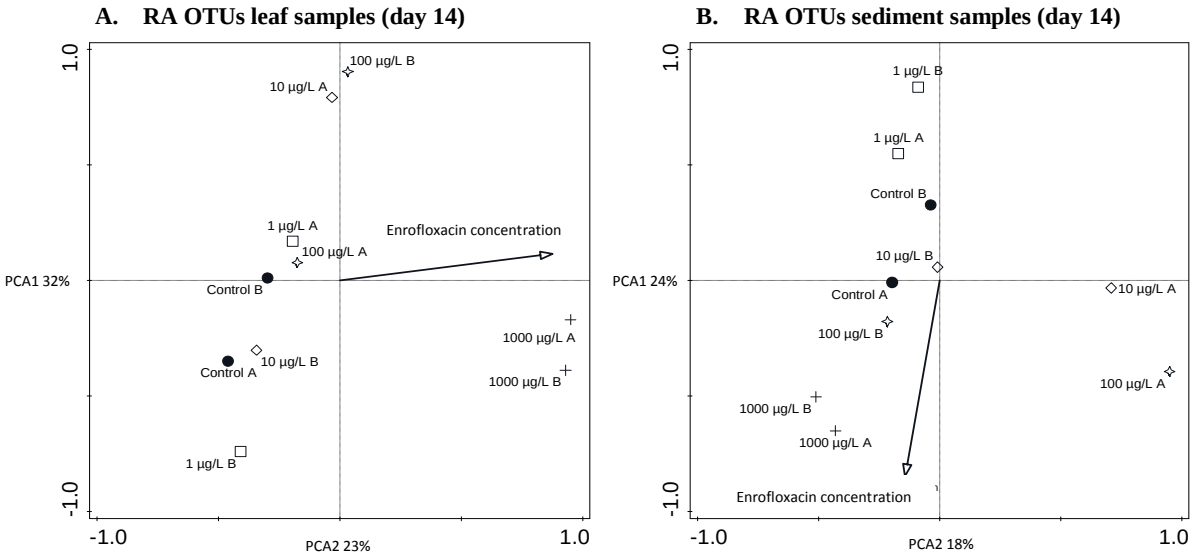


Figure 5. Principal component ordination diagrams of the Relative Abundance (RA) of the Operational Taxonomic Units (OTUs) datasets derived from the DGGE profiles for the leaf (**A**) and sediment (**B**) samples collected on day 14. The calculated NOECs are presented in Table 3. The letters A and B in the graphs refer to the two replicates in each treatment level.

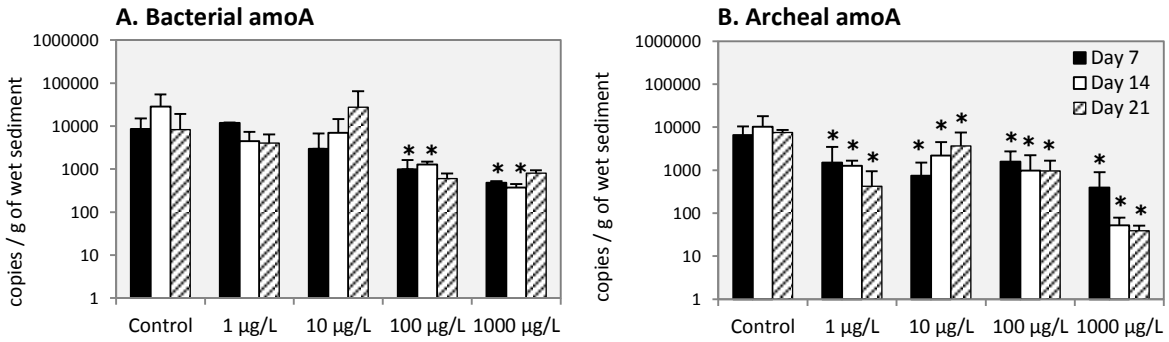


Figure 6. Bacterial (**A**) and archaeal (**B**) *amoA* gene abundance in the sediment samples collected on day 7, 14 and 21 after the first antibiotic application (mean ± standard deviation). The asterisk indicates significant differences with controls (Williams test; $p \leq 0.05$).

4. Discussion

4.1. Dissipation of enrofloxacin

The results of our experiment showed that the dissipation of enrofloxacin from the water column and the formation of its by-product ciprofloxacin were quick processes. Several semi-field studies have demonstrated that photodegradation and sorption to organic matter are the main processes influencing the dissipation of fluoroquinolone antibiotics from surface waters (Cardoza et al., 2005; Knapp et al., 2005). Knapp et al. (2005) evaluated the dissipation and transformation of enrofloxacin under different light conditions (i.e., full-light exposure, partial shading, and almost complete shading) in a mesocosm experiment performed during autumn in Kansas (USA). The enrofloxacin DT50 calculated by Knapp et al. (2005) (approximately 19 h) in the mesocosms with full-light exposure was slightly higher than the value calculated in our experiment (DT50 = 11.7 h), suggesting that tropical environmental conditions favour the dissipation of enrofloxacin from the aquatic environment, probably due to higher photodegradation.

4.2. Enrofloxacin effects on primary producers

In our experiment, the phytoplankton community and the biomass of the established periphyton community did not show a significant response to the antibiotic treatment. Laboratory toxicity studies have reported short-term growth inhibition EC50 values for enrofloxacin and ciprofloxacin in the range of 10 to 173 µg/L for cyanobacteria (*Microcystis aeruginosa* and *Anabaena flos-aquae*) and 3,100-18,700 µg/L for green algae (*Pseudokirchneriella subcapitata* and *Desmodesmus subspicatus*) (Robinson et al., 2005; Ebert et al., 2011). Wilson et al. (2004) found a concentration-dependent reduction in the abundance and species richness of phytoplankton, with cyanobacteria and cryptophyta/dinophyta being the most affected populations, in microcosms that were chronically exposed to a mixture of four tetracycline antibiotics, which have a similar toxicity to primary producers than fluoroquinolone antibiotics (Park and Choi, 2008). The significant effects observed by Wilson et al. (2004) 7 days after the start of the treatment period occurred in the microcosms that were exposed to an antibiotic concentration that was 2-3 times lower than the highest enrofloxacin concentration tested in our study. On the basis of this data, we expected to find a decline of the cyanobacterial population in the microcosms with the highest enrofloxacin concentration and a potential shift in the overall phytoplanktonic community structure, however, such trend could not be identified. A potential explanation for the absence of effects on the phytoplankton community in our experiment could be related to the high water pH measured in the microcosms. Enrofloxacin is a weak acid and the pH range measured in the test microcosms was rather alkaline (8.0-10.7). Based on the ionic component distributions shown in Kim et al. (2010), about 80 to 100% of the compound could have remained in its anionic form during the experimental period. Several studies have demonstrated that the bioaccumulation and toxicity of ionizable organic substances decreases when the molecule is in its ionized form (Rendal et al., 2011 and references therein). For example, Fahl et al. (1995) found that the toxicity of chlorsulfuron, an ionizable herbicide, on *Chlorella fusca* growth was enhanced 25-fold by lowering the pH of the growth medium from 6.5 to 5.0, and Kim et al. (2010) found that the toxicity of enrofloxacin and ciprofloxacin to *Daphnia magna* increased in waters with lower pH. Thus, studies aimed at assessing the effects of pH on the toxicity of enrofloxacin and other ionizable antibiotics on primary producers, especially cyanobacteria, are recommended in order to confirm this hypothesis and to quantify the variability of the sensitivity to antibiotic exposure under different pH ranges. Another potential explanation for the lack of effects on primary producers resides in the dominance of Chlorophyceae species and the variability observed in the occurrence and abundance of potentially sensitive taxa (cyanobacteria) in the studied microcosms. Daam and Van den Brink (2011) argued that the phytoplankton community structure of tropical ecosystems largely depends on seasonally-related weather conditions. And cyanobacterial taxa, typically *Microcystis*, tends to dominate during situations of nutrient scarcity and/or light limitations, the latter most commonly occurring during the rainy season. Therefore, in order to better observe

potential phytoplankton structure damages by antibiotic exposure under tropical conditions, further semi-field tests with cyanobacteria-dominated systems during rainy season are recommended.

4.3. Enrofloxacin effects on invertebrates

The analysis of the zooplankton and macroinvertebrate communities did not show a significant response to the enrofloxacin application. Sporadic significant responses of certain taxa were observed, but were isolated and did not show a concentration response relationship. Acute toxicity studies with freshwater cladocerans and macroinvertebrates show acute EC50 values higher than 50 mg/L (Park and Choi, 2008; Rico et al., Submitted). Long-term studies assessing the effects of enrofloxacin and ciprofloxacin on reproduction (Park and Choi, 2008) and life-history traits (Martins et al., 2012) of *Daphnia magna* found NOEC values higher than the highest antibiotic concentration tested in our study. Furthermore, previous microcosm experiments performed in temperate regions have not been able to identify negative responses of invertebrate communities to environmentally relevant antibiotic exposure concentrations (Wilson et al., 2004; Maul et al., 2006). Therefore, based on the available literature and the results of this study we can conclude that (tropical and temperate) aquatic invertebrate communities are highly tolerant to enrofloxacin under realistic exposure conditions (i.e., several micrograms per litre).

4.4. Enrofloxacin effects on microorganisms and ecosystem metabolism

Enrofloxacin clearly affected the structure of leaf-associated bacterial communities and reduced bacterial abundance at concentrations higher than 100 µg/L, however little or no effects were identified for the sediment bacterial community (Table 3). Observed differences between the sensitivity of both bacterial communities could be related to differences in exposure patterns and characteristics of these bacterial communities. The bacterial community of our (3 cm) depth integrated sediment samples might have been exposed to a gradient of antibiotic exposure concentrations (from higher concentrations in the top layer, to lower concentrations in the bottom layers) and environmental conditions (from aerobic conditions in the top layer, to less aerobic or anaerobic conditions in the deeper layers). The higher richness of the sediment bacterial community compared to the leaf one (as shown by the number of OTUs in the DGGE profiles; Table S4; Fig. S7), might make them more resilient to antibiotic exposure (Girvan et al., 2005) and is likely to hamper the identification of effects on less dominant species due to the fact that DGGE in general only allows to analyse populations of at least 1% in relative abundance (Muyzer et al., 1993). Knapp et al. (2005) did not find significant effects of enrofloxacin on water-living bacterial communities in microcosms exposed to a single dose of 25 µg/L, but suggested that effects could be more prominent on organic matter surfaces, where prolonged exposures are more likely, as shown in our study. Maul et al. (2006) demonstrated a shift in carbon source utilization of leaf-associated microbial communities repeatedly exposed for 12 days to 100 µg/L of ciprofloxacin. In our study, the alteration of the bacterial community structure and decrease in total bacterial abundance observed at the highest enrofloxacin concentration did not influence the organic matter decomposition rates in the leaf samples, however, such trend could have been masked by the influence of invertebrates on the leaf breakdown. Therefore, we recommend to include such endpoint in further microcosm experiments with antimicrobial substances, but to lower the mesh size of the litter bags containing the decomposing material to 300 µm or less to prevent any interaction with invertebrates.

Our study demonstrated that sediment bacterial and archaeal ammonia oxidizers are highly sensitive to enrofloxacin (NOEC = 10 and 1 µg/L, respectively), and a causal link with their nitrification function could be demonstrated, showing an increase in the ammonia concentrations and a trend towards inhibition of the nitrate formation during the antibiotic exposure period at 1000 µg/L. Several studies have demonstrated that nitrification is largely inhibited in aquatic systems exposed to therapeutic doses of antibiotics used in aquaculture (several mg/L), suggesting potential toxic effects for aquatic organisms due to the accumulation of ammonia

(Klaver and Matthews, 1994; Nimenya et al., 1999). Nimenya et al. (1999) estimated that ammonia oxidation and nitrate production will be reduced by about 1% and 2.3%, respectively, within 24h by enrofloxacin concentrations of 1 mg/L. This might explain the changes in nutrient concentrations observed at the highest treatment level of our experiment. Despite the reduction in sediment-born ammonia oxidizing microorganisms that was observed at almost all treatment levels, a significant increase in the microcosm ammonia concentrations was only demonstrated for the microcosms exposed to 1000 µg/L, returning to levels similar to controls within 2 weeks after the treatment. This suggests that water-living microorganisms (which were not evaluated) could have recovered faster than sediment microorganisms (potentially due to a lower exposure and damage), denoting a high resilience of the whole water-sediment microbial community and a fast recovery from antibiotic exposure. In conclusion, our study confirms that microbial functions such as nitrification might be affected in aquatic systems exposed to therapeutic concentrations of enrofloxacin such as those used in aquaculture bath treatments, but are not likely to be affected in natural aquatic ecosystems that are exposed to antibiotic residual concentrations, which typically are 2 to 3 orders of magnitude lower than therapeutic concentrations (Rico and Van den Brink, 2014).

4.5. Study limitations

To our knowledge, this is the first study that evaluated the fate and ecological effects of an antibiotic in tropical freshwater model ecosystems. The experimental set-up and methodological approach followed the recommendations provided for the ecotoxicological assessment of pesticides in tropical microcosms (see Daam and Van den Brink, 2011). However, we found some limitations that is worth to discuss in order to improve the methodological approach for testing the ecological effects of antibiotics. For example, nutrient additions have been recommended in order to sustain the plankton-dominated status of tropical model ecosystems (Daam and Van den Brink, 2011). In our experiment, biweekly pulsed nutrient (nitrogen and phosphorus) applications were performed, which could have masked the antibiotic effects on nitrogen transformation rates. In addition, aeration was constantly supplied to prevent temperature stratification in the microcosm water under such hot conditions and to avoid critical oxygen drops at night. We believe that such nutrient applications and aeration system were crucial to maintain the planktonic communities in such eutrophic systems, but could have hampered the observation of effects on microbial functional endpoints and ecosystem metabolism (e.g. nitrogen transformation, microbial respiration and aerobic organic matter mineralization). This suggests that worst-case effects of antibiotics in ecosystem functional endpoints should be better evaluated in less eutrophic systems and during the rainy season in which, as discussed previously, solar radiation and water temperatures are lower and the dominance of sensitive cyanobacteria is more likely. In addition, the metabolism of bacteria is known to be generally higher in tropical aquatic ecosystems with high temperatures (Amado et al., 2013), and the recovery potential of microorganisms exposed to non-selective bacteriostatic compounds is also expected to be higher, supporting the use of lower temperature systems to observe microbial-related effects.

During the first three weeks of the pre-treatment period, about 20% of the microcosm water was exchanged biweekly in order to homogenize the microcosms, however, this turned out to be not enough to prevent differences in dominating periphyton and suspended algae taxa that competed for light and nutrients. We tried to avoid that by exchanging more than 50% of the microcosm water during the week before the antibiotic treatment. However, after a few days the microcosms often returned to their original states. This probably influenced the diversity of the planktonic and microbial communities in the microcosms (data not shown) and increased the variability between replicates, lowering the power of the statistical test. Therefore, future experiments should try to provide intensive mixing during the whole pre-treatment period (more than 50%, if possible every two-three days) and increase replication.

4.6. Threshold concentrations and implications for risk assessment

Of all endpoints investigated in the current study, the abundance of bacterial and archaeal ammonia oxidizers were found to be the most sensitive (NOECs of 10 and < 1 µg/L, respectively). Therefore, according to the results of this study, the cut-off value used in the first-tier risk assessment of veterinary medicinal products (1 µg/L; VICH, 2000) provides a sufficient protection level for plant and invertebrate aquatic communities, and microbial-associated function (i.e., nitrification), but fails to protect the relative abundance of important microbial groups in sediments. Most of the second-tier threshold concentrations derived from toxicity data for standard test species and assessment factors appear to ensure a sufficient protection level for aquatic primary producers, invertebrate and microorganism communities, and for nitrification, whereas the threshold concentration derived from toxicity data for *Microcystis aeruginosa* (0.49 µg/L) ensures the most conservative protection for key sediment microorganisms (i.e. nitrifiers) (Table 4). Table 4 also shows that the threshold concentration derived from the luminescence inhibition test performed with the marine bacterium *Vibrio fischeri*, which is often used as surrogate for aquatic bacterial communities in risk assessments, does not result in a sufficient level of protection for all aquatic bacterial taxa, and probably neither for microbial-associated functions. Furthermore, an assessment factor of at least 10 is recommended when safe concentrations are calculated from median HC5 values (hazardous concentration for the 5th sensitivity percentile of species) derived with Species Sensitivity Distributions (SSDs) for primary producers, including species of green algae and cyanobacteria (Table 4).

Table 4. Threshold concentrations for enrofloxacin derived from laboratory toxicity data for bacteria, primary producers, invertebrates and fish. The last column indicates whether these threshold concentrations are protective or not for the abundance of ammonia oxidizing microorganisms (calculated NOEC < 1 µg/L) and their associated ecological function (calculated NOEC = 100 µg/L).

Taxonomic group	Species	Endpoint	Toxicity value (µg/L)	Assessment factor [a]	Threshold (µg/L)	Protective for ammonia oxidizing microbes/function?
Bacteria	*Vibrio fischeri*	IC50-15min (luminescence inhibition)	326,800 [b]	100	327	No/No
Primary producers	*Pseudokirchneriella subcapitata*	EC50-3d (growth inhibition)	3,100 [c]	100	31	No/Yes
	Microcystis aeruginosa	EC50-5d (growth inhibition)	49 [c]	100	0.49	Yes/Yes
	Assemblage	Median HC5 from SSD	8.80[d]	10	0.88	Yes/Yes
Invertebrates	*Daphnia magna*	EC50-2d (immobility)	56,700 [b]	1,000	57	No/Yes
	Daphnia magna	NOEC-21d (reproduction)	5,000 [b]	10	500	No/No
	Assemblage	Median HC5 from SSD	28,190[d]	10	2,819	No/No
Fish	*Oryzias latipes*	EC50-4d (mortality)	> 100,000 [b]	1,000	100	No/Yes

[a] Assessment factors for standard test species of primary producers, invertebrates and fish based on the international guidelines for the environmental risk assessment of veterinary medicinal products (VICH, 2004). The assessment factors for the bacteria IC50 and for the HC5 for species assemblages were based on authors judgement.
[b] Park and Choi (2008).
[c] Robinson et al. (2005).
[d] Rico et al. (Submitted).

The majority of the fluoroquinolone antibiotic concentrations monitored in tropical surface waters are below the µg/L range. Some studies, however, have measured concentrations up to several µg/L in rivers impacted by aquaculture pollution (Rico et al., Submitted), and in effluents of animal farms and hospitals (see Suzuki and Hoa, 2012 for a review). At such environmental concentrations, enrofloxacin is likely to impact, at least temporarily, the structure and function of bacterial and archaeal communities, particularly in sediments, but not to directly affect algal primary producers or invertebrates. One of the aims of our experiment was to assess whether the effects of antibiotic pollution on microbial communities and important ecosystem functions could result in side-effects on primary producers and invertebrates. This experiment did not suggest

indirect effects at higher trophic levels, however, the exposure period used in this study was relatively short, and the recovery of the ecosystem function impairment was relatively quick. Therefore, more studies are required with prolonged exposure periods and using other antibiotics (if possible, with higher environmental persistence) and under different environmental and biological conditions (with lower temperatures and with higher dominance of cyanobacteria). Such experiments should also include fish, as they have been demonstrated to show a lower tolerance to ammonia accumulation in surface waters than invertebrates (Arthur et al., 1987).

Acknowledgments

Mauricio R. Dimitrov is supported through funding from the Strategic Research Fund of the WIMEK graduate school (project: "Adaptive capacity and functionality of multi-trophic aquatic ecosystems"), as well as through the EU-FP7 project EvoTar (contract number: 282004). The authors would like to thank Apisit Jiraseve for antibiotic analysis, Soradakorn Pimla for phytoplankton identification, Hatairat Soodta for water quality analysis, and Chairat Thammachit for assistance during the preparation of the experiment.

Supporting Information

The supporting information of this chapter can be downloaded from:
http://dx.doi.org/10.1016/j.aquatox.2013.12.008.

Chapter 9

Predicting antibiotic resistance in aquaculture production systems and surrounding environments

Andreu Rico, Paul J. van den Brink, Alfredo Tello

Abstract

Intensive aquaculture production can constitute a relevant source of antibiotic pollution and antibiotic resistance (AR) genes. Assessing the risks associated with antibiotic pollution and AR is critical to derive measures aiming at preventing and mitigating AR risks. In this study we introduce a framework to predict and assess the development of AR in aquaculture production systems and their adjacent environments. The framework we propose mechanistically links antibiotic exposure and bacterial species sensitivity distributions using probabilistic risk assessment, and yields a quantitative estimate of the probability that the application of a given antibiotic results in the development of resistance in bacteria inhabiting aquaculture ponds and the outside environment. We apply this framework to predict the AR risks posed by the use of 12 antibiotics in intensive *Pangasius* catfish aquaculture. This study shows that most antibiotics, even when used according to recommendations, may increase the prevalence of AR in bacteria associated to sediments of aquaculture ponds. Although the framework we propose still requires field evaluations, it sets the ground for the inclusion of relevant AR endpoints in the prospective, screening-level risk assessment of aquaculture antibiotics and can be easily applied to other antibiotic pollution scenarios.

Article in preparation

1. Introduction

Antibiotic resistance (AR) is widely recognized as one of the major challenges facing global public health. The increasing prevalence of AR in hospital and community acquired infections along with the scarcity of new antibiotics in the drug development pipeline paint an overall worrying picture of our current and future ability to effectively treat bacterial infections (WHO, 2012). It has been clearly established that the wider environment is the ultimate reservoir of AR genes (D'Costa et al., 2006; D'Costa et al., 2011), and there is evidence supporting the transfer of AR genes from environmental bacteria to human pathogens (e.g. Olson et al., 2005; Poirel et al., 2002; Poirel et al., 2005). Recent research has also shown that selection of AR genes can occur at very low antibiotic concentrations (Gullberg et al., 2011) and that antibiotic pollution has the potential to increase the prevalence of AR in clinically-relevant bacteria in the environment (Tello et al., 2012).

Aquaculture production is considered an important source of antibiotic pollution and AR genes. Antibiotic treatments used in aquaculture production can exert a selective pressure on bacteria associated with diseases of aquatic animals and on environmental bacteria exposed to antibiotic residues (Heuer et al., 2009; Cabello et al., 2013). Monitoring studies performed in different parts of the world have demonstrated that aquaculture production systems and surrounding aquatic environments act as a sink and source of AR genes of clinical concern (Schmidt et al., 2001; Le et al., 2005; Buschmann et al., 2012). In Asia - which currently supplies approximately 90% of global aquaculture production (FAO, 2012) - several of the major classes of antibiotics are used in inland aquaculture (Rico et al., 2012). Growth projections for Asian aquaculture (Bostock et al., 2010) and the scarce and finite nature of freshwater resources (Carpenter et al., 2011) suggest that freshwater aquaculture will increasingly interact with multiple users of water resources, thereby increasing their role as a potential source of AR with clinical relevance. Assessing the risks associated with antibiotic pollution and AR in aquaculture and its surrounding environment is therefore critical to advice sound environmental management and policy.

Yet it is unclear whether AR in aquaculture production environments is predominantly generated by the spread of resistant bacteria from the fish mucus or intestines, by the selective pressure caused by antibiotic residues in water and sediments, or by a combination of both (Cabello et al., 2013). In this article we build on previous research on the environmental fate of aquaculture antibiotics (Rico et al., 2013a) and the selective pressure of antibiotic pollution (Tello et al., 2012), and introduce a framework to assess and predict the development of AR in aquaculture production systems and their adjacent environment. The risk assessment framework is based on the assessment of antibiotic exposure in water and sediments, and the modeling of bacterial sensitivities to characterize the selective pressure of antibiotics. By mechanistically linking antibiotic exposure to bacterial sensitivities using probabilistic risk assessment, the method derives quantitative estimates of the probability that the application of a given antibiotic may result in the development of resistance in environmental bacteria. The framework is conceived as a valuable tool to perform first-tier risk assessments that enable the comparison of different antibiotic compounds and treatment regimes, and the identification of aquaculture and environmental compartments that may act as hotspots for the development of AR. As a proof of principle, we apply the framework to predict the development of AR associated with the use of 12 antibiotics in *Pangasius* catfish (*Pangasianodon hypophthalmus*) farming in Vietnam.

2. Methodology

2.1. Antibiotic exposure assessment

Exposure concentration distributions were modelled for 12 antibiotics used in *Pangasius* catfish production, which include antibiotics that are listed as highly important for human medicine (Table 1). The distributions were conservatively based on maximum 18 h-average antibiotic exposure concentrations over the *Pangasius* grow-out period and were calculated for the pond

water (PWCs), the pore water in the pond sediment ($PSC_{pore\ water}$), the surface water in the effluent mixing area (EWCs), and the pore water in the sediment of the effluent mixing area ($ESC_{pore\ water}$). The time span of 18 h was chosen in order to match the exposure period used by the bacterial susceptibility tests (Andrews, 2001).

Table 1. List of antibiotics included in the present study.

Antibiotic name	Antibiotic group	Mode of action [a]	Resistance mechanism [a]	Use by catfish farmers (%) [b]	Important for human health? [c]
Amoxicillin	Penicillin	Cell wall synthesis	Reduced permeability/antibiotic inactivation	19	Yes
Ampicillin	Penicillin	Cell wall synthesis	Reduced permeability/antibiotic inactivation	3.1	Yes
Cephalexin	Cephalosporin	Cell wall synthesis	Alteration of target	16	No
Ciprofloxacin	Quinolone	DNA gyrase	Alteration of target	3.1	Yes
Colistin	Polymyxin	Cytoplasmatic membrane	Reduced permeability/pumping out of cell (efflux)	2.3	Yes
Doxycycline	Tetracycline	Protein synthesis (30S inhibitor)	Pumping out of cell (efflux)	34	No
Florfenicol	Amphenicol	Protein synthesis (50S inhibitor)	Antibiotic inactivation	63	No[d]
Kanamycin	Aminoglycoside	Protein synthesis (30S inhibitor)	Antibiotic inactivation	6.3	Yes
Levofloxacin	Quinolone	DNA gyrase	Alteration of target	3.1	Yes
Rifampicin	Rifamycin	DNA-directed RNA polymerase	Alteration of target	6.3	Yes
Sulfamethoxazole	Sulfonamide	Folic acid metabolism	Development of resistant biochemical pathway	44	No
Trimethoprim	Sulfonamide	Folic acid metabolism	Development of resistant biochemical pathway	44	No

[a] Madigan et al. (2003); [b] Rico et al. (2013b); [c] WHO (2012); [d] Veterinary use only.

Antibiotic exposure concentrations in the pond water (PWC), the effluent mixing point (EWC), and the pond sediment (PSC) and the environmental sediment (ESC) (sorbed fraction in dry weight basis) were calculated using the ERA-AQUA model (Rico et al., 2012 and 2013a). The ESC were modelled by implementing Equation 2 of the Supporting Information in the ERA-AQUA model, and assuming that concentration dynamics in the effluent mixing point are dominated by sorption/desorption and degradation processes. The model was run based on data on recommended antibiotic therapeutic dosages (Table S1), antibiotic physico-chemical properties (Table S2), and with the *Pangasius* grow-out pond scenario described in Table S3, which represents typical aquaculture management practices (see Rico and Van den Brink, 2014 for a detailed description). In order to account for the variability of the antibiotic exposure, 1000 model runs were performed with time steps of 10 min, based on 1000 Monte Carlo scenario samples obtained by varying the six most important scenario parameters highlighted by a previous sensitivity analysis (see Rico et al., 2013a). The variation of these six parameters (i.e., organic matter fraction, water temperature, fish stocking density, fish mortality fraction, percentage of water discharge per event, time interval between water discharge events, and duration of the effluent discharge event) was done by re-sampling the data distributions shown in Rico et al. (2014), which were generated with literature data or with survey data on aquaculture production characteristics. The exposure concentration in the pore water of the pond sediment compartment ($PSC_{pore\ water}$) and in the sediment of the effluent mixing area ($ESC_{pore\ water}$) were calculated based on the sorbed sediment concentrations provided by the ERA-AQUA model (PSC and ESC) and using Equation 2 of the Supporting Information, which assumes equilibrium conditions between the pore water concentration and the (sorbed) sediment concentrations.

Finally, a normal distribution was fitted to the log-transformed concentrations for PWCs, $PSC_{pore\ water}$, EWCs and $ESC_{pore\ water}$ (i.e., 1000 samples). The fitting was performed by maximum likelihood

estimation of the exposure distribution parameters (mean: μ_{logEC}, standard deviation: σ_{logEC}) and their 95% confidence intervals were calculated by parametric bootstrap with 1000 resamples of the original dataset using the @Risk 6.0 software (Palisade corporation, Ithaca, New York, USA).

2.2. Bacterial species sensitivity distributions

Minimum Inhibitory Concentration (MIC) distributions for each of the 12 antibiotics listed in Table 1 were obtained from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) MIC and zone diameter distribution website (EUCAST, 2010; Kahlmeter et al., 2003). Because the data generated by MIC tests is interval censored, we considered the antibiotic concentration immediately below the median as the conservative estimation of the MIC50 for each bacterial species. Antibiotic concentrations greater than or equal to the MIC50 are likely to inhibit approximately half of the wild-type population of any given bacterial taxon and will thus – assuming equal growth rates of non-resistant and resistant populations – favour an increase in the prevalence of resistance (Tello et al., 2012). SSDs were derived using MIC50 values from the MIC distributions of every bacterial taxa available for each antibiotic. To minimize the lack of independence between individual observations, MIC50 values were aggregated within bacterial genera. The rationale behind including every bacterial taxon – as opposed to only those for which there is evidence to suggest that they may grow in the environment – is that (1) studies have shown that predictions based on EUCAST MIC values are within the concentrations that negatively affected environmental bacteria (Singer et al., 2011), and (2) the magnitude of bacterial diversity suggests that the sensitivity distributions of many environments are likely to encompass the range of sensitivities represented in the EUCAST MIC dataset.

The MIC50 dataset used to derive the antibiotic SSDs consisted of 44 genera, including bacteria that are known to be widely distributed in the environment (e.g. *Pseudomonas* spp., *Acinetobacter* spp., *Burkholderia* spp.), and some genera that are amongst the most common aquaculture fish pathogens (e.g. *Escherichia* spp., *Salmonella* spp., *Streptococcus* spp.) (Table S4). SSDs were derived for each antibiotic by fitting a normal distribution to the log-transformed MIC50 values. The median hazardous concentration for the 5% (HC5) and 50% (HC50) of the bacterial genera, and their lower (95%) and upper (5%) confidence limits, were calculated according to Aldenberg and Jaworska (2000). The goodness of fit of the MIC50 data to the log-normal distribution were assessed by the Anderson–Darling and the Kolmogorov-Smirnov tests (α=0.05). The calculation of the SSD parameters (mean: μ_{logSS}, standard deviation: σ_{logSS}), the HC5 and HC50 values, and the goodness-of-fit tests were performed using the ETX 2.0 software (Van Vlaardingen et al., 2004). In order to compare the bacterial sensitivities to the different antibiotics, SSDs were built using molar-mass corrected log MIC50s. Statistical differences between the molar-mass corrected log MIC50 datasets for each antibiotic were assessed with the two-sided Kolmogorov-Smirnov test (α=0.05) using the GenStat 15th Edition software (VSN International Ltd, Hemel Hempstead, UK).

2.3. Linking antibiotic exposure to bacterial resistance

The link between antibiotic exposure and bacterial SSDs, and the calculation of the bacterial resistance risk were performed using probabilistic risk assessment. Probabilistic risk assessment is based on the estimation of the likelihood and extent of adverse environmental effects as a result of exposure to a substance by comparing an exposure concentration distribution with a SSD derived from a sample of toxicity data (Solomon et al., 2000). In probabilistic risk assessments the risk is usually represented as the overlap between the exposure concentration and the species sensitivity probability density functions, and such overlap is graphically represented by Joint Probability Curves (JPCs) (Fig. 1). The Area Under the Curve (AUC) of the JPC is used as a measure of risk, and can be defined as the risk of some log Exposure Concentration (EC) to exceed some log Species Sensitivity (SS) (Aldenberg et al., 2002; Fig. 1).

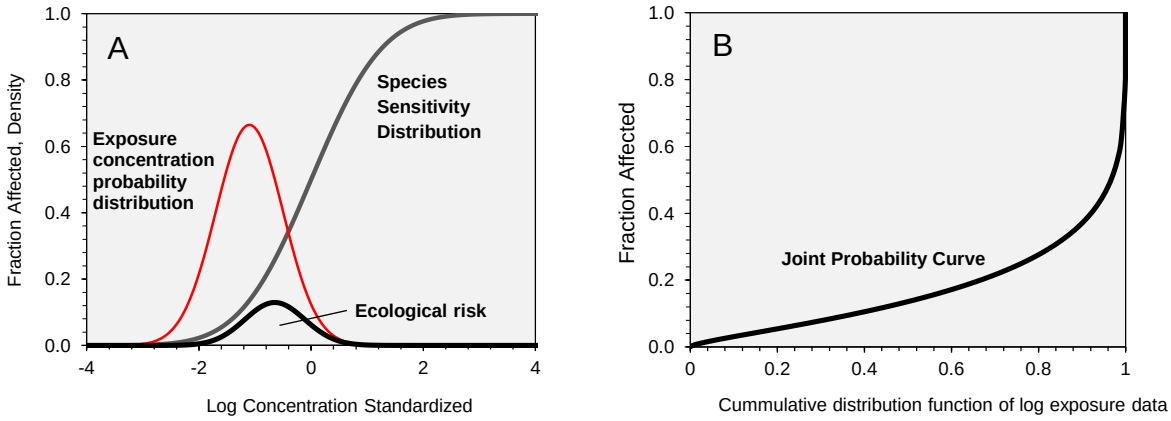


Figure 1. Graphical description of the fundamentals of probabilistic risk assessment. **A**. Example of normal log exposure concentration (EC) probability distribution function (scaled to the SSD), standard normal SSD cumulative distribution function, and calculated risk distribution. **B**. Corresponding Joint Probability Curve (JPC). The area under the curve of the JPC corresponds with the risk estimate.

In this study, we define the Resistance Development Risk (RDR) as the risk that the antibiotic exposure exerts a selective pressure favouring the development of AR, and can be calculated as the probability that the antibiotic exposure distribution exceeds the bacterial MIC50 SSD. In the case of two independent normal distributions, this probability (i.e., area under the curve of the JPC) can be calculated according to Equation 1 (Aldenberg et al., 2002).

$$p\,(\log \mathrm{EC} > \log \mathrm{SS}) = \emptyset_{0,1}\left(\frac{\mu_{\log EC} - \mu_{\log SS}}{\sqrt{\sigma^2_{\log EC} + \sigma^2_{\log SS}}}\right) \qquad \textit{Eq. 1}$$

where $\emptyset_{0,1}(x)$ is the cumulative distribution function of x with mean 0 and standard deviation 1.

The JPCs and the median RDRs were calculated for the 12 antibiotics applied in *Pangasius* catfish production in the four compartments (i.e., pond water, pond sediment, effluent discharge point and environmental sediment). The JPCs were calculated according to the methods described in Aldenberg et al. (2002), and the RDRs by using Equation 1. The AR risk for the bacterial community in each compartment was defined as high, moderate and low when the probability that some random selected exposure concentration resulting in AR was higher than 50% (RDR > 50%), between 5% and 50% (5% ≥ RDR ≥ 50%), and below 5% (RDR < 5%), respectively.

We complemented this assessment with a second estimate of the potential resistance risk posed by antibiotic exposure, which is more conservative than the described RDR. This second resistance risk estimate is based on wild-type cut-off values (CO_{WT}). CO_{WT} values separates wild-type (i.e., non-resistant) from resistant bacterial populations (Kahlmeter et al., 2003). Therefore, antibiotic concentrations above the CO_{WT} will increase the prevalence of resistance in the bacterial population to 100% (for rationale see Tello et al., 2012). We performed a direct comparison of the 50th and 90th percentiles of the antibiotic exposure concentrations in each compartment with the available wild-type cut-off values (CO_{WT}) of bacterial species (Table S5), and calculated the percentage of bacterial CO_{WT} values that were exceeded by each of these two exposure concentrations.

3. Results and discussion

3.1. Antibiotic exposure assessment

Modelled antibiotic exposure concentrations in the pond water, in the effluent mixing point and in the sediment of the effluent mixing area ranged from few to several hundred ppbs, whereas concentrations in pond sediments were found to fall in the low ppm range (Table S6). Uncertainty in the exposure modelling may arise from the use of antibiotic physico-chemical properties (e.g. organic-carbon sorption coefficient, water and sediment degradation rate) from QSARs when experimental data was not available (see Table S2). Furthermore, environmental sediment concentrations were calculated based on sediment sorption from the dissolved water concentrations at the effluent discharge area, whereas some studies have indicated that a large portion of antibiotics applied to *Pangasius* pond effluents are discharged into the environment attached to particulate organic matter and result in peak exposure concentrations several meters down-stream the effluent discharge point (Andrieu et al., Submitted). This is also supported by other studies, which also found higher antibiotic concentrations in sediment samples collected from aquaculture drainage systems than the ones we calculated in our study (Lalumera et al., 2004; Le and Munekage, 2004). Therefore, the environmental sediment concentration values we used for the risk assessment might not reflect the worst-case exposure scenarios for environmental sediments. The enrofloxacin concentrations monitored in *Pangasius* catfish pond effluents during medication (0.2-3.2 µg/L) appear to correspond reasonably well with the exposure concentration estimated for a similar compound, ciprofloxacin, in our study: 0.4 (0.1-1.4) µg/L (Table S6). Also, the environmental concentrations of antibiotics monitored in aquatic ecosystems exposed to aquaculture pollution that have been reported by other authors fall within the exposure concentration distributions calculated in our study (Managaki et al., 2007; Zou et al., 2011).

3.2. Bacterial resistance susceptibility

The bacterial genera included in the dataset showed a large variation in antibiotic susceptibility to the 12 antibiotics, with MIC50s ranging from 1 ppb (for *Clostridium* exposed to rifampicin) to 128,000 ppb (for *Enterobacter* exposed to amoxicillin). None of the bacterial genera were systematically found to be positioned in the lower tail of the SSDs, indicating that bacterial resistance susceptibilities vary between chemicals and are not consistent across antibiotic compounds, as was also shown by the SSD analysis performed by Tello et al. (2012). In addition, fish pathogenic bacterial genera (e.g. *Escherichia* spp*., Salmonella* spp*., Streptococcus* spp.) did not show a markedly lower susceptibility when compared to other genera, suggesting that the development of resistance in target pathogens is likely to be accompanied by resistance in non-target bacteria. The log-normal conformity tests (Kolmogorov-Smirnov and Anderson-Darling) of the SSDs were accepted for 6 out of the 12 evaluated antibiotics. However, we did not consider this as an obstacle to fit the data to the log-normal model. Instead, we assumed that the distribution of bacterial sensitivities is truly bell-shaped and that the failure of these statistical tests is an artifact caused by the double-dilution steps that are standardly used in the MIC tests (see Kahlmeter et al., 2003 and references therein). Calculated SSDs showed notable differences in the susceptibility of bacterial species as a whole to different antibiotics, with median HC50 values ranging from 23.3 ppb for rifampicin to 8000 ppb for sulfamethoxazole (Table S6). Figure 2 and the results of the two-sided Kolmogorov-Smirnov test (Table S7) show that, at the same exposure concentrations, the AR pressure exerted by antibiotics such as rifampicin, ciprofloxacin and levofloxacin, which act by inhibiting the synthesis of RNA or DNA in bacteria (Table 1), is considerably higher than that of the rest of the studied antibiotics. This indicates that resistance susceptibility of bacteria is largely conditioned by the antibiotic's mode of action, and that those antibiotics that directly affect gene replication seem to exert the highest resistance selective pressure.

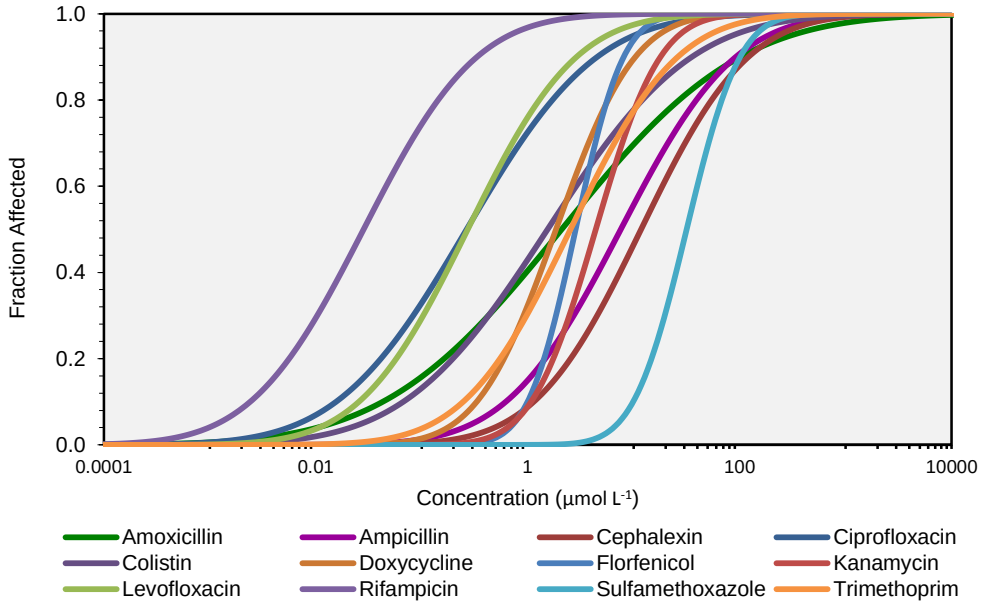


Figure 2. Bacterial MIC50 Species Sensitivity Distributions for the 12 evaluated antibiotics. The distribution parameters are provided in Table S6.

3.3. Antibiotic resistance risks

The highest RDRs were calculated for the pond sediment compartment. In this compartment, high risks were calculated for 5 antibiotics (kanamycin > levofloxacin > florfenicol > rifampicin > ampicillin), and moderate risks were calculated for 4 antibiotics (ciprofloxacin > cephalexin > amoxicillin > trimethoprim) (Table 2; Fig. 3). The median exposure concentrations calculated for the sediment compartment for kanamycin, levofloxacin, florfenicol, rifampicin, ampicillin and ciprofloxacin were found to exceed several CO_{WT} values. The percentages of exceedance ranged from 4% for ciprofloxacin to 100% for kanamycin. Moreover, percentages of exceedance were up to 100% for three antibiotics when the conservative 90th percentile of exposure was used (Table 3). In the pond water compartment, the majority of the antibiotics showed low RDR values, with the exception of rifampicin (RDR: 28%), levofloxacin (RDR: 17%) and amoxicillin (RDR: 12%), which showed moderate risks (Table 2; Fig. 3). In this compartment, the median exposure levels of the majority of the antibiotics did not exceed the CO_{WT} values, with the exception of rifampicin. However, the CO_{WT} values of 5-8% of bacteria were exceeded for three antibiotics when the 90th percentile of the exposure distribution was used (Table 3). The calculated RDR values for the evaluated antibiotics in environmental water and in environmental sediment were very low or insignificant (Table 2) and did not exceed any of the available bacterial CO_{WT} values (Table 3).

The application of our modelling approach to the *Pangasius* aquaculture scenario shows that antibiotic residues accumulated in pond sediments constitute a potential cause of antibiotic resistance genes in fish pathogenic bacteria and in other clinically relevant bacteria for most antibiotics. In addition, the residual concentrations of some antibiotics in pond water may be high enough to induce resistance in up to 20-30% of the exposed bacteria. High prevalence of (multiple) antimicrobial resistance has been monitored in fish samples collected from *Pangasius* catfish ponds and cages in the Mekong Delta (Sarter et al., 2007; Bartie et al., 2012), however, less effort has been put on monitoring resistance in the pond or in the surrounding environment of these farms. The increased levels of AR modelled in our study after antibiotic medication in intensive aquaculture production systems is supported by several AR monitoring studies (DePaola et al., 1995; Tendencia and De la Peña, 2001; Buschmann et al., 2012). These studies demonstrated prevalence of resistance genes in several pathogenic bacteria (e.g. *Vibrio* sp., *Aeromonas* sp.) isolated from samples collected in aquaculture farms and surrounding environments. A direct comparison of the findings of these studies with the results of our

modelling approach is hardly possible due to the differences in antibiotic use practices and monitored aquaculture species and environments, and due to other limitations inherent to the differences between the approach described here and the methods traditionally used in monitoring studies, which are discussed below.

Table 2. Calculated antibiotic Resistance Development Risks (%) associated with the pond water concentration, pond sediment concentration, and the antibiotic concentrations in water and sediment of the effluent mixing area.

	Resistance Development Risk			
	Pond water	**Pond sediment**	**Effluent mixing area (water)**	**Effluent mixing area (sediment)**
Amoxicillin	12.2	17.3	1.51	0.01
Ampicillin	2.72	55.1	0.04	<0.01
Cephalexin	2.77	29.9	0.03	<0.01
Ciprofloxacin	1.00	36.5	0.01	<0.01
Colistin	3.34	2.87	0.12	0.01
Doxycycline	0.08	0.64	<0.01	<0.01
Florfenicol	0.04	92.3	<0.01	<0.01
Kanamycin	0.46	99.4	<0.01	<0.01
Levofloxacin	17.2	95.3	0.66	0.08
Rifampicin	27.6	85.6	1.49	0.17
Sulfamethoxazole	0.00	0.06	<0.01	<0.01
Trimethoprim	2.01	13.9	0.02	<0.01

Table 3. Number of bacterial genera for which there are available CO_{WT} values (*n*), and percentage of exceedance of the bacterial CO_{WT} values calculated for the 50^{th} and 90^{th} percentiles of the antibiotic exposure distributions associated with pond water, pond sediment, and water and sediment of the effluent mixing area.

	n	**Pond water**		**Pond sediment**		**Effluent mixing area (water)**		**Effluent mixing area (sediment)**	
		50^{th}	**90^{th}**	**50^{th}**	**90^{th}**	**50^{th}**	**90^{th}**	**50^{th}**	**90^{th}**
Amoxicillin	18	0	0	0	17	0	0	0	0
Ampicillin	26	0	7.7	23	100	0	0	0	0
Cephalexin	8	0	0	0	0	0	0	0	0
Ciprofloxacin	52	0	0	4	17	0	0	0	0
Colistin	7	0	0	0	0	0	0	0	0
Doxycycline	20	0	0	0	0	0	0	0	0
Florfenicol	8	0	0	38	75	0	0	0	0
Kanamycin	2	0	0	100	100	0	0	0	0
Levofloxacin	44	0	4.5	95	100	0	0	0	0
Rifampicin	12	8.3	8.3	83	83	0	0	0	0
Sulfamethoxazole	2	0	0	0	0	0	0	0	0
Trimethoprim	7	0	0	0	0	0	0	0	0

3.4. Approach limitations and strengths

The framework we propose allows a standardized assessment of the likelihood that an antibiotic treatment may result in the development of antibiotic resistance by effectively linking two central factors influencing the selection of antibiotic resistance: exposure concentration and bacterial susceptibility. The processes involved in the selection, maintenance and spread of antibiotic resistance, however, are varied and complex (Martinez, 2008) and several of them are not accounted for in the methodology we describe e.g., horizontal gene transfer, co and cross-selection of antibiotic resistance genes (Miranda et al., 2013). Additionally, uncertainty in our results arises from the potential differences in antibiotic bioavailability between environmental compartments and the agar medium used in the MIC tests, and the extrapolation of *in vitro* data to the field. Furthermore, the representativity of MIC50 SSDs derived for clinically relevant bacteria to assess resistance in bacteria inhabiting aquatic environments has not been evaluated. In this respect, laboratory and semi-field experiments (e.g. microcosms) could be used to evaluate the predictions made by the bacterial MIC50 SSDs. For example, Stepanauskas et al. (2006) exposed different species of bacterioplankton to a gradient of ampicillin concentrations using freshwater microcosms and demonstrated that the 85% of the bacterial isolates exposed to a concentration of 10 mg/L acquired resistance after an incubation period of 7 days. The fraction of resistant bacteria corresponding to this concentration in the ampicillin MIC50 SSD used in this study is 75(62-85)% (median and 95% CI), suggesting that in this case the use of the SSD concept

with clinically relevant bacteria offers a reasonably good estimation. However, this requires further investigations. Another limitation of our modelling approach relates to the fact that, in the environment, bacteria are likely exposed to multiple antibiotics simultaneously, in conjunction with other stressors such as disinfectants or heavy metals that may significantly affect the selection of resistance (D'Costa et al., 2006; Stepanauskas et al., 2006). In this context, and regarding the potential emergence of multi-drug resistance and the mobility of resistance genes (Cabello et al., 2013), the environmental resistance risks may even be greater than the ones calculated here.

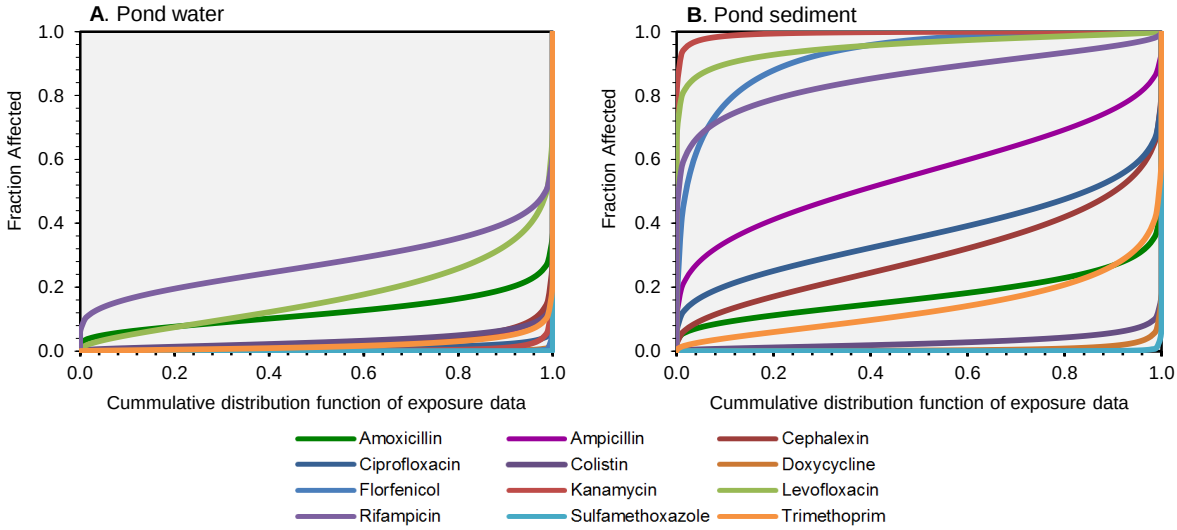


Figure 3. Joint Probability Curves for the pond water (**A**) and for the pond sediment (**B**) compartments built with the antibiotic exposure concentration distributions and the MIC50 Species Sensitivity Distributions. The closer to the upper left corner the curve is displayed, the higher the antibiotic resistance risk.

Despite these apparent caveats, however, the framework we propose offers the first link between antibiotic use practices, predicted exposure patterns, and resistance development risks in aquaculture. This framework overcomes two major limitations that are often discussed in AR monitoring studies. One of them is the difficulty to establish a causal relationship between certain antibiotic use practices and the levels of monitored antibiotic resistance, frequently hampered by the lack of information on the exposure history of the bacterial isolates obtained from water and sediment samples (Le et al., 2005; Buschmann et al., 2012). Another one is the narrow view obtained from monitoring studies, which are able to evaluate resistance on a small proportion of cultivable bacteria as compared to the actual diversity of environmental bacterial (Amann et al., 1995). We believe that the use of MIC50 SSDs based on data collated from thousands of worldwide sources, gives the framework a level of applicability, and our results a level of generalizability, that is hard to achieve by a single monitoring study.

One of the major strengths of the proposed framework is that it takes into account a wide range of environmental and aquaculture factors that influence antibiotic exposure, and ultimately affect antibiotic resistance. Such relationships allow the study and development of a range of broad-scale, as well as farm-scale, management options to minimize the risk of antibiotic resistance in aquaculture production. Regarding, for example, the calculated RDRs for antibiotics used in *Pangasius* catfish ponds, it can be concluded that risk managers should try to restrict the use of antibiotics for which the predicted RDRs are higher (e.g. kanamycin, levofloxacin; Table 2), and/or try to limit the use of antibiotics that are likely to result in a high resistance selective pressure for bacteria in the waters that are directly discharged into the environment from aquaculture facilities. At a farm-scale, the models and concepts used in this paper can be useful in evaluating potential impacts of different antibiotic administration regimes or aquaculture practices (e.g. water exchange, sediment removal). For instance, Figure 4 shows that the application of three consecutive florfenicol treatments (with two weeks interval) compared to one single treatment

might increase the risk of antibiotic resistance in pond sediments from 25% to more than 95%. Another advantage of this approach is the capacity to dynamically predict resistance pressure for non-target bacteria, as compared to the point measurements obtained from monitoring studies (Fig. 4). Thus, this approach can be used effectively to support decisions on relevant moments and compartments to be monitored in order to obtain worst-case estimates of resistance levels in the environment.

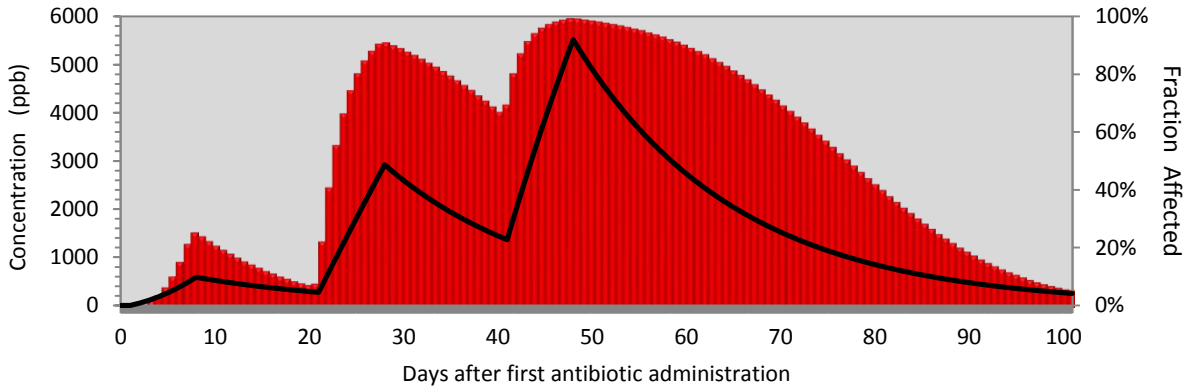


Figure 4. Concentration dynamics of florfenicol in the pond sediment compartment, $PSC_{pore\ water}$ (line), and corresponding affected fraction of bacteria for which the MIC50 is exceeded (bars). The simulation was performed with the ERA-AQUA model using the average *Pangasius* catfish scenario described in Table S3 and assuming that 3 florfenicol treatments are applied with a time interval of 2 weeks between treatments. Each florfenicol treatment consists of 7 in-feed applications of 10 mg/kg fish for 7 consecutive days.

4. Conclusions

The development of antibiotic resistance in aquaculture environments is an issue of concern, first and foremost, to aquaculture itself (Smith, 2012). Minimizing the development of resistance in bacterial fish pathogens is crucial for the effective treatment of diseases, and therefore essential for fish welfare and the economic viability of the majority of aquaculture enterprises. Moreover, concerns have been raised by multiple stakeholders regarding the potential human health risks associated with the use of antibiotics in aquaculture (FAO/OIE/WHO, 2006). Quantitative risk assessments on AR in aquaculture environments have been considered challenging due to the lack of data and methods to characterize antibiotic exposure (Miranda et al., 2013), to limitations regarding the extrapolation of *in vitro* bacterial susceptibility data to the field (Smith, 2012), and to the complexity of assessing gene flows between aquatic species and environmental compartments (Pruden et al., 2013). Despite these challenges the need to consider antibiotic resistance in the prospective risk assessment of human and veterinary antibiotics has received increasing support (Montforts, 2005; Boxall et al., 2012; Tello et al., 2012; Ashbolt et al., 2013). In this context, the development of methods to define exposure standards protective of background resistance levels and to assess antibiotic treatments regarding their resistance potential is urgently required.

The framework we propose effectively links antibiotic use to exposure and resistance. Its application to the *Pangasius* aquaculture production scenario has shown that most antibiotics, even when used according to recommendations, may increase the prevalence of AR in bacteria associated to aquaculture ponds. Furthermore, our modelling calculations have indicated that sediments of intensive aquaculture earthen ponds may constitute hotspots for antibiotic resistance development. The suitability of the RDR approach as a prospective tool in the initial phase of a risk assessment provides an efficient means to benchmark multiple antibiotics and treatment regimes with regards to their potential to develop resistance in different environmental compartments. Follow-up studies based on in-situ quantification of antibiotic residues and antibiotic resistance genes may then be used to validate, refine and – if necessary – complement management actions. Importantly, the framework we propose may be used as a

management tool to promote the prudent use of antibiotics in aquaculture and makes a step forward towards the determination of safer management practices and exposure levels. Furthermore, this framework, with the help of appropriate exposure modelling approaches, can also be used to quantify resistance development risks in other antibiotic pollution scenarios such as waste-water treatment plants, manure amended agricultural soils or livestock farms.

Supporting Information

The environmental sediment concentrations (ESC) were calculated by implementing Equation 1 in the ERA-AQUA model. The sediment-water partition coefficient (k_d) and degradation rate coefficient in sediment (k_{sed}) were calculated according to the methods described in Rico et al. (2012), assuming that the temperature and the sediment characteristics in the effluent discharge point were the same as in the aquaculture pond. In this study, the sediment desorption rate coefficient used in Equation 1 and also the one used by the ERA-AQUA model to calculate the pond sediment concentration (PSC) was set to 0.03 d^{-1} according to the average value reported by Birdwell et al. (2007), because the approach used by the ERA-AQUA model (Equation 2 of Birdwell et al., 2007) was found to overestimate desorption rate coefficients for antibiotics with low *Koc*.

$$\frac{\partial ESC}{\partial t} = [k_s \cdot (k_d \cdot EWC_{diss} - ESC)] - [k_{sed} \cdot ESC] \qquad \textit{Eq. 1}$$

ESC = antibiotic exposure sediment concentration in the effluent discharge point (µg kg^{-1} d.w.)
k_s = first-order desorption rate coefficient (d^{-1})
k_d = sediment-water partition coefficient (L kg^{-1})
EWC_{diss} = antibiotic dissolved concentration in water at the effluent discharge point (µg L^{-1})
k_{sed} = first-order degradation rate coefficient in sediment (d^{-1})

The pore water concentration in the pond ($PSC_{pore\ water}$) and in the environmental sediment ($ESC_{pore\ water}$) compartments were calculated based on the PSC and the ESC provided by the ERA-AQUA model and using Equation 2.

$$C_{pore\ water} = \frac{C_{sorbed}}{Koc \cdot foc} \qquad \textit{Eq. 2}$$

$C_{pore\ water}$ = calculated pore water concentration (µg/L): $PSC_{pore\ water}$ or $ESC_{pore\ water}$.
C_{sorbed} = calculated sediment concentration (µg/kg d.w.): PSC or ESC.
K_{oc} = organic carbon sorption coefficient of the antibiotic (L/kg)
f_{oc} = fraction of organic carbon in sediment (-)

Table S1. Antibiotic treatment dose and duration used in the exposure assessment. The data is based on the recommended antibiotic treatments reported by Rico et al. (2013a).

Antibiotic	Treatment dose and duration
Amoxicillin	50 mg/kg body weight, daily for 5 days
Ampicillin	50 mg/kg body weight, daily for 5 days
Cephalexin	50 mg/kg body weight, daily for 5 days
Ciprofloxacin	10 mg/kg body weight, daily for 7 days
Colistin	10 mg/kg body weight, daily for 7 days
Doxycycline	10 mg/kg body weight, daily for 5 days
Florfenicol	10 mg/kg body weight, daily for 7 days
Kanamycin	50 mg/kg body weight, daily for 7 days
Levofloxacin	10 mg/kg body weight, daily for 7 days
Rifampicin	10 mg/kg body weight, daily for 7 days
Sulfamethoxazole	50 mg/kg body weight, daily for 7 days
Trimethoprim	10 mg/kg body weight, daily for 7 days

Table S2. Physico-chemical and pharmacokinetic properties used to perform the ERA-AQUA model simulations. For data selection methods see Rico et al. (2014).

		Amoxicillin		Ampicillin		Cephalexin		Ciprofloxacin		Colistin		Doxycycline	
M	g/mol	3.65E+02	FOOTPRINT (2012)	3.49E+02	FOOTPRINT (2012)	3.47E+02	FOOTPRINT (2012)	3.31E+02	FOOTPRINT (2012)	8.13E+02	FOOTPRINT (2012)	4.44E+02	FOOTPRINT (2012)
k_{ow}	(-)	7.41E+00	FOOTPRINT (2012)	2.24E+01	FOOTPRINT (2012)	4.47E+00	FOOTPRINT (2012)	1.07E+01	FOOTPRINT (2012)	3.98E-03	FOOTPRINT (2012)	9.55E-01	FOOTPRINT (2012)
k_{oc}	L/kg	8.66E+02	FOOTPRINT (2012)	8.51E+01	Kim et al. (2009)	6.63E+02	US EPA (2012)	6.10E+03	FOOTPRINT (2012)	3.54E+04	FOOTPRINT (2012)	7.28E+03	FOOTPRINT (2012)
$SOL\ (T_{ref})$	mg/L	3.43E+03	FOOTPRINT (2012)	1.01E+04	FOOTPRINT (2012)	1.79E+03	FOOTPRINT (2012)	3.00E+03	FOOTPRINT (2012)	5.64E+03	FOOTPRINT (2012)	6.30E+02	FOOTPRINT (2012)
T_{refSOL}	°C	2.00E+01	FOOTPRINT (2012)	2.00E+01	FOOTPRINT (2012)	2.00E+01	FOOTPRINT (2012)	2.00E+01	FOOTPRINT (2012)	2.00E+01	FOOTPRINT (2012)	2.00E+01	FOOTPRINT (2012)
ΔH_{SOL}	J/mol	2.50E+04	Rico et al. (2012b)	2.50E+04	Rico et al. (2012b)	2.50E+04	Rico et al. (2012b)	2.50E+04	Rico et al. (2012b)	2.50E+04	Rico et al. (2012b)	2.50E+04	Rico et al. (2012b)
$VP(T_{ref})$	mPa	6.24E-12	FOOTPRINT (2012)	1.16E-14	FOOTPRINT (2012)	4.31E-10	FOOTPRINT (2012)	2.19E-07	FOOTPRINT (2012)	9.19E-20	FOOTPRINT (2012)	1.89E-18	FOOTPRINT (2012)
T_{refVP}	°C	2.50E+01	FOOTPRINT (2012)	2.50E+01	FOOTPRINT (2012)	2.50E+01	FOOTPRINT (2012)	2.50E+01	FOOTPRINT (2012)	2.50E+01	FOOTPRINT (2012)	2.50E+01	FOOTPRINT (2012)
ΔH_p	J/mol	1.14E+05	FOOTPRINT (2012)	1.05E+05	FOOTPRINT (2012)	9.70E+04	FOOTPRINT (2012)	9.15E+04	Rico et al. (2012b)	9.70E+04	Rico et al. (2012b)	1.06E+05	Rico et al. (2012b)
$DT50_{water}$	d	3.00E+00	Lee et al. (2008)	3.75E+01	US EPA (2012)	3.75E+01	US EPA (2012)	5.00E-02	Cardoza et al. (2004)	1.80E+02	US EPA (2012)	3.56E+00	Sanderson et al. (2005)
T_{refw}	°C	2.44E+01	Lee et al. (2008)	2.50E+01	US EPA (2012)	2.50E+01	US EPA (2012)	1.79E+01	Cardoza et al. (2004)	2.50E+01	US EPA (2012)	1.90E+01	Sanderson et al. (2005)
$DT50_{sediment}$	d	2.30E-01	FOOTPRINT (2012)	3.50E+00	Maki et al. (2006)	3.38E+02	US EPA (2012)	5.42E+02	US EPA (2012)	1.62E+03	US EPA (2012)	5.00E+00	Maki et al. (2006)
T_{refs}	°C	2.00E+01	FOOTPRINT (2012)	1.90E+01	Maki et al. (2006)	2.50E+01	US EPA (2012)	2.50E+01	US EPA (2012)	2.50E+01	US EPA (2012)	2.00E+01	Maki et al. (2006)
E	J/mol	6.54E+04	Rico et al. (2012b)	6.54E+04	Rico et al. (2012b)	6.54E+04	Rico et al. (2012b)	6.54E+04	Rico et al. (2012b)	6.54E+04	Rico et al. (2012b)	6.54E+04	Rico et al. (2012b)
Fish $BioT_{1/2(Mref,Tref)}$	d	1.79E+00	B	1.79E+00	B	1.13E+01	C	7.86E+00	A	1.13E+01	C	1.48E+01	A

		Florfenicol		Kanamycin		Levofloxacin		Rifampicin		Sulfamethoxazole		Trimethoprim	
M	g/mol	3.58E+02	FOOTPRINT (2012)	4.85E+02	FOOTPRINT (2012)	3.61E+02	FOOTPRINT (2012)	8.23E+02	US EPA (2012)	2.53E+02	PEIAR Database (2012)	2.90E+02	FOOTPRINT (2012)
k_{ow}	(-)	1.10E+00	Kim et al. (2009)	2.00E-07	FOOTPRINT (2012)	1.26E+02	FOOTPRINT (2012)	1.74E+04	US EPA (2012)	7.76E+01	Kim et al. (2009)	8.13E+00	FOOTPRINT (2012)
k_{oc}	L/kg	3.80E+01	FOOTPRINT (2012)	1.00E+01	US EPA (2012)	4.44E+01	US EPA (2012)	6.92E+02	US EPA (2012)	1.86E+03	Kim et al. (2009)	2.84E+03	FOOTPRINT (2012)
$SOL\ (T_{ref})$	mg/L	1.32E+03	FOOTPRINT (2012)	1.00E+06	FOOTPRINT (2012)	6.76E+05	US EPA (2012)	1.40E+03	US EPA (2012)	6.10E+02	FOOTPRINT (2012)	1.82E+04	FOOTPRINT (2012)
T_{refSOL}	°C	2.00E+01	FOOTPRINT (2012)	2.00E+01	FOOTPRINT (2012)	2.50E+01	US EPA (2012)	2.50E+01	US EPA (2012)	3.70E+01	FOOTPRINT (2012)	2.00E+01	FOOTPRINT (2012)
ΔH_{SOL}	J/mol	2.50E+04	Rico et al. (2012b)	2.50E+04	Rico et al. (2012b)	2.50E+04	Rico et al. (2012b)	2.50E+04	Rico et al. (2012b)	2.50E+04	Rico et al. (2012b)	2.50E+04	Rico et al. (2012b)
$VP(T_{ref})$	mPa	5.54E-20	FOOTPRINT (2012)	4.39E-18	FOOTPRINT (2012)	2.37E-17	US EPA (2012)	3.99E-38	US EPA (2012)	2.49E-13	FOOTPRINT (2012)	1.31E-03	FOOTPRINT (2012)
T_{refVP}	°C	2.50E+01	FOOTPRINT (2012)	2.50E+01	FOOTPRINT (2012)	2.50E+01	US EPA (2012)	2.50E+01	US EPA (2012)	2.50E+01	FOOTPRINT (2012)	2.50E+01	FOOTPRINT (2012)
ΔH_p	J/mol	9.63E+04	Rico et al. (2012b)	9.70E+04	Rico et al. (2012b)	9.01E+04	FOOTPRINT (2012)	9.70E+04	Rico et al. (2012b)	7.47E+04	FOOTPRINT (2012)	8.00E+04	Rico et al. (2012b)
$DT50_{water}$	d	3.45E+01	SPAH Schering-Plough Animal Health (2004)	8.70E+00	US EPA (2012)	6.00E+01	US EPA (2012)	1.80E+02	US EPA (2012)	1.90E+01	Lam et al. (2004)	5.70E+00	Lam et al. (2004)
T_{refw}	°C	2.20E+01	SPAH Schering-Plough Animal Health (2004)	2.50E+01	US EPA (2012)	2.50E+01	US EPA (2012)	2.50E+01	US EPA (2012)	2.00E+01	Lam et al. (2004)	2.00E+01	Lam et al. (2004)
$DT50_{sediment}$	d	2.72E+01	SPAH Schering-Plough Animal Health (2004)	7.79E+01	US EPA (2012)	5.42E+02	US EPA (2012)	1.62E+03	US EPA (2012)	2.00E+00	Liu et al. (2010)	1.10E+02	FOOTPRINT (2012)
T_{refs}	°C	2.20E+01	SPAH Schering-Plough Animal Health (2004)	2.50E+01	US EPA (2012)	2.50E+01	US EPA (2012)	2.50E+01	US EPA (2012)	2.50E+01	Liu et al. (2010)	2.00E+01	FOOTPRINT (2012)
E	J/mol	6.54E+04	Rico et al. (2012b)	6.54E+04	Rico et al. (2012b)	6.54E+04	Rico et al. (2012b)	6.54E+04	Rico et al. (2012b)	6.54E+04	Rico et al. (2012b)	6.54E+04	Rico et al. (2012b)
Fish $BioT_{1/2(Mref,Tref)}$	d	3.61E+00	A	1.13E+01	C	7.86E+00	A	1.13E+01	C	6.83E+00	A	1.22E+01	A

M: Relative molecular mass of the substance; k_{ow}: Octanol/water partition coefficient of the substance; k_{oc}: Sorption coefficient of the substance on organic carbon; $SOL\ (T_{ref})$: Solubility of the substance in water at reference temperature; T_{refSOL}: Reference temperature at which $SOL(T_{ref})$ was determined; ΔH_{SOL}: Enthalpy of dissolution; $VP(T_{ref})$: Saturated vapour pressure of the substance at reference temperature; T_{refVP}: Reference temperature at which VP(T_{ref}) was determined; ΔH_p: Enthalpy of vaporization; $DT50_{water}$: Half-life degradation of the substance in water; T_{refw}: Reference temperature at which $DT50_{water}$ was determined; $DT50_{sediment}$: Half-life degradation of the substance in sediment; T_{refs}: Reference temperature at which $DT50_{sediment}$ was determined; *E*: Molar Arrhenius activation energy; *Fish* $BioT_{1/2(Mref,Tref)}$: Biological half-life of the drug in the cultured species (fish) (Mref: 0.1 kg,Tref: 20°C), A = ninety percentile of the data distribution derived for the given chemical class in the aquaculture species group. More than 4 data points were available; B = maximum value for the specific chemical class in the aquaculture species group. Less than 4 data points were available; C = ninety percentile of the overall data distribution for all chemical classes for fish. For a detailed description of the pharmacokinetic data sources and rationale see Rico and Van den Brink (2014).

Table S3. Parameters of the aquaculture pond scenario used to perform the ERA-AQUA model simulations. For a description of the data sources see Rico and Van den Brink (2014).

Scenario Parameters	Value	Data source	Remarks
Duration of the culture cycle (d)	216	SEAT project database	Mean value of data distribution (*n* = 211)
Aquaculture pond			
Pond area (m^2)	4437	SEAT project database	Mean value of data distribution (*n* = 212)
Pond water depth (m)	4.08	SEAT project database	Mean value of data distribution (*n* = 212)
Mass concentration of suspended solids in pond water (mg/L)	63.0	Giang et al. (2008)	Based on a study on water quality conducted in 64 intensive catfish ponds in the Mekong Delta (Vietnam).
Mass fraction of organic matter in suspended solids (-)	0.5	Assumption	
Top sediment layer depth (m)	0.1	Assumption	
Mass fraction of organic matter in sediment (-)	0.09 ± 0.02	Boyd et al. (1994)	Based on a distribution built for organic carbon content with 358 soil samples from freshwater ponds. The value chosen was 5% of carbon content, based on the approximate 75th percentile of the distribution considering the high intensity of the Pangasius production. The standard deviation was chosen based on the organic content measured in shrimp ponds by Boyd et al. (1992) (mean: 0.0862; SD: 0.0228)
Sediment porosity (v/v)	0.51	Egna and Boyd (1997)	Based on the sediment porosity values proposed for a silty clay sediment texture by Egna and Boyd (1997)
Sediment bulk density (kg/L)	0.76	Boyd (1995)	Based on the averaged bulk density of the 0-15 cm top sediment layer of 22-year-old fish pond
Average water temperature (°C)	28.0 ± 4.2	WMO (2013)	Based on monthly averages for a 30-year period from the station of Ho Chi Min (mean: 28.0; SD: 4.2)
Average percolation rate (mm/d)	0.87	Nhan et al. (2008)	
Average rainfall rate (mm/d)	5.36	WMO (2013)	Based on monthly averages for a 30-year period from the station of Ho Chi Min
Average evaporation rate (mm/d)	4.50	Nhan et al. (2008)	
Cultured species characteristics			
Organism weight at stocking (g)	19.1	SEAT project database	Mean value of data distribution (*n* = 176)
Organism weight at harvest (g)	981	SEAT project database	Mean value of data distribution (*n* = 172)
Maximum organism weight (g)	1010	Assumption	Parameter calculated for the calibration of the growth sub-model of the ERA-AQUA model
Cultured species density at stocking (g/m^2)	792 ± 353	SEAT project database	Mean value and SD of data distribution (*n* = 200)
Lipid fraction of cultured organisms (-)	0.03	Ho and Paul (2009)	
Mortality fraction during the culture period (-)	0.24 ± 0.11	SEAT project database	Mean value and SD of data distribution (*n* = 181)
Feed input to the cultured species			
Daily specific feeding rate (SFR) (kg food/kg cultured species · d)	0.06	Phan et al. (2009)	
Organism's weight at which SFR was determined (g cultured species)	78.0	Phan et al. (2009)	
Lipid fraction of feed (-)	0.06	Boyd (1995)	
Feed conversion ratio in the cultured species (kg food/kg cultured species)	1.69	Phan et al. (2009)	
Fraction of eaten feed (-)	0.95	Boyd (1995)	
Effluent discharge management			
Water discharge per event (%)	36.2 ± 16.2	SEAT project database	Mean value and SD of data distribution (*n* = 199)
Time interval between discharge events (d)	1.00 ± 0.14	SEAT project database	Mean value and SD of data distribution (*n* = 211)
Duration of the effluent discharge event (h)	6.48 ± 3.05	SEAT project database	Mean value and SD of data distribution (*n* = 32)
Effluent dilution factor	10	Assumption	

Table S4. Bacterial genera MIC50 values (ppb) used to build the Species Sensitivity Distributions.

Bacterial genera	Amoxicillin	Ampicillin	Cephalexin	Ciprofloxacin	Colistin	Doxycycline	Florfenicol	Kanamycin	Levofloxacin	Rifampicin	Sulfamethoxazole	Trimethoprim
Acinetobacter	-	16000	-	125	375	64	-	-	95	-	-	4000
Alcaligenes	-	-	-	2000	-	-	-	-	-	-	-	-
Anaerobic cocci gram negative	32	-	-	-	-	-	-	-	-	-	-	-
Bacteroides	16000	16000	-	4000	-	-	-	-	1000	-	-	-
Bifidobacterium	-	125	-	-	-	-	-	-	-	-	-	-
Burkholderia	-	-	-	2000	-	-	-	-	1000	-	-	-
Campylobacter	3000	1750	-	64	-	2125	500	-	-	-	-	-
Chryseobacterium	-	-	-	1250	-	-	-	-	-	-	-	-
Citrobacter	64000	16000	-	16	125	1000	-	-	48	-	8000	125
Clostridium	266	-	-	250	-	-	-	-	-	1	-	-
Corynebacterium	-	-	-	16000	-	-	-	-	-	6	-	-
Enterobacter	128000	16000	16000	16	250	1000	-	1500	32	-	8000	250
Enterococcus	-	750	-	750	-	6000	1500	-	1500	500	-	-
Escherichia	4000	2000	2000	8	250	2000	2000	2000	16	-	8000	250
Fusobacterium	32	-	-	-	-	-	-	-	-	-	-	-
Hafnia	-	8000	-	16	-	-	-	-	-	-	-	-
Heamophilus	188	188	8000	6	-	-	-	-	8	125	-	64
Helicobacter	8	-	-	64	-	-	-	-	125	125	-	-
Klebsiella	64000	16000	2000	16	250	500	-	1000	32	-	16000	250
Kluyvera	-	6000	-	8	-	-	-	-	-	-	-	-
Legionella	1000	500	-	-	-	2000	-	-	4	8	-	-
Listeria	-	250	-	500	-	-	-	1000	500	-	16000	64
Mannheima	125	-	-	-	-	-	500	-	-	-	-	-
Mannheimia	-	-	-	-	-	-	-	-	-	-	-	-
Moraxella	8000	2000	-	16	-	250	-	-	16	-	-	8000
Morganella	64000	-	64000	8	64000	2000	-	-	32	-	-	2000
Mycobacterium	-	-	-	250	-	2000	-	1000	125	125	-	-
Neisseria	64	157	375	3	-	-	-	-	4	8	-	-
Pasteurella	125	125	-	8	-	125	250	4000	-	-	-	-
Peptostreptococcus	-	-	-	250	-	-	-	-	500	-	-	-
Porphyromonas	32	-	-	-	-	-	-	-	-	-	-	-
Prevotella	500	-	-	-	-	-	-	-	500	-	-	-
Propionibacterium	32	-	-	-	-	-	-	-	-	-	-	-
Proteus	500	16000	34000	16	64000	4000	-	4000	32	-	-	1500
Providencia	-	12000	-	512	-	-	-	-	-	-	-	-
Pseudomonas	-	-	-	95	500	-	-	32000	1125	-	-	-
Raoultella	-	8000	-	8	125	-	-	-	-	-	-	-
Salmonella	500	1000	-	16	500	1000	2000	1250	32	-	16000	250
Serratia	36000	16000	-	64	40000	4000	2000	-	125	-	-	3000
Shigella	-	16000	-	-	-	-	-	-	-	-	-	-
Staphylococcus	-	1000	1500	219	-	157	1500	1000	750	8	8000	32250
Stenotrophomonas	-	16000	-	1000	4000	1000	-	-	500	-	-	-
Streptococcus	16	79	500	500	-	95	-	-	500	16	1000	1063
Yersinia	-	32000	-	24	-	500	-	-	16	-	-	500

Table S5. CO_{WT} values (ppb) for the 12 evaluated antibiotics.

Bacteria species	Amoxicillin	Ampicillin	Cephalexin	Ciprofloxacin	Colistin	Doxycycline	Florfenicol	Kanamycin	Levofloxacin	Rifampicin	Sulfamethoxazole	Trimethoprim
Acinetobacter anitratus	-	-	-	1000	-	-	-	-	500	-	-	-
Acinetobacter baumannii	-	-	-	1000	-	-	-	-	500	-	-	-
Acinetobacter calcoaceticus	-	-	-	1000	-	-	-	-	-	-	-	-
Acinetobacter lwoffii	-	-	-	1000	-	-	-	-	500	-	-	-
Acinetobacter spp	-	-	-	1000	-	-	-	-	500	-	-	-
Campylobacter coli	8000	8000	-	500	-	1000	4000	-	-	-	-	-
Campylobacter jejuni	16000	8000	-	500	-	500	4000	-	-	-	-	-
Citrobacter freundii	-	8000	-	-	-	8000	-	-	-	-	-	-
Citrobacter spp	-	-	-	125	-	-	-	-	125	-	-	-
Clostridium difficile	-	-	-	-	-	-	-	-	-	4	-	-
Enterobacter aerogenes	-	-	-	125	2000	8000	-	-	-	-	-	-
Enterobacter cloacae	-	-	-	125	2000	8000	-	-	-	-	-	-
Enterobacter spp	-	-	16000	125	-	8000	-	-	250	-	-	-
Enterococcus avium	-	4000	-	-	-	-	-	-	-	-	-	-
Enterococcus casseliflavus	-	4000	-	-	-	-	-	-	-	-	-	-
Enterococcus faecalis	-	4000	-	4000	-	500	8000	-	4000	4000	-	-
Enterococcus faecium	-	4000	-	4000	-	500	8000	-	4000	-	-	-
Enterococcus gallinarum	-	4000	-	-	-	-	-	-	-	-	-	-
Escherichia coli	8000	8000	16000	64	2000	4000	16000	8000	250	-	64000	2000
Haemophilus influenzae	2000	1000	64000	64	-	-	-	-	64	1000	-	500
Haemophilus parainfluenzae	1000	-	-	64	-	-	-	-	64	-	-	-
Hafnia alvei	-	-	-	125	-	-	-	-	-	-	-	-
Helicobacter pylori	125	-	-	500	-	-	-	-	500	-	-	-
Klebsiella oxytoca	-	8000	-	125	2000	4000	-	-	250	-	-	-
Klebsiella pneumoniae	-	8000	-	125	2000	4000	-	-	250	-	-	-
Klebsiella spp	-	8000	16000	125	-	-	-	-	250	-	-	-
Listeria monocytogenes	-	1000	-	-	-	-	-	-	-	-	-	-
Mannheimia haemolytica	500	-	-	-	-	-	-	-	-	-	-	-
Moraxella catarrhalis	125	125	-	125	-	1000	-	-	125	-	-	-
Morganella morganii	8000	-	16000	125	-	8000	-	-	250	-	-	-
Neisseria gonorrhoeae	-	-	-	16	-	-	-	-	16	-	-	-
Neisseria meningitidis	-	-	-	16	-	-	-	-	32	-	-	-
Pasteurella multocida	1000	1000	-	64	-	1000	1000	-	-	-	-	-
Proteus mirabilis	2000	8000	16000	64	-	-	-	-	250	-	-	-
Proteus spp	-	-	-	-	-	-	-	-	250	-	-	-
Proteus vulgaris	-	-	16000	64	-	-	-	-	250	-	-	-
Pseudomonas aeruginosa	-	-	-	500	4000	-	-	-	2000	-	-	-
Salmonella enteritidis	-	8000	-	-	-	-	-	-	-	-	-	-
Salmonella paratyphi	-	8000	-	-	-	-	-	-	-	-	-	-
Salmonella spp	4000	8000	-	-	2000	8000	16000	-	250	-	-	2000
Salmonella typhi	-	8000	-	-	-	-	-	-	-	-	-	-
Salmonella typhimurium	-	8000	-	-	-	-	-	-	-	-	-	-
Serratia spp	-	-	-	64	-	-	-	-	500	-	-	-
Shigella flexneri	-	8000	-	-	-	-	-	-	-	-	-	-
Shigella sonnei	-	8000	-	-	-	-	-	-	-	-	-	-
Staphylococcus aureus	-	-	8000	1000	-	500	8000	8000	1000	32	128000	2000
Staphylococcus aureus MRSA	-	-	-	-	-	-	-	-	-	32	-	2000

Table S5. Continued.

Bacteria species	Amoxicillin	Ampicillin	Cephalexin	Ciprofloxacin	Colistin	Doxycycline	Florfenicol	Kanamycin	Levofloxacin	Rifampicin	Sulfamethoxazole	Trimethoprim
Acinetobacter anitratus	-	-	-	1000	-	-	-	-	500	-	-	-
Staphylococcus capitis	-	-	-	1000	-	-	-	-	500	-	-	-
Staphylococcus coagulase negative	-	-	-	1000	-	-	-	-	500	64	-	-
Staphylococcus coagulase negative MRSE	-	-	-	-	-	-	-	-	-	64	-	-
Staphylococcus cohnii	-	-	-	1000	-	-	-	-	-	-	-	-
Staphylococcus epidermidis	-	-	-	1000	-	-	-	-	500	64	-	-
Staphylococcus epidermidis MSSE	-	-	-	-	-	-	-	-	-	64	-	-
Staphylococcus haemolyticus	-	-	-	1000	-	-	-	-	500	64	-	-
Staphylococcus hominis	-	-	-	1000	-	-	-	-	500	-	-	-
Staphylococcus intermedius	-	-	-	1000	-	-	-	-	-	-	-	-
Staphylococcus lugdunensis	-	-	-	1000	-	-	-	-	-	-	-	-
Staphylococcus saprophyticus	-	-	-	1000	-	-	-	-	500	-	-	-
Staphylococcus sciuri	-	-	-	1000	-	-	-	-	-	-	-	-
Staphylococcus simulans	-	-	-	1000	-	-	-	-	-	-	-	-
Staphylococcus warneri	-	-	-	1000	-	-	-	-	500	-	-	-
Stenotrophomonas maltophilia	-	-	-	-	-	8000	-	-	-	-	-	-
Streptococcus agalactiae	250	250	-	2000	-	-	-	-	2000	-	-	-
Streptococcus anginosus	250	-	-	-	-	-	-	-	-	-	-	-
Streptococcus bovis	-	-	-	-	-	-	-	-	2000	-	-	-
Streptococcus group C	-	-	-	2000	-	-	-	-	1000	-	-	-
Streptococcus group G	64	-	-	2000	-	-	-	-	1000	-	-	-
Streptococcus intermedius	-	-	-	2000	-	-	-	-	-	-	-	-
Streptococcus milleri	-	-	-	2000	-	-	-	-	-	-	-	-
Streptococcus mitis	-	-	-	4000	-	-	-	-	2000	-	-	-
Streptococcus oralis	125	-	-	-	-	-	-	-	2000	-	-	-
Streptococcus pneumoniae	64	64	-	2000	-	500	-	-	2000	125	-	-
Streptococcus pyogenes	64	-	-	1000	-	500	-	-	2000	-	-	-
Streptococcus sanguinis	-	-	-	-	-	-	-	-	2000	-	-	-
Streptococcus, viridans group	-	-	-	-	-	-	-	-	2000	-	-	-
Yersinia enterocolitica	-	-	-	250	-	2000	-	-	125	-	-	4000

Table S6. Calculated parameters of the antibiotic exposure distributions and bacterial Species Sensitivity Distributions. Concentrations are expressed in ppbs with their 95% confidence intervals between brackets. The PSC and the ESC refer here to (sorbed) sediment concentrations in µg/kg d.w.

	Amoxicillin	Ampicillin	Cephalexin	Ciprofloxacin	Colistin	Doxycycline	Florfenicol	Kanamycin	Levofloxacin	Rifampicin	Sulfamethoxazole	Trimethoprim
Exposure concentration distributions												
PWC												
μ_{logEC}	1.30 (1.27-1.32)	1.65 (1.62-1.67)	1.92 (1.90-1.95)	-0.43(-0.45--0.41)	1.05 (1.02-1.07)	0.76 (0.73-0.78)	1.26 (1.24-1.29)	1.76 (1.74-1.79)	1.15 (1.13-1.18)	0.85 (0.84-0.87)	1.64 (1.61-1.66)	1.09 (1.07-1.11)
σ_{logEC}	0.35 (0.34-0.37)	0.37 (0.35-0.38)	0.39 (0.37-0.40)	0.34 (0.32-0.36)	0.35 (0.34-0.37)	0.36 (0.34-0.37)	0.38 (0.37-0.40)	0.36 (0.35-0.38)	0.38 (0.36-0.40)	0.24 (0.23-0.25)	0.34 (0.32-0.35)	0.35 (0.34-0.37)
50th percentile	19.8 (18.8-20.8)	44.2 (41.9-46.6)	84.0 (79.3-88.7)	0.37 (0.35-0.39)	11.1 (10.6-11.7)	5.70 (5.41-5.99)	18.4 (17.4-19.4)	58.0 (55.0-61.1)	14.3 (13.5-15.1)	7.13 (6.89-7.38)	43.3 (41.2-45.4)	12.3 (11.7-12.9)
90th percentile	56.1 (53.3-59.0)	131 (124-138)	263 (245-279)	1.02 (0.97-1.07)	31.5 (30.0-33.1)	16.26 (15.43-17.11)	56.9 (53.9-60.2)	170 (161-179)	43.6 (41.2-46.0)	14.2 (13.9-14.9)	117 (111-123)	35.0 (33.2-36.7)
PSC												
μ_{logEC}	3.21 (3.19-3.24)	4.15 (4.13-4.18)	4.66 (4.64-4.68)	4.06 (4.04-4.08)	4.20 (4.18-4.22)	3.75 (3.73-3.77)	3.97 (3.95-3.99)	4.49 (4.47-4.51)	3.79 (3.76-3.81)	3.86 (3.83-3.88)	4.14 (4.12-4.16)	4.07 (4.04-4.09)
σ_{logEC}	0.35 (0.34-0.37)	0.34 (0.33-0.36)	0.33 (0.32-0.35)	0.33 (0.32-0.34)	0.33 (0.32-0.35)	0.34 (0.33-0.36)	0.33 (0.31-0.34)	0.33 (0.32-0.35)	0.33 (0.32-0.35)	0.33 (0.31-0.34)	0.33 (0.31-0.34)	0.33 (0.31-0.34)
50th percentile	1637 (1555-1722)	14285 (13586-15007)	45772 (43591-48017)	11500 (10957-12055)	15959 (15198-16738)	5580 (5310-5858)	9313 (8877-9761)	30697 (29241-32196)	6102 (5813-6399)	7161 (6826-7506)	13852 (13210-14514)	11614 (11069-12176)
90th percentile	4455 (4221-4897)	39423 (37493-41414)	122859 (117005-128886)	30478 (29039-91951)	42722 (40687-44808)	15247 (14507-16007)	24508 (23363-25687)	81935 (78049-85935)	16279 (15507-17069)	18868 (17986-19776)	36270 (34589-38005)	30737 (29292-32222)
EWC												
μ_{logEC}	-0.11 (-0.13--0.08)	0.22 (0.19-0.25)	0.48 (0.45-0.51)	-2.08 (-2.11--2.05)	-0.39 (-0.42--0.37)	-0.67 (-0.69--0.64)	-0.17 (-0.20--0.15)	0.33 (0.31-0.36)	-0.28 (-0.31--0.25)	-0.57 (-0.59--0.55)	0.22 (0.19-0.24)	-0.35 (-0.37--0.32)
σ_{logEC}	0.41 (0.39-0.42)	0.43 (0.41-0.45)	0.45 (0.43-0.47)	0.45 (0.43-0.47)	0.43 (0.41-0.45)	0.43 (0.41-0.44)	0.44 (0.42-0.46)	0.42 (0.40-0.44)	0.44 (0.42-0.46)	0.32 (0.30-0.33)	0.41 (0.39-0.43)	0.42 (0.40-0.44)
50th percentile	0.78 (0.74-0.83)	1.66 (1.56-1.77)	3.03 (2.84-3.23)	0.01 (0.01-0.01)	0.40 (0.38-0.43)	0.22 (0.20-0.23)	0.67 (0.63-0.71)	2.15 (2.03-2.29)	0.53 (0.49-0.56)	0.27 (0.26-0.28)	1.65 (1.55-1.75)	0.45 (0.42-0.48)
90th percentile	2.61 (2.45-2.76)	5.92 (5.56-6.30)	11.4 (10.6-12.1)	0.03 (0.03-0.03)	1.45 (1.36-1.54)	0.76 (0.71-0.81)	2.45 (2.30-2.61)	7.50 (7.05-7.97)	1.95 (1.83-2.08)	0.68 (0.65-0.72)	5.53 (5.21-5.87)	1.57 (1.48-1.67)
ESC												
μ_{logEC}	-0.55 (-0.58--0.53)	-0.50 (-0.53--0.47)	1.35 (1.32-1.38)	-0.37 (-0.40--0.34)	2.11 (2.08-2.13)	0.52 (0.49-0.55)	-0.75 (-0.78--0.72)	-0.73 (-0.76--0.70)	-0.57 (-0.60--0.54)	0.26 (0.24-0.28)	0.72 (0.69-0.75)	1.02 (0.99-1.05)
σ_{logEC}	0.41 (0.39-0.43)	0.44 (0.42-0.46)	0.46 (0.44-0.48)	0.49 (0.47-0.51)	0.44 (0.42-0.46)	0.45 (0.43-0.47)	0.46 (0.44-0.48)	0.45 (0.43-0.47)	0.47 (0.45-0.49)	0.35 (0.33-0.36)	0.41 (0.40-0.43)	0.45 (0.43-0.47)
50th percentile	0.28 (0.26-0.30)	0.32 (0.30-0.34)	22.4 (20.9-23.9)	0.40 (0.40-0.46)	127 (120-136)	3.32 (3.11-3.54)	0.18 (0.17-0.19)	0.19 (0.17-0.20)	0.27 (0.25-0.29)	1.83 (1.74-1.92)	5.26 (4.95-5.58)	10.5 (9.82-11.18)
90th percentile	0.94 (0.89-1.00)	1.16 (1.09-1.24)	87.3 (81.6-93.3)	1.79 (1.67-1.92)	463 (435-493)	12.5 (11.7-13.3)	0.69 (0.64-0.74)	0.71 (0.67-0.76)	1.07 (1.00-1.15)	5.10 (4.85-5.36)	17.8 (16.8-18.9)	39.7 (37.2-42.3)
Species sensitivity distributions												
Number of genera	24	27	9	34	12	19	8	10	26	10	8	15
μ_{logSS}	2.88 (2.42-3.34)	3.42 (3.14-3.70)	3.61 (3.12-4.10)	1.94 (1.66-2.22)	3.10 (2.55-3.65)	2.91 (2.68-3.15)	3.00 (2.77-3.24)	3.33 (3.05-3.60)	1.99 (1.72-2.26)	1.37 (0.88-1.85)	3.90 (3.64-4.17)	2.87 (2.51-3.23)
σ_{logSS}	1.33 (0.95-1.72)	0.85 (0.62-1.09)	0.79 (0.43-1.18)	0.96 (0.73-1.19)	1.06 (0.63-1.51)	0.58 (0.39-0.78)	0.35 (0.17-0.54)	0.48 (0.26-0.71)	0.80 (0.58-1.03)	0.83 (0.46-1.23)	0.39 (0.19-0.60)	0.79 (0.51-1.08)
median HC5	4.87 (0.69-19.4)	103 (32.1-239)	182 (16.4-673)	2.23 (0.71-5.27)	20.0 (1.55-93.1)	86.6 (31.6-172)	250 (75.5-465)	329 (87.4-698)	4.55 (1.47-10.2)	0.89 (0.09-3.31)	1681 (443-3353)	34.5 (6.90-96.8)
median HC50	764 (264-2207)	2639 (1391-5006)	4080 (1320-12611)	87.9 (46.2-167)	1253 (352-4455)	822 (481-1404)	1015 (588-1752)	2130 (1127-4023)	98.2 (52.9-182)	23.3 (7.67-71.1)	8000 (4356-14692)	735 (321-1681)
Kolmogorov Smirnov Test	Accepted	Rejected	Accepted	Rejected	Rejected	Accepted	Rejected	Accepted	Rejected	Accepted	Rejected	Accepted
Anderson Darling Test	Accepted	Rejected	Accepted	Rejected	Rejected	Accepted	Rejected	Rejected	Accepted	Accepted	Rejected	Accepted

Table S7. Results of the two-sided Kolmogorov-Smirnov test (α=0.05) performed to compare bacterial MIC50 species sensitivity distribution between antibiotics. The results are expressed as *p*-values. A *p*-value below 0.05 (marked in bold) denotes statistical differences between the evaluated distributions.

	Amoxicillin	**Ampicillin**	**Cephalexin**	**Ciprofloxacin**	**Colistin**	**Doxycycline**	**Florfenicol**	**Kanamycin**	**Levofloxacin**	**Rifampicin**	**Sulfamethoxazole**	**Trimethoprim**
Amoxicillin												
Ampicillin	**0.036**											
Cephalexin	0.054	0.310										
Ciprofloxacin	**0.027**	**<0.001**	**<0.001**									
Colistin	0.256	**0.038**	**0.010**	**0.007**								
Doxycycline	0.165	**0.002**	**0.042**	**0.004**	0.091							
Florfenicol	0.080	**0.013**	**0.023**	**0.007**	0.060	0.460						
Kanamycin	**0.008**	0.078	0.259	**<0.001**	**0.008**	0.169	0.287					
Levofloxacin	**0.030**	**<0.001**	**<0.001**	0.271	**0.030**	**0.007**	**0.003**	**<0.001**				
Rifampicin	**0.005**	**<0.001**	**<0.001**	0.091	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.034**			
Sulfamethoxazole	**0.009**	0.067	0.083	**<0.001**	**0.014**	**<0.001**	**0.002**	**0.005**	**<0.001**	**<0.001**		
Trimethoprim	0.208	0.057	0.086	**0.003**	0.301	0.464	0.296	**0.033**	**0.012**	**<0.001**	**<0.001**	

Chapter 10

General discussion and conclusions

With the increasing worldwide demand for seafood products and the decline of fishery stocks (Naylor et al., 2000), Asian aquaculture has been immersed into decades of fast-paced growth (Bostock et al., 2010). The growth and expansion of the Asian aquaculture sector is accompanied by an increasing demand to produce new species and to intensify its production. In a context of production diversification and intensification, aquatic disease management has become one of the major challenges to the development of the industry, and chemotherapy has played a fundamental role in reducing mortality rates and in maintaining a good health status in the cultured animals (Bondad-Reantaso et al., 2005).

Residues of veterinary medicines applied in aquaculture production may enter the environment by several routes including effluent discharges, leaching from medicated feeds, excretion from treated animals (Boxall et al., 2004; Kümerer, 2009), and due to inappropriate disposal of chemical containers. Environmental pollution with aquaculture medicines is often regarded by Asian producers as a less relevant issue compared to other negative problems such as food safety issues, occupational health hazards or the development of antimicrobial resistance (e.g. Somga et al., 2012; Yuan and Chen, 2012). However, environmental contamination with veterinary medicines may lead to biodiversity loss and may be the cause of water quality problems that may in turn lead to increased animal mortalities and unaffordable economic losses. Research dedicated to assess and to minimize their environmental risks is urgently required in order to support the sustainability of Asian aquaculture production, and to protect biodiversity and important ecosystem services provided by surrounding aquatic ecosystems (e.g. organic matter decomposition, nutrient cycling).

The Environmental Risk Assessment (ERA) of aquaculture medicines is a rather unexplored field that has hardly looked beyond the environmental impacts caused by some chemotherapeutic agents applied in the salmon industry (e.g. Haya et al., 2005; Burridge et al., 2010). The general aim of this thesis was to increase our understanding on the potential environmental risks posed by the use of aquaculture medicines in Asia. Building the foundations of this research area for the Asian continent required a first evaluation of local chemical use and production practices as well as the development of basic tools to facilitate preliminary risk assessments. The work carried out within this thesis has been pioneer in assessing the ecological risks of aquaculture medicines used in a selected group of species and countries with major export potential, and in monitoring exposure and effects of common aquaculture antibiotics for tropical aquatic ecosystems. However, given the diversity and the extension of the Asian aquaculture production sector, it is evident that much still remains to be done. This section discusses the results of this thesis in light of the research objectives proposed in **Chapter 1**, and highlights further research needs to improve the scientific knowledge and methods that underpin the ERA of veterinary medicines in Asian aquaculture.

1. Environmental exposure to veterinary medicines applied in Asian aquaculture

Because the majority of Asian aquaculture is produced in ponds and freshwater cages (Bostock et al., 2010), this thesis focused on the assessment of chemical exposure in these two production

systems. This section provides an overview of the exposure patterns observed in water and sediment.

As expected, **Chapter 7** showed that peak exposure concentrations of the antibiotic enrofloxacin applied to a *Pangasius* catfish pond occurred at the effluent discharge point. Concentrations measured few meters down-stream the discharge point dropped significantly to undetected levels, suggesting that worst-case exposure only occurs in small areas and that dilution immediately reduces exposure concentrations (**Chapter 7**). Magnitude and duration of the exposure in effluent discharge points largely depends on differences in dosage and treatment duration, water exchange practices, production and species characteristics, and dissipation rates of chemotherapeutants in aquaculture ponds (**Chapter 5**). Antibiotics applied mixed with feed to cage-based farms follow a similar exposure pattern as the one found in pond systems. This is characterized by a peak water concentration caused by leaching from feeds during (or shortly after) administration, and a subsequent drop, with as many peaks as antibiotic applications (**Chapter 6**). It should be noted that Asian aquatic ecosystems impacted by aquaculture generally show a high concentration of suspended organic material (**Chapter 6**), and therefore chemical's bioavailability to most aquatic organisms is expected to be lower than in undisturbed ecosystems, except for filter-feeders and benthic orgnanisms.

Different exposure patterns were observed for the sediments in the proximity of aquaculture ponds and cages. Whereas enrofloxacin residues discharged by *Pangasius* catfish effluents were found to increase down-stream the effluent discharge point up to a distance of 100 m (**Chapter 7**), residual concentrations of oxytetracycline and enrofloxacin applied to tilapia cage farms were measured at highest concentrations in sediments right underneath the cages and decreased in the down-stream areas (**Chapter 6**). Differences in accumulation patterns can be explained by the hydrologic conditions of the system, i.e. settling of particulate material downstream the effluent discharge point propitiated by decreased water flow velocity. In both studies, antibiotic accumulation seemed to be caused by the settling of faeces and particulate material (feed waste) onto the sediment, and antibiotics were spread along large sediment areas (> 100 m down-stream the farm). The sediment exposure concentrations revealed by these studies were considerably high (up to 10-20% of the therapeutic dose), and were found to persist for weeks to months (**Chapter 6**).

In conclusion, the environmental exposure pattern of aquaculture medicines in tropical aquatic ecosystems of Asia largely varies among production systems and characteristics of the chemotherapeutic treatment. Overall, medical disinfectants (e.g. benzalkonium chloride, glutaraldehyde, formaldehyde) and some ecto-parasiticides (trichlorfon, deltamethrin), which are principally applied in ponds, result in short-term water exposure since their treatment durations are shorter (1 to 3 applications) and normally have a higher solubility and lower organic matter sorption coefficient than other substances such as antibiotics (**Chapters 4** and **5**). Exposure to antibiotics is generally short-term in water (i.e. characterized by several short-lasting peaks), and longer-term and generally higher for sediments (especially for fluoroquinolones and tetracyclines). Consequently, for antibiotics and other hydrophobic substances (e.g. ivermectin), chemical monitoring studies should include collection of sediment samples in the proximity of cage farms or several meters downstream pond effluent discharge points (**Chapters 6** and **7**).

2. Modelling environmental exposure to aquaculture medicines: what are the next steps?

Monitoring chemical exposure in aquaculture producing regions of Asia is often limited by economic, technical or logistic reasons. The use of models has proven to be useful in assessing the environmental discharge and exposure of aquaculture medicines within this thesis (**Chapters 4, 5** and **9**), and elsewhere (Rose and Pedersen, 2005; Boxall et al., 2006a; Metcalfe et al., 2009). Risk

assessment models, such as the ERA-AQUA model (Rico et al. 2012b; **Chapter 4**), are generally based on theoretical assumptions, and therefore testing their accuracy under field conditions is a critical but most needed step in order to increase their trust, as well as to refine their underlying processes and equations (**Chapter 4**). Within this thesis, a preliminary evaluation of the concentration dynamics predicted by the ERA-AQUA model was performed. This evaluation was carried out in a commercial *Pangasius* catfish grow-out pond (described in **Chapter 7**) that had received a treatment with the antibiotic enrofloxacin, administered at a dose of 10 mg/kg fish for 5 consecutive days. During the field trial, data on environmental parameters (e.g. rainfall, temperature), aquaculture management practices (e.g. water replacement, feeding), and fish population dynamics (e.g. growth, mortality) were recorded. Furthermore, water and sediment samples were collected to analytically verify enrofloxacin concentrations, and additional samples were taken to measure water and sediment quality parameters (e.g. suspended solids, fraction of organic matter). The results of this preliminary investigation suggest that the model reflects antibiotic dynamics in the evaluated pond compartments reasonably well (Fig. 1). Accuracy of the model predictions was somehow acceptable, but not optimal. Measured water concentrations were generally within two-fold measured concentrations, and measured sediment concentrations were slightly underestimated by the model (Fig. 1). It should be noted, however, that enrofloxacin residues were already detected in the water and sediment samples collected before the start of the treatment. It could not be determined whether these contamination levels originated from residues of previous antibiotic administrations or whether they could be caused by irrigation from surrounding aquaculture ponds. A possible factor influencing the overestimation of water concentrations and the underestimation of sediment concentrations could be related to the use of an enrofloxacin organic-carbon sorption coefficient from a study in which sediment characteristics (e.g. texture, pH) were slightly different to the ones measured in the aquaculture pond. A lesson learned from this experiment was that the evaluation of the ERA-AQUA model predictions should preferably be carried out in smaller scale experimental ponds with a more precise control of the dosing and environmental conditions, and also assuring no previous use of the studied substance or pond contamination before and during treatment. In any case, for a more widespread use of the risk assessment models such as the ERA-AQUA (e.g. in regulatory risk assessment) and for detecting potential improvements, it is evident that more field evaluations are required. These should also take into account different compounds and aquaculture species such as shrimps, for which different chemical uptake routes are anticipated.

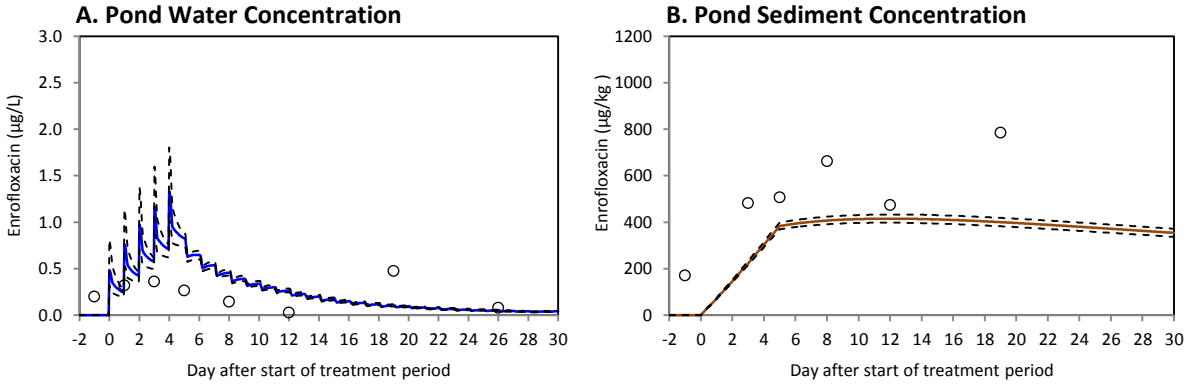


Figure 1. Comparison of measured (dots) and median predicted concentration (lines) of enrofloxacin in water and sediment of a *Pangasius* catfish pond, Vietnam. The dashed lines represent the 95th confidence interval of the predicted concentration, which was based on 1000 Monte Carlo permutations generated from several input parameter distributions (i.e. temperature, initial water volume, and water and sediment quality parameters).

The majority of the aquatic exposure assessments performed within this thesis were performed on a pond-based scale (**Chapter 4, 5** and **9**) and followed a single-compound approach assuming that ponds were isolated from each other. However, Asian aquaculture shows an increasing trend towards high spatial farm aggregation (e.g. Fig. 2; Ha et al., 2013). In areas with high level of farm clustering, it is most likely that several veterinary medicinal treatments are applied at the same

time (or within short time intervals). This can make that concentrations build-up in the environment and that down-stream aquatic ecosystems are exposed to chemical mixtures, as has already been documented in larger-scale monitoring studies performed within this thesis (**Chapter 6**) and elsewhere (Le and Munekage, 2004; Managaki et al., 2007; Zou et al., 2011). Accordingly, it is expected that some of the environmental risk evaluations performed within this thesis (**Chapters 4, 5** and **8**) somehow underestimated the real risks, especially when considering the potential for long-term exposure to chemical mixtures in aquatic sediments (**Chapter 6**). More realistic exposure assessments should be carried out by adopting larger time (e.g. months to years) and spatial scales (e.g. watershed level). This could be done, for example, by coupling ERA-AQUA model outputs to hydrological or GIS based modelling tools with suitable temporal and spatial configurations. Such an approach should pay special attention to accurately predict transport and deposition of veterinary medicinal residues through particulate material, as also recommended by Rose and Pedersen et al. (2005). The proposed next-step modelling approach will not only help in the identification of relevant chemical mixtures and representative time windows to be tested in refined ecotoxicological assessments, but will also contribute to identify specific areas on which monitoring should be focused and where environmental remediation and restoration is required. Priority areas for up-scaled exposure assessments are the Mekong Delta in Vietnam, where about 38 tons of antibiotics are estimated to be yearly discharged by *Pangasius* pond effluents (based on FAO 2012, and **Chapter 5**), and the coastal shrimp production areas of Guangxi and Guangdong provinces in China (Fig. 2; **Chapter 5**).

Figure 2. Satellite image of a shrimp production area in Guangxi province, south-east China, 2005.

3. Options for reducing the environmental discharge of aquaculture medicines

The mass balance calculations performed in **Chapter 5** demonstrated that about 25% of the chemotherapeutants applied in Asian pond aquaculture end-up in the environment through effluent discharges. They also showed that the main factors influencing the effluent discharge of aquaculture medicines are the mode of application (directly to water *vs* mixed with feed), the density of the cultured species in the aquaculture pond, the frequency and duration of the effluent discharge events, and the organic-matter sorption and bioaccumulation potential of the applied compound. Taking this into consideration, the only options that are within the reach of aquaculture farmers and operators to minimize the environmental discharge of aquaculture medicines are the reduction of cultured species density and of water discharge. For compounds applied mixed with feed, reducing cultured species biomass in aquaculture ponds logically reduces the amount of chemical quantities required per unit of water volume, and therefore also reduces the mass of chemical residues available for environmental discharge. Water discharge

from ponds can be managed in such a way that minimizes the environmental release of residues, for example by limiting water discharge during, and shortly after, chemical administration and allowing degradation of the compound within the pond before environmental release. Chemical fate and degradation modelling tools could be useful in indicating optimal timing and volume of effluent discharge to minimize environmental risks (**Chapter 4**). Environmental discharge of veterinary medicines could also be reduced by waste-water treatment methods such as sedimentation and phytoremediation. Sedimentation ponds have proven to be cost-effective water treatment methods for removing biosolids and particulate organic material from water (Boyd, 1998), and therefore also antibiotics residues that generally tend to be attached to them (Rose and Pedersen, 2005; **Chapters 6** and **7**). Recent studies have shown that wetland macrophytes could reduce the concentration of antibiotics in water and sediment samples up to 40% (Hoang et al., 2012, 2013). Thus, it is recommended that the removal efficiency and feasibility in the implementation of sedimentation and phytoremediation methods are further investigated. Such methods are particularly needed in ponds with high fish densities in which water quality deterioration dictates the need of water renewal (e.g. *Pangasius* catfish ponds in Vietnam), and high chemotherapeutant use and discharge constitute an important environmental problem (**Chapters 4** and **5**).

Because a large portion of the chemicals applied to earthen ponds end up in the sediment compartment (**Chapter 5**), the pumping of pond sediments and their disposal in surrounding ecosystems may also contribute to the contamination with veterinary medicines. This practice is most regularly done in intensive production areas such as the *Pangasius* producing region in the Mekong Delta (Anh et al., 2010). Due to the large amounts of sludge that are being generated and the large environmental impacts of this practice, pond sludge is being more and more utilized as fertilizer in agricultural production (Lin and Yi, 2003; Anh et al., 2010). Given the high persistence of veterinary medicinal residues (e.g. antibiotics) in pond sediments (**Chapter 4**), it is expected that such practice contributes to the spread of antibiotics over agricultural soils. Research has shown that veterinary medicinal residues contained in manure applied to agricultural fields may accumulate in vegetables, posing a potential hazard for human consumers, and may also support a pathway for antibiotic resistance genes to humans (Kumar et al., 2005; Boxall et al., 2006b). Thus, it is necessary to quantify residues of veterinary medicines in sludge before it is disposed into the environment or reused in agriculture. A practical way to avoid expensive and time-consuming chemical monitoring will be to use chemical fate models such as the ERA-AQUA (**Chapter 4**) in advising a proper sludge removal planning for farmers that have made use of chemotherapy. However, this will not solve the problem of the spread of antimicrobial resistance genes.

It has been estimated that the percentage of antibiotics applied to cage systems that is released into the environment amounts to approximately 75% of the applied dose (Lalumera et al., 2004). This percentage, however, may vary a lot depending on the coating agent used, the amount of uneaten feed, and the biotransformation of the compound in the cultured species (Duis et al., 1995; Metcalfe et al., 2009). Similarly to what has been found in marine cage studies (Coyne et al., 1994; Capone et al., 1996), **Chapter 6** showed that leaching from medicated feeds and the deposition of uneaten feeds and faeces in sediments underneath freshwater cages are the most likely routes of environmental contamination. Antibiotics applied in Asian aquaculture are manually or mechanically mixed with feeds in the farm before administration, and water is typically used to allow the antibiotic to attach to the feed pellets. This differs to the methods used in salmon producing countries, where medicated feeds prepared with oil coating agents are industrially made and sold ready-to-use in the market (Duis et al., 1995; Rigos et al., 1999). The methods used by Asian farmers are expected to result in higher leaching rates, especially when fish are already infected and uptake rates decrease. Due to the open nature of the cage production systems, options to reduce the environmental discharge of chemotherapeutants are very limited (e.g. improved antibiotic coating). Therefore the only alternative to improve the

environmental performance of these farms is to reduce the risks of disease that require medication, or simply the migration of the fish production into (semi-)closed systems (i.e. ponds, tanks), as already seems to be happening in some areas (e.g. Thailand; Belton et al., 2009b).

4. Toxicological effects of aquaculture medicines for tropical aquatic ecosystems

The current literature lacks examples that show the ecological effects in the field caused by the contamination with aquaculture medicines in Asia. Therefore, the discussion on their toxicological effects is currently based on results from laboratory and, to a lesser extent, model ecosystem experiments, and needs to take into consideration the (potential) limitations of the extrapolation of these experimental results to assess the ecological effects for tropical aquatic ecosystems of Asia.

Parasiticides have been identified as the group of compounds with higher toxicity potential for non-target aquatic organisms after use in Asian aquaculture as compared to medical disinfectants or antibiotics (**Chapter 5**). Results of studies reviewed within **Chapter 2** show that for parasiticides the sensitivity of aquatic organisms inhabiting different geographical areas (temperate *vs* tropical) and habitats (freshwater *vs* marine), does not show marked differences. However, ecological effects of anthelmintics and parasiticides applied to aquaculture ponds in Asia might be higher than those shown by experiments performed in the marine environment (see Burridge et al., 2010), mainly because of lower dilution. Based on the microcosm experiments conducted by Sanderson et al. (2007) and Boonstra et al. (2011), and also the results of **Chapter 5**, residues from the anthelmintic ivermectin applied in aquaculture ponds are likely to temporally affect the structure of invertebrate communities, with cladocerans being the most sensitive taxa. Also high effects are expected by antiparasitic treatments with deltamethrin on invertebrate communities (**Chapter 5**) according to the results of the microcosm experiment described in Schanné and Van der Kolk (2001). According to the results of this thesis (**Chapter 5**), priority should be given to the assessment of the effects of some anthelmintics and parasiticides (e.g. ivermectin and deltamethrin) on benthic invertebrate communities exposed to tilapia or *Pangasius* pond effluents. Such experiments should assess the stress caused by pulsed exposure concentrations rather than the more-static exposure patterns typically used in standard laboratory experiments. In this regard, the parameterization and implementation of toxicokinetic and toxicodynamic models could help in approaching a more realistic understanding of the effects of pulsed aquaculture effluent discharges and on the extrapolation across different exposure patterns (Ashauer et al., 2007; Jager et al., 2011).

Results of **Chapter 5** show that benzalkonium and hydantoin compounds used for water disinfection and as medical disinfectants can pose a high acute toxicological risk for invertebrates, fish and primary producers next to effluent discharge points. These risk calculations are rather conservative due to a lack of available data regarding their environmental degradation, as well as ecotoxicological studies assessing their effects on non-standard aquatic organisms (**Chapter 5**). Future research should address these data gaps by evaluating their persistence and the formation of transformation products in organic matter rich environments, and should also assess their acute toxicity for tropical aquatic organisms including microorganisms.

This thesis has significantly contributed to increase our knowledge on the sensitivity of tropical aquatic organisms to two of the most widespread antibiotics used in Asian aquaculture, oxytetracycline and enrofloxacin (**Chapters 6, 7** and **8**). It has been demonstrated that the acute sensitivity of tropical freshwater zooplankton (i.e. *Moina macrocopa*) is similar to that of their temperate counterparts (i.e. *Daphnia magna*), and that tropical macroinvertebrates (i.e. molluscs, worms, insects, crustaceans) are, in most cases, less sensitive than zooplankton species (**Chapters 6** and **7**). Those experiments also confirmed the high tolerance of aquatic invertebrates to

aquaculture antibiotics that had previously been reported by other authors (Robinson et al., 2005; Park and Choi, 2008). According to the available toxicity data (e.g. Holten-Lützhøft et al., 1999, Ebert et al., 2011), and the risk assessments performed in **Chapters 5, 6** and **7**, primary producers, and more specifically cyanobacteria populations, are expected to be affected by environmental concentrations of antibiotics. This, however, could not be demonstrated in the microcosm experiment conducted with the antibiotic enrofloxacin (**Chapter 8**), probably due to (1) the low compound bioavailability, explained by the high water pH and the consequent ionization of the test compound, and (2) the variable abundance of cyanobacteria in the test systems. Wilson et al. (2004) demonstrated that long-term exposure to antibiotic mixtures can severely suppress the growth of cyanobacteria and other phytoplanktonic groups. The available literature reveals that antibiotic concentrations in the µg/L range can affect the relative abundance of biofilm microorganisms involved in important ecological functions such as photosynthesis, and carbon and nitrogen cycling in biological reactors (Yergeau et al., 2012; **Chapter 8**), though effects on biological processes at environmentally relevant concentrations have not yet been demonstrated (Wunder et al., 2013; Yan et al., 2013; **Chapter 8**). The extrapolation of these experimental results with regard to the differences in species sensitivity among the tested species and those inhabiting Asian tropical ecosystems becomes less relevant since most sensitive species of phytoplankton (e.g. *Microcystis* sp.) and aquatic microbes are cosmopolitan (Dolan, 2005). The extrapolation of these results to flow-through ecosystems as those impacted by aquaculture antibiotic residues still remains to be investigated, but it is envisaged that the bioavailability of antibiotics in natural ecosystems will be much lower than in the test systems that have been currently utilized.

Based on the results of this thesis and the published literature it can be concluded that the environmental exposure to aquaculture antibiotics is not expected to pose a direct toxicological risk for invertebrate and fish communities. Antibiotic exposure is likely to result in subtle and transient effects on the structure of microbial communities. However, due to the high ecological redundancy and recovery potential of microbial communities under tropical conditions (e.g. **Chapter 8**), effects on water quality and side effects at higher trophic levels are most unlikely to be detected in lotic aquatic ecosystems. More investigations are required to develop sensitive endpoints to assess microbial-related effects on ecosystems, and to investigate the effects of long-term (> 30 days) exposure to antibiotic mixtures on sediment biofilm structure and functions under tropical conditions. For the latter, the abundance of nitrifying bacteria and archea (**Chapter 8**), in combination with other microbial phenotypes involved in organic matter mineralization (Yergeau et al., 2012; Wunder et al., 2013) are recommended to be used as potentially sensitive endpoints. Research into the ecotoxicology of aquaculture antibiotics should also move towards the investigation of sub-lethal effects on aquatic fauna and flora caused by the disruption of ecological associations with microorganisms such as symbiosis (e.g. nitrogen fixation in macrophytes) and facilitation (e.g. digestive efficiencies in invertebrates or fish, or disease susceptibility caused by the alteration of mucus or epithelial bacteria).

Ecotoxicological assessment of aquaculture medicines should also pay attention to other substances that have not been studied within this thesis. For example, the hormone 17α-methyltestoreone used for sex-reversal in tilapia hatcheries has been detected in water samples collected in the surroundings of aquaculture facilities of Thailand (Barbosa et al., 2013) at concentrations that are up to 200 times higher than the concentrations that affect reproductive endpoints of *Danio rerio* such as vitellogenin concentration and 11-ketotestosterone content (Andersen et al., 2006). This suggests that the pollution with this hormone may induce endocrine disrupting effects and may affect the sex-ratio and the reproduction rates of wild fish populations. This thesis showed that a wide range of probiotics are currently used in Asian aquaculture (**Chapters 2** and **3**). The exact chemical and biological composition of the probiotic cocktails that are extensively used in Asian aquaculture is still relatively unknown (Wang et al., 2008) and the ecological effects posed by these residues and the introduction of probiotic microorganisms into aquatic ecosystems is a matter of concern that has not yet been addressed. The environmental

introduction of these probiotics is not likely to result in negative effects for water quality, but it is expected to affect the equilibrium and structure of aquatic bacterial communities.

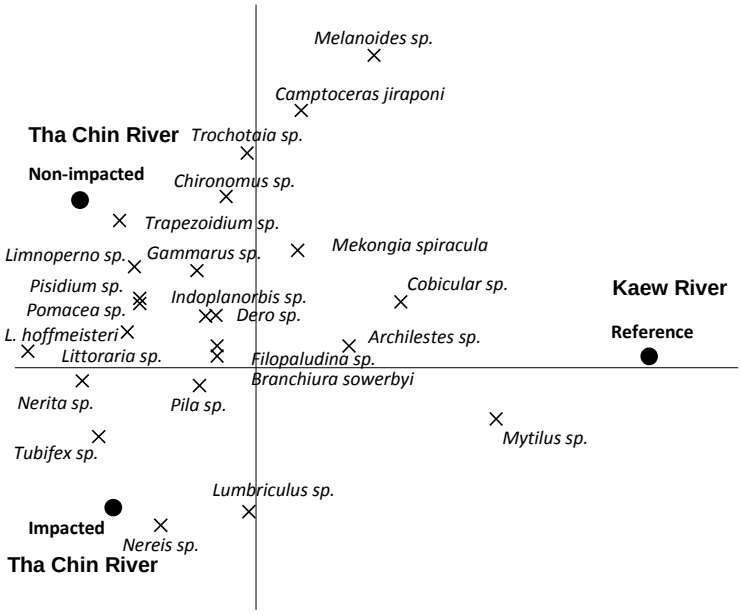


Figure 3. Redundancy analysis biplot showing the difference in species composition between sites with a different ecological impact, i.e. impacted, non-impacted and reference site. Ecological impact explained 22% of the difference in species composition between the 35 sites, of which 79% is displayed on the first axis and the remainder 21% on the second one. The samples collected in the Tha Chin River differed significantly in species composition ($p \leq 0.001$) from the samples collected in the Kaew River (reference site), while the difference in species composition between the aquaculture impacted and non-impacted sites of the Tha Chin River was marginally significant ($p = 0.063$).

5. Challenges for the monitoring of the effects of aquaculture medicines in the field

As already discussed, there are very few examples of studies evaluating the ecological effects of veterinary medicinal residues from aquaculture farms. This can be explained by the lack of economic investments in this research area but also by other factors related to environmental and technical limitations that will be discussed here. One of them is the difficulty to assess effects on pelagic organisms due to the flow-through conditions of the impacted ecosystems, which can be solved by concentrating efforts towards the assessment of benthic communities. Another one is related to the fact that intensive aquaculture generally releases high loads of organic material and nutrients. In this way, natural aquatic ecosystems are not only exposed to chemotherapeutant residues but also to a range of other water quality disturbances generated by increased nutrient levels and eutrophication (e.g. lower oxygen concentrations, increased turbidity). The accumulation of aquaculture wastes generates a phenomenon traditionally called as 'organic enrichment'. This generally results in a reduction of the number of invertebrate taxa and an increase in the abundance of the few invertebrate species with high tolerance to low oxygen levels in the surroundings of the aquaculture farms. This alteration of the benthic fauna composition hampers the identification of the specific effects caused by chemotherapeutant residues. This phenomenon has been previously reported in marine cage aquaculture (e.g. Telfer et al., 2006) and has also been investigated during the field monitoring study performed in Thailand (**Chapter 6**). Here, the invertebrate composition of 35 sediment samples was analysed. The samples were collected from directly impacted (underneath the tilapia cages; *n*=14) and less directly or non-impacted areas (at least 1 km down-stream from the tilapia cages; *n*=11) of the Tha Chin River, and from a reference area in the Kaew River (*n*=10), which apparently was not impacted by any anthropogenic source of pollution. This analysis showed that the benthic invertebrate composition of the Tha Chin River was significantly different to that of the Kaew

River, and a trend was observed towards a higher abundance of worm taxa (*Nereis* sp.*, Tubifex* sp.*, Lumbriculus* sp.*)* in the sediments next to the tilapia cages (Fig. 3). As expected, differences in benthic invertebrates composition did not show a significant correlation to measured antibiotic concentrations, expressed as the sum of oxytetracycline and enrofloxacin (redundancy analysis; Monte Carlo test: $p = 0.7$).

Another challenge for the field monitoring of effects of veterinary medicines is the lack of suitable biomarkers to assess exposure and effects on non-target aquatic organisms. Whereas effect biomarkers such as cholinesterase inhibition have proven to be useful for assessing the effects of some antiparasitic compounds (Tu et al., 2009b; Coelho et al., 2011), most studied biomarkers for aquaculture antimicrobials are not sufficiently sensitive to quantify sub-lethal effects at environmentally relevant concentrations (Ambili et al., 2013; Oliveira et al., 2013; Pereira et al., 2014) and, hence, their use is often restricted to assess stress caused by therapeutic doses in aquaculture facilities (**Chapter 7**). The use of available biomarkers in field assessments also has other shortcomings such as the variability in control (enzymatic) levels and their limited stressor specificity, which often challenge the interpretation of exposure-related effects (Schmidt et al., 2012).

In conclusion, the evaluation of the effects of aquaculture veterinary medicines under field conditions is challenged by situations of mixture toxicity and multiple stress, and the lack of suitable biomarkers of exposure for some compounds (i.e., antimicrobials). Consequently, more ecologically relevant assessments must take into consideration the combined toxicological effects of the whole aquaculture discharge. The appropriate isolation of the specific chemoterapeutant effects will only be accomplished through controlled laboratory and/or semi-field experiments that use 'nutrient and non-nutrient' treatments and controls.

6. Developing ecological thresholds for aquaculture medicines

Nowadays, the monitoring of the environmental impacts of Asian aquaculture is based on the assessment of the compliance of measured water quality parameters (e.g. nutrients, oxygen concentrations, pH) with available effluent standards (e.g. Boyd, 2003). The inclusion of veterinary medicines in these monitoring assessments requires the definition of ecological thresholds for these substances. In this respect, **Chapter 5** offers the first set of ecological thresholds (Predicted No Effect Concentrations: PNECs) for aquaculture medicines with a potential for use in environmental monitoring in Asian aquaculture producing countries. Such PNECs are based on a tiered approach that uses contemporary ecotoxicological methods and up-to-date data for each compound. The lower tiers of this approach have been extensively validated for agricultural pesticides using results of micro- and mesocosm experiments (Brock et al., 2006), and therefore it is expected that they also offer a sufficient level of protection for aquaculture compounds with similar mode of action such as parasiticides and some fungicides. PNECs for most aquaculture antimicrobials are based on lower-tier calculations due to the limited amount of available toxicological data for non-standard test species and model ecosystem experiments (**Chapter 5**). Results of **Chapter 8**, as well as results from other semi-field studies (e.g. Wilson et al., 2004), suggest that lower-tier antibiotic PNECs are sufficiently protective for ecosystem structure (not including microorganisms) and functioning, however more experiments need to be performed with other compounds to confirm this.

Besides the potential ecotoxicological effects, a major concern related to the environmental pollution with aquaculture antibiotics is the development of antibiotic resistance in environmental bacteria and their potential consequences for human health (Heuer et al., 2009; Miranda et al., 2013; Pruden et al., 2013; **Chapter 9**). A relevant question is to ask whether the use of current action limits and PNECs used in ERA provide a sufficient protection level for resistance development in environmental bacteria. To shed light on this issue, the Phase I action

limit established by the international environmental risk assessment guidelines for veterinary medicines (VICH, 2000) and the acute PNECs derived in **Chapter 5** were compared to the bacterial MIC50 SSDs derived in **Chapter 9**. It was found that the median percentage of bacterial genera affected by the Phase I action limit (1 ppb) is close to or well below the 5% for all 12 evaluated antibiotics. The comparison of ecological thresholds was only performed for 8 antibiotics, because for the other 4 the PNECs had not been derived from experimental data but using QSARs. The results of this comparison show that, with the exception of trimethoprim, the fraction of bacterial genera affected is close to or well below 1%. The PNEC for trimethoprim had been derived with green algae instead of cyanobacteria and resulted in an affected fraction of 10% (Fig. 4). According to this comparison, it can be concluded that the current antimicrobial action limits and the ecological threshold concentrations, as proposed by **Chapter 5** and derived with cyanobacteria toxicity data (instead of green-algae), could induce resistance in about (or in less than the) 5% of bacteria. It might be questionable, however, whether the SSD 5% cut-off value (or its lowest confidence limit) traditionally used in risk assessment to protect aquatic ecosystems (Aldenberg et al., 2002; Brock et al., 2006; Maltby et al., 2009) would also result in a sufficient protection level for antibiotic resistance because of two main reasons. First, because the non-protected 5% of bacteria might include animal and human pathogens of critical relevance in epidemiology. Second, because the potential mobility of resistance genes, and the horizontal and vertical gene transfer between environmental compartments, could lead to the colonization of resistant pathogens in animals and humans, and might result in health risks even when protecting the largest percentage of environmental bacteria. Experimental research is needed to evaluate the suitability of the resistance susceptibility endpoints used and discussed within this thesis (**Chapter 9**). However, based on the information that is currently available, it can be concluded that the action limits and the threshold values proposed by ERA seem to provide a conservative protection for antibiotic resistance development in the aquatic environment.

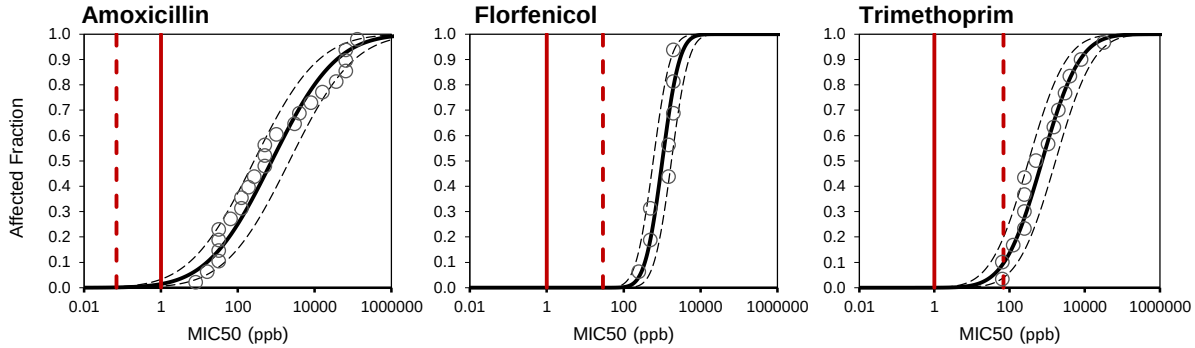


Figure 4. Comparison of bacterial MIC50 SSDs with the VICH Phase I action limit for aquaculture effluents (solid vertical lines) and the acute PNECs derived in **Chapter 5** (dashed vertical lines) for amoxcillin, florfenicol and trimethoprim.

7. Options for a better control of the use of veterinary medicines in Asian aquaculture

The literature review performed in **Chapter 2** made evident that detailed and up-to-date information on the use of veterinary medicines in Asian aquaculture was hardly available as an open access resource. Efforts have been made in **Chapter 3** to fill this data gap for a number of important aquaculture species with high export potential. To continue delivering the appropriate information to perform ERAs of aquaculture medicines, Asian countries should aim to improve their mechanisms that control the use of veterinary medicines at a national level and at a farm-level. At the national level, governments must encourage sellers, importers and producers of veterinary medicines to keep records trying to, as much as possible, separate the amounts of drugs labelled for aquaculture purposes from those labelled for other animal producing commodities such as poultry or livestock. They should also make this data available to aquaculture authorities and researchers. Although progress has recently been made to improve

these mechanisms in some countries such as China (Yuan and Chen, 2012), Thailand (Baoprasertkul et al., 2012), Vietnam (Tai, 2012) and Philippines (Somga et al., 2012), it is evident that in some cases (e.g. Vietnam) more efficient measures should be implemented by, for example, monitoring active ingredient quantities instead of product names, and by better defining the role and responsibility of the different competent authorities involved in the process (Tai, 2012). The Norwegian Institute of Public Health offers a good example of a successful tracking system for veterinary medicine sales used in salmonid aquaculture that has been working since 2001 (NIPH, 2009).

At the production level monitoring is, at varying degrees, executed to assess compliance to regulatory lists of approved and restricted or prohibited chemicals. The enforcement of such regulations becomes useful in promoting the prudent use of chemotherapeutants regarding international food safety controls. In some cases, such as the case of the Vietnamese aquaculture industry (VMARD, 2009), such lists contain a wide range of antibiotic classes, some of them with critical importance for human medicine (WHO, 2012). Thus, it is recommended that regulatory lists of approved chemicals are urgently revised and that antibiotics used for human disease treatments are completely separated from aquaculture uses. Results of the chemical use survey performed in **Chapter 3** show that, with few exceptions, most of the chemicals in use comply with national regulations. It should be noted, however, that the majority of the farms surveyed in **Chapter 3** were mainly export oriented. In **Chapter 6**, the excessive antibiotic use reported by cage-based farmers that serve the domestic market show an indication of the importance of monitoring and improving the knowledge on appropriate chemical use in non-exporting (small-scale) farms.

Third party certification bodies have played a major role in distributing information on prudent use practices and checking compliance to national and international regulations (Corsin et al., 2007). However, because certification is more widespread among large-scale export oriented farms, such kind of support is less frequently arriving to small holders (Bush et al., 2013). Authorities should see third-party certification efforts as a contribution to this, but should not consider them as the only mean for proper chemical use control. Stronger commitment to the inspection and monitoring of the chemical use at the farm level is required by Asian governments regarding the tracking of active ingredients and yearly quantities used by farmers. For this, governments should encourage farmers to keep records. In the future, farm-level chemical use inspections should take into account farms oriented to all kinds of market sectors, and should also include nurseries and hatcheries, for which their chemical use practices as well as environmental impacts have been less investigated (**Chapter 2**).

8. Concluding remarks and recommendations

According to the results of this thesis it can be concluded that the introduction of biosecurity measures and new advances in water quality control, feed technology and disease diagnosis seem to have resulted in a decline of the use of veterinary medicinal treatments in (semi-)intensive shrimp production in some important producing countries of Asia such as China, Thailand, and Vietnam (**Chapter 3**). Nevertheless, veterinary medicines are essential in some critical phases of the aquaculture production (e.g. egg disinfection, stocking of ponds), and are regularly used in intensive aquaculture production systems with high water exchange and low biosecurity potential e.g. *Pangasius* catfish ponds and freshwater tilapia cage farms (**Chapters 3** and **6**). Therefore, it is recommended that aquaculture-producing countries of Asia increase the control on their chemical production, sales and imports, and that continued monitoring is carried out to assess compliance to regulations and to assess chemical use practices at the farm level. Also more knowledge should be provided to aquaculture farmers on disease diagnosis, appropriate application methods and disposal of used chemotherapeutant containers, particularly to small-holders (**Chapter 2**).

Regulatory ERAs should be implemented in the registration and post-registration evaluation of aquaculture chemicals in Asian countries, as already done in most developed countries. For this, the risk assessment guidelines set by the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Products (VICH, 2000, 2004) or similar approaches should be used. The risk assessment models and scenarios developed within this thesis provide a valuable platform to perform compound and species-specific preliminary evaluations and to guide scientific studies aimed at improving the knowledge that supports ERA (**Chapters 4** and **5**).

This thesis has shown that a significant amount of veterinary medicines applied to intensive aquaculture ponds and freshwater tilapia cages is released into the environment by waste and waste-water discharges (**Chapter 5** and **6**). High ecological risks are expected by the current application of chemotherapeutants in intensive aquaculture production of *Pangasius* catfish in Vietnam and, to a lesser extent, in intensive shrimp production scenarios of China. Regarding the range of evaluated compounds, it can be concluded that the highest predicted ecological risk is posed by the application of some antiparasitic compounds due to their high toxic potential for non-target invertebrate communities (**Chapter 5**).

Asian aquatic ecosystems neighbouring aquaculture production are not only exposed to chemotherapeutant residues but to a range of other stressors (e.g. nutrients, water quality treatments, agrochemicals, industrial pollution). This challenges the identification of chemotherapeutant-specific biological effects in the field, and suggests that a weight-of-evidence approach based on multiple lines of evidence should be used to assess effects of chemotherapeutant residues. Such an approach must include the monitoring of contaminant levels in the environment, and an adequate suite of biomarkers and bioindicators derived from laboratory and (semi-field) experiments performed at different levels of biological organization. In this regard, this thesis has made significant progress in assessing exposure patterns and relevant biological effect measures for two commonly used antibiotics applied to different aquaculture scenarios of Asia. Field monitoring studies have shown that aquaculture antibiotics accumulate in sediments surrounding fish cage farms and pond effluent discharge points at relatively high concentrations (**Chapters 6** and **7**). The assessment of toxicological effects of antibiotics on different tropical aquatic organisms and at different levels of biological organization (molecular, individual, population, community) have shown that the short-term risks seem to be mild for aquatic ecosystems, and are not expected to go beyond transient effects on the structure of microbial communities. However, further research is required to assess the ecological effects of antibiotic mixtures and long-term effects on sediment dwelling invertebrates, and in sediment microbial communities and their mediated ecological functions.

The regular use of antibiotics in some aquaculture farms of Asia and the high levels of environmental pollution detected in aquatic ecosystems raise concerns about their contribution to the development of resistance in environmental bacteria. Although with limited or yet unknown ecological consequences, the increased levels of antibiotic resistance are expected to reduce the future ability to effectively treat bacterial infections in aquaculture and might have dramatic consequences for human health. Water threshold values for ecological protection seem to provide a sufficient protection level to avoid resistance development in bacteria. However, this thesis has demonstrated that antibiotics, even when used according to recommendations, may increase the prevalence of antibiotic resistance in water and sediments of aquaculture ponds with high fish densities (**Chapter 9**). This paints an overall worrying picture about the consequences of antibiotic pollution for human populations in aquaculture production environments and indicates an urgent need to invest in research towards the reduction of antibiotic use - through the development of improved biosecurity methods and vaccination - and the implementation of cost-effective effluent treatment methods to reduce environmental pollution (e.g. sedimentation ponds, phytoremediation).

To conclude, the future of intensive aquaculture in Asia may be confronted with the degradation of the aquatic ecosystems that constitute the primary water source for their production activities. The assessment of chemotherapy methods and their potential environmental risks is a challenging and timely issue that requires further attention by governments and researchers all over the world. This thesis has made an attempt to provide the tools and insights to approach some relevant issues, however further work is required (1) to assess more realistic exposure patterns of veterinary medicines in aquaculture production areas, (2) to explore new ecological endpoints and to refine existing ecotoxicological approaches for veterinary medicines, and (3) to incorporate the antibiotic resistance issue into the risk assessment paradigm.

References

Adriaanse, P.I. 1996. Fate of pesticides in field ditches: the TOXSWA simulation model. Winand Staring Centre, Report 90, Wageningen, The Netherlands.

Albabouch, L., Gandini, G., Ryder, J. 2005. Causes of detentions and rejections in international fish trade. FAO fisheries technical paper 473. FAO, Rome, Italy, pp. 110.

Alday-Sanz, V., Corsin, F., Irde, E., Bondad-Reantaso, M.G. 2012. Survey on the use of veterinary medicines in aquaculture. In: Bondad-Reantaso, M.G., Arthur, J.R., Subasinghe, R.P., eds. Improving biosecurity through prudent and responsible use of veterinary medicines in aquatic food production, pp. 29-44. FAO Fisheries and Aquaculture Technical Paper No. 547. Rome, FAO. 207 pp.

Aldenberg, T., Jaworska, J.S. 2000. Uncertainty of the hazardous concentration and fraction affected for normal species sensitivity distributions. Ecotoxicology and Environmental Safety 46, 1-18.

Aldenberg, T., Jaworska, J.S., Traas, T.P. 2002. Normal species sensitivity distributions and probabilistic ecological risk assessment. In: Species sensitivity distributions in ecotoxicology. Posthuma L, Suter II GW, Traas TP (eds). CRC Press, Lewis, Boca Raton, FL. pp 49-102.

Amado, A.M., Meirelles-Pereira, F., Vidal, L.O., Sarmento, H., Suhett, A.L., Farjalla, V.F., Cotner, J.B., Roland, F. 2013. Tropical freshwater ecosystems have lower bacterial growth efficiency than temperate ones. Frontiers in Microbiology 4, 167.

Amann, R.I., Ludwig, W., Schleifer, K.H. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiology Reviews 59: 143-69.

Amaraneni, S.C. 2006. Distribution of pesticides, PAHs and heavy metals in prawn ponds near Kolleru lake wetland, India. Environment International 32, 294-302.

Ambili, T.R., Saravanan, M., Ramesh, M., Abhijith, D.B., Poopal, R.K. 2013. Toxicological effects of the antibiotic oxytetracycline to an Indian major carp *Labeo rohita*. Archives of Environmental Contamination and Toxicology 64, 494-503.

Ambrose, R.B., Wool, T.A., Connolly, J.P., Schanz, R.W. 1988. WASP4, a Hydrodynamic and Water Quality Model, Model Theory, User's Manual and Programmer's Guide. USEPA/600/3-87/039, Athens, Georgia.

Ambrose, R.B., Wool, T.A., Martin, J.L. 1993. The water-quality analysis simulation program, WASP5. U.S. Environmental protection Agency, Athens, GA.

Andersen, L., Goto-Kazeto, R., Trant, J.M., Nash, J.P., Korsgaard, B., Bjerregaard, P. 2006. Short-term exposure to low concentrations of the synthetic androgen methyl- testosterone affects vitellogenin and steroid levels in adult male zebrafish (*Danio rerio*). Aquatic Toxicology 76, 343-352.

Anderson, D.P. 1992. Immunostimulants, adjuvants, and vaccine carriers in fish: applications to aquaculture. Annual Reviews of Fish Diseases 2, 281-307.

Ando, T., Nagase, H., Eguchi, K., Hirooka, T., Nakamura, T., Miyamoto, K., Hirata, K. 2007. A novel method using cyanobacteria for ecotoxicity tests of veterinary antimicrobial agents. Environmental Toxicology and Chemistry 26, 601-606.

Andrews, J.M. 2001. Determination of minimum inhibitory concentrations. Journal of Antimicrobial Chemotherapy 48, 5-16.

Andrieu, M., Rico, A., Phu, T.M., Huong, D.T.T., Phuong, N.T., Van den Brink, P.J. Submitted. Ecological risk assessment of the antibiotic enrofloxacin applied to *Pangasius* catfish farms in the Mekong delta, Vietnam.

Anh, P.T., Bush, S.R., Mol, A.P.J., Kroeze, C. 2011. The multi-level environmental governance of Vietnamese aquaculture: global certification, national standards, local cooperatives. Journal of Environmental Policy and Planning 13, 373-397.

Anh, P.T., Kroeze, C., Bush, S.R., Mol, A.P.J. 2010. Water pollution by intensive brackish shrimp farming in south east Vietnam: Causes and options for control. Agricultural Water Management 97, 872-882.

Anh, P.T., Kroeze, C., Bush, S.R., Mol, A.P.J. 2010. Water pollution by *Pangasius* production in the Mekong Delta, Vietnam: causes and options for control. Aquaculture Research 42, 108-128.

Ankley, G.T., Brooks, B.W., Huggett, D.B., Sumpter, J.P. 2007. Repeating history: pharmaceuticals in the environment. Environmental Science and Technology 41, 8211-8217.

APHA, American Public Health Association. 1996. Standard method for the examination of water and waste water. American Water Works Association and Federal Water Pollution Control Administration. Washington, DC, pp 1193.

APHA, American Public Health Association. 2005. Standard methods for the examination of water and waste water. American Water Works Association and Federal Water Pollution Control Administration. 21st Edition. Washington, DC, USA. pp 1193.

Arnot, J.A., Meylan, W., Tunkel, J., Howard, P.H., Mackay, D., Bonnell, M., Boethling S. 2009. A quantitative structure-activity relationship for predicting metabolic biotransformation rates for organic chemicals in fish. Environmental Toxicology and Chemistry 28, 1168-1177.

Arthur, J.R., Lavilla-Pitogo, C.R., Subasinghe, R.P. 2000. Use of chemicals in aquaculture in Asia. Proceedings of the Meeting on the Use of Chemicals in Aquaculture in Asia. 20-22th May of 1996. Rigbauan, Iloilo, Philippines. SEAFDEC, Philippines. pp 235.

Arthur, J.W., West, C.W., Allen, K.N., Hedtke, S.F. 1987. Seasonal toxicity of ammonia to five fish and nine invertebrate species. Bulletin of Environmental Contamination and Toxicology 38, 324-331.

Ashauer, R., Boxall, A.B.A., Brown, C.D. 2007. New ecotoxicological model to simulate survival of aquatic invertebrates after exposure to fluctuating and sequential pulses of pesticides. Environmental Science and Technology 41, 1480-1486.

Ashbolt, N.J., Amézquita, A., Backhaus, T., Borriello, P., Brandt, K.K., Collignon, P., Coors, A., Finley, R., Gaze, W.H., Heberer, T., Lawrence, J.R., Larsson, D.G.J., McEwen, S.A., Ryan, J.J., Schönfeld, J., Silley, P., Snape, J.R., Van den Eede, C., Topp, E. 2013. Human health risk assessment (HHRA) of environmental development and transfer of antibiotic resistance. Environmental Health Perspectives 121, 993-1001.

Baoprasertkul, P., Somsiri, T., Boonyawiwat, V. 2012. Use of veterinary medicines in Thai aquaculture: current status. In: Bondad-Reantaso M.G., Arthur, J.R., Subasinghe, R.P., eds. Improving biosecurity through prudent and responsible use of veterinary medicines in aquatic food production, pp. 83-89. FAO Fisheries and Aquaculture Technical Paper No. 547. Rome, FAO. pp 207.

Barbosa, I.R., Lopes, S., Oliveira, R., Domingues, I., Soares, A.M.V.M., Nogueira, A. 2013. Determination of 17α-methyltestosterone in freshwater samples of tilapia farming by high performance liquid chromatography. American Journal of Analytical Chemistry 4, 207-211.

Barg, U., Lavilla-Pitogo, C. 1996. The use of chemicals in aquaculture: a summary brief of two international expert meetings. Food and Agriculture Organization of the United Nations. FAO Aquaculture Newsletter 14, 12-13.

Bartie, K.L., Austin, F.W., Diab, A., Dickson, C., Dung, T.T., Giacomini, M., Crumlish, M. 2012. Intraspecific diversity of *Edwardsiella ictaluri* isolates from diseased freshwater catfish, *Pangasianodon hypophthalmus* (Sauvage), cultured in the Mekong Delta, Vietnam. Journal of Fish Diseases 35, 671-682.

Baudhuin, P., Baufay, H., Rahman-Li, Y., Sellinger, O.Z., Wattiaux, R., Jacques, P., De Duve, C. 1964. Tissue fractionation studies. 17. Intracellular distribution of monoamine oxidase, aspartate aminotransferase, alanine aminotransferase, D-amino acid oxidase and catalase in rat-liver tissue. Biochemical Journal 92, 179-184.

BDOF, Bangladeshi Department of Fisheries. 2011. Fish hatchery act of 2011. Available at: http://www.fisheries.gov.bd

Bebak-Williams, J., Bullock, G., Carson, M.C. 2002. Oxytetracycline residues in freshwater recirculating systems. Aquaculture 205, 221-230.

Beltman, W.H.J., Adriaanse, P.I., Van Elswijk, M.J.B. 1996. TOXSWA 1.0 : user's manual. Technical document / DLO-Staring Centrum, 33, SC-DLO, Wageningen.

Belton, B., Little, D., Grady, K. 2009b. Is responsible aquaculture sustainable aquaculture? WWF and the eco-certification of tilapia. Society and Natural Resources: An International Journal 22, 840-855.

Belton, B., Turongruang, D., Bhujel, R., Little, D.C. 2009a. The history, status, and future prospects of monosex tilapia culture in Thailand. Aquaculture Asia 14, 16-19.

Benford, D. 2000. The acceptable daily intake. A tool for ensuring food safety. International Life Sciences Institute (ILSI). ILSI Europe Concise Monograph Series. ILSI Press, Washington DC, US. pp 38.

Bernard, M., Latgea, J.-P. 2001. *Aspergillus fumigatus* cell wall: composition and biosynthesis. Medical Mycology 39, 9-17.

Beveridge, M.C.M., Phillips, M.J., Macintosh, D.J. 1997. Aquaculture and the environment: the supply of and demand for environmental goods and services by Asian aquaculture and the implications for sustainability. Aquaculture Research 28, 797-807.

Birdwell, J., Cook, R.L., Thibodeaux, L.J. 2007. Desorption kinetics of hydrophobic organic chemicals from sediment to water: a review of data and models. Environmental Toxicology and Chemistry 26, 424-434.

Bondad-Reantaso, M.G., 2010. FAO expert workshop on improving biosecurity through prudent and responsible use of veterinary medicines in aquatic food production. Food and Agriculture Organization of the United Nations. FAO Aquaculture Newsletter 45, 37-39.

Bondad-Reantaso, M.G., Arthur, J.R., Subasinghe, R.P. 2012. Improving biosecurity through prudent and responsible use of veterinary medicines in aquatic food production. FAO Fisheries and Aquaculture Technical Paper. No. 547. Rome, FAO, 207 pp.

Bondad-Reantaso, M.G., Subasinghe, R.P., Arthur, J.R., Ogawa, K., Chinabut, S., Adlard, R., Tan, Z., Shariff, M. 2005. Disease and health management in Asian aquaculture. Veterinary Parasitology 132, 249-272.

Boonstra, H., Reichman, E.P., Van den Brink, P.J. 2011. Effects of the veterinary pharmaceutical ivermectin in indoor aquatic microcosms. Archives of Environmental Contamination and Toxicology 60, 77-89.

Bosma, R.H., Hanh, C.T.T., Potting, J. 2009. Environmental Impact Assessment of the Pangasius Sector in the Mekong Delta. Wageningen University. Available from: http://edepot.wur.nl/8332

Bostock, J., McAndrew, B., Richards, R., Jauncey, K., Telfer, T., Lorenzen, K., Little, D., Ross, L., Handisyde, N., Gatward, I., Corner, R. 2010. Aquaculture: global status and trends. Philosophical Transitions of the Royal Society B 365, 2897–2912.

Boxall, A.B., Fogg, L.A., Blackwell, P.A., Kay, P., Pemberton, E.J., Croxford, A. 2004. Veterinary medicines in the environment. Reviews in Environmental Contamination and Toxicology 180, 1-91.

Boxall, A.B.A., Fogg, L., Baird, D.J., Lewis, C., Telfer, T., Kolpin, D., Gravell, A., Pemberton, E.J., Boucard, T. 2006a. Targeted monitoring study for veterinary medicines in the environment. Science Report SC030183/SR. Environment Agency, Bristol, UK.

Boxall, A.B.A., Johnson, P., Smith, E.J., Sinclair, C.J., Stutt, E., Levy, L.S. 2006b. Uptake of veterinary medicines from soils into plants. Journal of Agricultural Food Chemistry 54, 2288-2297.

Boxall, A.B.A., Rudd, M., Brooks, B.W., Caldwell, D., Choi, K., Hickmann, S., Innes, E., Ostapyk, K., Staveley, J., Verslycke, T., Ankley, G.T., Beazley, K., Belanger, S., Berninger, J.P., Carriquiriborde, P., Coors, A., DeLeo, P., Dyer, S., Ericson, J., Gagne, F., Giesy, J.P., Gouin, T., Hallstrom, L., Karlsson, M., Larsson, D.G.J., Lazorchak, J., Mastrocco, F., McLaughlin, A., McMaster, M., Meyerhoff, R., Moore, R., Parrott, J., Snape, J., Murray-Smith, R., Servos, M., Sibley, P.K., Straub, J.O., Szabo, N., Tetrault, G., Topp, E., Trudeau, V.L., van Der Kraak, G. 2012. Pharmaceuticals and personal care products in the environment: What are the big questions? Environ Health Perspectives 120, 1221–1229.

Boyd, C.E. 1995a. Bottom soils, sediment, and pond aquaculture. Chapman and Hall. US. pp 348.

Boyd, C.E. 1995b. Soil and water quality management in aquaculture ponds. Infofish International 5, 29-36.

Boyd, C.E. 2003. Guidelines for aquaculture effluent management at the farm-level. Aquaculture 226, 101-112.

Boyd, C.E., Massaut, L. 1999. Risks associated with the use of chemicals in pond aquaculture. Aquaculture Engineering 20, 113-132.

Boyd, C.E., McNevin, A.A., Clay, J., Johnson, H.M. 2005. Certification issues for some common aquaculture species. Reviews in Fisheries Science 13: 231-279.

Boyd, C.E., Tanner, M.E., Madkour, M., Masuda, K. 1994. Chemical characteristics of bottom soils from freshwater and brackishwater aquaculture ponds. Journal of the World Aquaculture Society 25, 517-534.

Boyd, C.E., Tucker, C.S. 1998. Pond aquaculture water quality management. Fluwer Academic publishers. Masachussets US. pp 700.

Bozdogan, H. 1987. Model selection and akaike's information criterion (AIC): the general theory and its analytical extensions. Psychometrika 52, 345-370.

Brain, R.A., Hanson, M.L., Solomon, K.R., Brooks, B.W. 2008. Aquatic plants exposed to pharmaceuticals: effects and risks. Reviews in Environmental Contamination and Toxicology 192, 67-115.

Brain, R.A., Johnson, D.J., Richards, S.M., Sanderson, H., Sibley, P.K., Solomon, K.R. 2004. Effects of 25 pharmaceutical compounds to *Lemna gibba* using a seven day static-renewal test. Environmental Toxicology and Chemistry 23, 371-382.

Bravo, S., 2012. Environmental impacts and management of veterinary medicines in aquaculture: the case of salmon aquaculture in Chile. In: Bondad-Reantaso, M.G., Arthur, J.R., Subasinghe, R.P. (eds). Improving biosecurity through prudent and responsible use of veterinary medicines in aquatic food production. FAO Fisheries and Aquaculture Technical Paper No. 547. Rome, FAO. pp 207.

Brinke, M., Höss, S., Fink, G., Ternes, T.A., Heininger, P., Traunspurger, W. 2010. Assessing effects of the pharmaceutical ivermectin on meiobenthic communities using freshwater microcosms. Aquatic Toxicology 99, 126-137.

Brock, T.C.M., Arts, G.H.P., Maltby, L., Van den Brink, P.J. 2006. Aquatic risks of pesticides, ecological protection goals and common aims in European Union Legislation. Integrated Environmental Assessment and Management 2, 20–46.

Brooks, B.W., Ankley, G.T., Hobson, J.F., Lazorchak, J.M., Meyerhoff, R.D., Solomon, K.R. 2009. Assessing the aquatic hazards of veterinary medicines. In: Crane, M., Boxall, A.B.A., Barrett, K. (Eds). Veterinary medicines in the environment. SETAC, Pensacola, FL, USA, pp 97-128.

Brooks, B.W., Berninger, J.P., Kristofco, L.A., Ramirez, A.J., Stanley, J.K., Valenti, T.W. 2012. Pharmaceuticals in the environment: lessons learned for reducing uncertainties in environmental risk assessment. Progress in Molecular Biology and Translational Science 112, 231-258.

Burridge, L., Weis, J.S., Cabello, F., Pizarro, J., Bostick, K. 2010. Chemical use in salmon aquaculture: A review of current practices and possible environmental effects. Aquaculture 306, 7-23.

Buschmann, A.H., Tomova, A., López, A., Maldonado, M.A., Henríquez, L.A., Ivanova, L., Moy, F., Godfrey, H.P., Cabello, F.C. 2012. Salmon Aquaculture and Antimicrobial Resistance in the Marine Environment. PLoS ONE 7, e42724.

Bush, S.R., Belton, B., Hall, D., Van der Geest, P., Murray, F.J., Ponte, P., Oosterveer, P., Islam, M.S., Mol, A.P.J., Hatanaka, M., Kruijssen, F., Ha, T.T.T., Little, D.C., Kusumawati, R. 2013. Certify sustainable aquaculture?. Science 341: 1067-1068.

Cabello, F.C. 2006. Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. Environmental Microbiology 8, 1137-1144.

Cabello, F.C., Godfrey, H.P., Tomova, A., Ivanova, L., Dölz, H., Millanao, A., Buschmann, A.H. 2013. Antimicrobial use in aquaculture re-examined: its relevance to antimicrobial resistance and to animal and human health. Environmental Microbiology 15, 1917-1942.

Capone, D.G., Weston, D.P., Miller, V., Shoemaker, C. 1996. Antibacterial residues in marine sediments and invertebrates following chemotherapy in aquaculture. Aquaculture 145, 55-75.

Cardoza, L.A., Knapp, C.W., Larive, C.K., Belden, J.B., Lydy, M., Graham, D.W., 2005. Factors affecting the fate of ciprofloxacin in aquatic field systems. Water Air and Soil Pollution 161, 383-398.

Carpenter, S.R., Stanley, E.H., Vander Zanden, M.J. 2011. State of the world's freshwater ecosystems: physical, chemical and biological changes. Annual Review of Environment and Resources 36, 75-99.

Chinh, N. 2005. Survey on drug and chemical usages in striped catfish (*Pangasianodon hypophthalmus*) farming in An Giang, Dong Thap and Can Tho provinces, Vietnam (in Vietnamese). Master Thesis Report. College of Aquaculture and Fisheries, Can Tho University, Vietnam, pp. 75.

Choo, P.S. 1994. Degradation of oxytetracycline hydrochloride in fresh- and seawater. Asian Fisheries Science 7, 195-200.

Choo, P.S. 1995. Withdrawal time for oxytetracycline in red tilapia cultured in freshwater. Asian Fisheries Science 8, 169-176.

Chopra, I., Roberts, M., 2001. Tetracycline antibiotics: mode of action, applications, molecular biology and epidemiology of bacterial resistance. Microbiology and Molecular Biology Reviews 65, 232-260.

Christensen, A.M., Ingerslev, F., Baun, A. 2006. Ecotoxicity of mixtures of antibiotics used in aquacultures. Environmental Toxicology and Chemistry 25, 2208-2215.

Ciarlone, A.E., Fry, B.W., Ziemer, D.M. 1990. Some observations on the adsorption of tetracyclines to glass and plastic labware. Microchemical Journal 42, 250-255.

CMA, Chinese Ministry of Agriculture. 2002. Regulation NY 5071-2002 on Pollution free aquatic products - Fishery drugs application guideline.

Coelho, S., Oliveira, R., Pereira, S., Musso, C., Domingues, I., Bhujel, R.C., Soares, A.M.V.M., Nogueira, A.J.A. 2011. Assessing lethal and sub-lethal effects of trichlorfon on different trophic levels. Aquatic Toxicology 103, 191-198.

Corsin, F.S., Funge-Smith, S., Clausen, J. 2007. A qualitative assessment of standards and certification schemes applicable to aquaculture in the Asia-pacific region. Asia-Pacific Fisheries Commission (APFIC), Food and Agricultural Organization of the United Nations, FAO Regional Office for Asia and the Pacific, Bangkok, Thailand, pp 98.

Costanzo, S.D., O'Donohue, M.J., Dennison, W.C. 2004. Assessing the influence and distribution of shrimp pond effluent in a tidal mangrove creek in north-east Australia. Marine Pollution Bulletin 48, 514-525.

Costa-Pierce, B.A. 2002. Ecological aquaculture: the evolution of the blue revolution. Blackwell Science Ltd.

Costello, M.J., Grant, A., Davies, I.M., Cecchini, S., Papoutsoglou, S., Quigley, D., Saroglia, M. 2001. The control of chemicals used in aquaculture in Europe. Journal of Applied Ichthyology 17, 173-180.

Coyne, R., Hiney, M., O'Connor, B., Kerry, J., Cazabon, D., Smith, P. 1994. Concentration and persistence of oxytetracycline in sediments under a marine salmon farm. Aquaculture 123, 31-42.

Crumlish, M., Dung, T.T., Turnbull, J.F., Ngoc, N.T.N., Ferguson, H.W. 2002. Identification of *Edwardsiella ictaluri* from diseased freshwater catfish, *Pangasius hypophthalmus* (Sauvage), cultured in the Mekong Delta, Vietnam. Journal of Fish Diseases 25, 733-736.

Cruz-Lacierda, E.R., Corre, V.L., Yamamoto, A., Koyama, J., Matsuoka, J. 2008. Current status of the use of chemicals and biological products and health management practices in aquaculture farms in the Philippines. Memoirs of the Faculty of Fisheries of Kagoshima University 57, 37-45.

Cruz-Lacierda, E.R., De la Peña, L. 2000. The use of chemicals in aquaculture in the Philippines. In: Arthur, J.R., Lavilla-Pitogo, C.R., Subasinghe, R.P. (Eds). Proceedings of the meeting on the use of chemicals in aquaculture in Asia. Tigbauan, Philippines: Southeast Asian Fisheries Development Center, pp 155-184.

Cunningham, V.L., Buzby, M., Hutchinson, T., Mastrocco, F., Parke, N., Roden, N. Effects of human pharmaceuticals on aquatic life: next steps. Environmental Science and Technology 2006, 40, 3456-3462.

D'Costa, V.M., King, C.E., Kalan, L., Morar, M., Sung, W.W. L., Schwarz, C., Froese, D., Zazula, G., Calmels, F., Debruyne, R., Golding, G. B., Poinar, H.N., Wright, G.D. 2011. Antibiotic resistance is ancient. Nature 477, 457-461.

D'Costa, V.M., McGrann, K.M., Hughes, D.W., Wright, G.D. 2006. Sampling the antibiotic resistome. Science 311, 374-377.

Daam, M., Van den Brink, P. 2010. Implications of differences between temperate and tropical freshwater ecosystems for the ecological risk assessment of pesticides. Ecotoxicology 19, 24-37.

Daam, M.A., Crum, S.J.H., Van den Brink, P.J., Nogueira, A.J.A. 2008. Fate and effects of the insecticide chlorpyrifos in outdoor plankton-dominated microcosms in Thailand. Environmental Toxicology and Chemistry 27, 2530-2538.

Daam, M.A., Van den Brink, P.J. 2011. Conducting model ecosystem studies in tropical climate zones: lessons learned from Thailand and way forward. Environmental Pollution 159, 940-946.

De Knecht, J., Boucard, T., Brooks, B.W., Crane, M., Erikson, C., Gerould, S., Koschorreck, J., Scheef, G., Solomon, K.R., Yan, Z. 2009. Environmental risk assessment and management of veterinary medicines. In: Veterinary medicines in the environment. Crane, M., Boxall, A.B.A., Barret, K., (Eds), SETAC Publications. Pensacola, FL, pp 21-55.

De Oliveira-Filho, E.C., Lopes, R.M., Paumgartten, F.J.R. 2004. Comparative study on the susceptibility of freshwater species to copper-based pesticides. Chemosphere 56, 369-374.

De Orte, M.R., Carballeira, C., Viana, I.G., Carballeira, A. 2013. Assessing the toxicity of chemical compounds associated with marine land-based fish farms: The use of mini-scale microalgal toxicity tests. Chemistry and Ecology 29, 554-563.

De Silva, S.S. 2012. Aquaculture: a newly emergent food production sector – and perspectives of its impacts on biodiversity and conservation. Biodiversity and Conservation 21, 3187-3220.

De Silva, S.S., Davy, F.B. 2010. Success stories in Asian aquaculture. Sprincer Science and Business Media B.V. pp 221.

De Zwart, D. 2002. Observed regularities in species sensitivity distributions for aquatic species. In: Posthuma, L., Suter, G.W.I., Traas, T.P. (Eds). Species-Sensitivity Distributions in Ecotoxicology. Lewis, Boca Raton, FL, USA, pp 133-154.

Decamp, O., Moriarty, D.J.W., Lavens, P. 2008. Probiotics for shrimp larviculture: review of field data from Asia and Latin America. Aquaculture Research 39, 334-338.

DePaola, A., Peeler, J.T., Rodrick, G.E. 1995. Effect of oxytetracycline-medicated feed on antibiotic resistance of gram-negative bacteria in catfish ponds. Applied and Environmental Microbiology 61, 2335-2340.

Dietze, J.E., Scribner, E.A., Meyer, M.T., Kolpin, D.W. 2005. Occurrence of antibiotics in water from 13 fish hatcheries, 2001-2003. International Journal of Environmental Analytical Chemistry 85, 1141-1152.

Dobbs, M.G., Cherry, D.S., Scott, J.C., Petrille, J.C. 1995. Environmental assessment of an alkyl dimethyl benyzl ammonium chloride (ADBAC) based molluscicide using laboratory tests. Proceedings of The Fifth International Zebra Mussel and Other Aquatic Nuisance Organisms Conference. Toronto, Canada. pp 87-101.

Dolan, J.R. 2005. Biogeography of aquatic microbes. Aquatic Microbial Ecology 41, 39-48.

Duis, K., Inglis, V., Beveridge, M.C.M., Hammer, C. 1995. Leaching of four different antibacterials from oil- and alginate-coated fish-feed pellets. Aquaculture Research 26, 549-556.

Dung, T.T., 2011. Trial on vaccine against 'Bacillus Necrosis *Pangasius*'(BNP) in grow-out *Pangasius* culture. Vietfish International 8, 76-80.

Dung, T.T., Haesebrouck, F., Sorgeloos, P., Tuan, N.A., Pasmans, F., Smet, A., Decostere, A. 2009. Inc*K* plasmid-mediated tetracycline resistance in *Edwardsiella ictaluri* isolates from diseased freshwater catfish in Vietnam. Aquaculture 295, 157-159.

Dung, T.T., Haesebrouck, F., Tuan, N.A., Sorgeloos, P., Baele, M., Decostere, A. 2008. Antimicrobial susceptibility pattern of *Edwardsiella ictaluri* isolates from natural outbreaks of bacillary necrosis of *Pangasianodon hypophthalmus* in Vietnam. Microbial Drug Resistance 14, 311-316.

Ebert, I., Bachmann, J., Kühnenm, U., Küster, A., Kussatz, C., Maletzki, D., Schlütery, C. 2011. Toxicity of the fluoroquinolone antibiotics enrofloxacin and ciprofloxacin to photoautotrophic aquatic organisms. Environmental Toxicology and Chemistry 30, 2786-2792.

ECB. 2003. Technical Guidance Document in support of Commission Directive 93/67/EEC on Risk Assessment for new notified substances, Commission Regulation (EC) No 1488/94 on Risk Assessment for existing substances and Directive 98/8/EC of the European Parliament and of the Council concerning the placing of biocidal products on the market. In: European Chemicals Bureau, I., Italy (Eds).

Eckardt, N.A. 2010. The chlorella genome: big surprises from a small package. The Plant Cell 22 (2924).

ECOTOX. 2010. ECOTOXicology database of the Environmental Protection Agency of the United States. Available at: www.epa.gov/ecotox.

Egna, H., Boyd, C.E., 1997. Dynamics of pond aquaculture. CRC, Florida. pp 437.

Eguchi, K., Nagase, H., Ozawa, M., Endoh, Y.S., Goto, K., Hirata, K., Miyamoto, K., Yoshimura, H. 2004. Evaluation of antimicrobial agents for veterinary use in the ecotoxicity test using microalgae. Chemosphere 57, 1733-1738.

Ellman, G.L., Courtney, K.O., Anders, V., Featherstone, R.M. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology 7, 88–95.

EMEA, European Medicines Agency. 2000. Note for guidance on the risk analysis approach for residues of veterinary medicinal products in food of animal origin. Veterinary Medicines and Information Technology. EMEA/CVMP/187/00-Consultation.

EMEA, European Medicines Agency. 2007. Environmental impact assessment for veterinary medicinal products in support of the VICH guidelines GL6 (Phase I) and GL38 (Phase II). London (UK): European Medicines Agency. EMEA/CVMP/ERA/ 418282/2005.

EMEA, European Medicines Agency. 2008. Revised Guideline on Environmental Impact Assessment for Veterinary Medicinal Products in Support of the VICH Guidelines Gl6 And Gl 38. EMEA/CVMP/ERA/418282/2005-Rev.1. London.

Emmanuel, E., Keck, G., Blanchard, J.M., Vermande, P., Perrodin, Y. 2004. Toxicological effects of disinfections using sodium hypochlorite on aquatic organisms and its contribution to AOX formation in hospital wastewater. Environment International 30, 891-900.

Ervik, A., Thorsen, B., Eriksen, V., Lunestad, B.T., Samuelsen, O.B. 1994. Impact of administering antibacterial agents on wild fish and blue mussels *Mytilus edulis* in the vicinity of fish farms. Aquaculture 18, 45-51.

EU. 1998. Directive 98/8/EC of the European Parliament and of the Council of 16 February 1998 concerning the placing of biocidal products on the market. Official Journal of the European communities L123, 1-63.

EU. 2001. Directive 2001/82/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to veterinary medicinal products. Official Journal of the European communities L311, 1-66.

EU. 2010. Commission Regulation 37/2010 of 22 December of 2010 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. Official Journal of the European Union L15, 1-72.

EUCAST, European Committee on Antimicrobial Susceptibility Testing). 2010. MIC and Zone Diameter Distributions of Wild Type Microorganisms. Available at: http://www.srga.org/eucastwt/wt_eucast.htm.

Fahl, G.M., Kreft, L., Altenburger, R., Faust, M., Boedeker, W., Grimme, L.H. 1995. pH-Dependent sorption, bioconcentration and algal toxicity of sulfonylurea herbicides. Aquatic Toxicology 31, 175-187.

FAO, Food and Agriculture Organization of the United Nations, 2012a. State of the world fisheries and aquaculture in 2012. FAO fisheries and aquaculture department, Rome, Italy 230.

FAO, Food and Agriculture Organization of the United Nations, 2012b. FishStat Plus. Fishery statistical collections. Released: 18th April 2012. Italy, Rome. Available at: http://www.fao.org/fishery/statistics/software/fishstat/en

FAO, Food and Agriculture Organization of the United Nations. 2009. The State of World Fisheries and Aquaculture 2008. Fisheries and Aquaculture Department. Food and Agriculture Organization of the United Nations, Rome, Italy, pp. 176.

FAO, Food and Agriculture Organization of the United Nations. 2002. Joint FAO/WHO expert committee on food additives. Fifty-eight meeting held between 21-27 February of 2002 in Rome. Summary and conclusions report. pp 9. Available at: http://www.who.int/foodsafety/chem/jecfa/summaries/en/summary_58.pdf

FAO, Food and Agriculture Organization of the United Nations. 2010. FishStat, fishery statistical collections: aquaculture production (1950-2008). Food and Agriculture Organization of the United Nations. Rome, Italy. www.fao.org/fishery/statistics/software/fishstat/en

FAO/OIE/WHO. 2006. Expert consultation on antimicrobial use in aquaculture and antimicrobial resistance: Seoul, Republic of Korea, 13-16 June 2006. Department of food safety, zoonoses and foodborne diseases world health organization. Geneva, Switzerland. pp 107. Available at: http://www.who.int/foodborne_disease/resistance/aqua_jun06/en/index.html

Faruk, M.A.R., Ali, M.M., Patwary, Z.P. 2008. Evaluation of the status of use of chemicals and antibiotics in freshwater aquaculture activities with special emphasis to fish health management. Journal of Bangladesh Agricultural University 6, 381-390.

Faruk, M.A.R., Sultana, N., Kabir, M.B. 2005. Use of chemicals in aquaculture activities in Mymensingh area, Bangladesh. Bangladesh Journal of Fisheries 29, 1-10.

Fernando, C.H. 2002. A guide to tropical freshwater zooplankton. Identification, ecology and impact on fisheries. Backhuys Publishers, Leiden, The Netherlands. pp 290.

Ferreira, C.S., Nunes, B.A., de Melo Henriques-Almeida, J.M., Ghilhermino, L. 2007. Acute toxicity of oxytetracycline and florfenicol to the microalgae *Tetraselmis chuii* and to the crustacean *Artemia parthenogenetica*. Ecotoxicology and Environmental Safety 67, 452-458.

FOOTPRINT Database. 2012. Available at: http://sitem.herts.ac.uk/aeru/vsdb/index.htm.

Francis, C.A., Roberts, K.J., Beman, J.M., Santoro, A.E., Oakley, B.B. 2005. Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. Proceedings of the National Academy of Sciences of the United States of America 102, 14683-14688.

Fu, W., Franco, A., Trapp, S. 2009. Methods for estimating the bioconcentration factor of ionizable organic chemicals. Environmental Toxicology and Chemistry 28, 1372-1379.

Gagliano, G., Mc Namara, F. 1996. Environmental assessment for enrofloxacin - BAYTRIL 3. 23% Concentrate Antimicrobial Solution. Guidel. 21 CFR Part 25.

Galic, N., Hommen, U., Baveco, J.M., Van den Brink, P.J. 2010. Potential application of population models in the European ecological risk assessment of chemicals II: Review of models and their potential applications to address environmental protection aims. Integrated Environmental Assessment and Management 6, 338-360.

Ge, L., Chen, J., Wei, X., Zhang, S., Qiao, X., Cai, X., Xie, Q. 2010. Aquatic photochemistry of fluoroquinolone antibiotics: kinetics, pathways, and multivariate effects of main water constituents. Environmental Science and Technology 44, 2400-2405.

GESAMP. 1997. Towards safe and effective use of chemicals in coastal aquaculture. Reports and Studies, no. 65. Food and Agricultural Organization of the United Nations, Rome, Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection, IMO/FAO/UNESCO-IOC/WMO/WHO/ IAEA/UN/UNEP. pp 40.

Giang, H.T., Ut, G.V., Phuong, N.T. 2008. Study on water quality of intensive catfish culture (*Pangasionodon hypophthalmus*) ponds in An Giang province. Scientific Journal of Can Tho University 1, 1-9 (In Vietnamese).

Girvan, M.S., Campbell, C.D., Killham, K., Prosser, J.I., Glover, L.A. 2005. Bacterial diversity promotes community stability and functional resilience after perturbation. Environmental Microbiology 7, 301-313.

Gräslund, S., Bengtsson, B.E. 2001. Chemicals and biological products used in south-east Asian shrimp farming, and their potential impact on the environment - A review. Science of the Total Environment 280, 93-131.

Gräslund, S., Holmström, K., Wahlström, A. 2003. A field survey of chemicals and biological products used in shrimp farming. Marine Pollution Bulletin 46, 81-90.

Grave, K., Torren-Edo, J., Mackay, D. 2010. Comparison of the sales of veterinary antibacterial agents between 10 European countries. Journal of Antimicrobial Chemotheraphy 5, 2037-2040.

Gregory, K.J., Sexton, P.M., Christopoulos, A. 2007. Allosteric modulation of muscarinic acetylcholine receptors. Current Neuropharmacology 5, 157-167.

Grisez, L., Tan, Z. 2005. Vaccine development for Asian aquaculture. In: Walker P, Lester R, Bondad-Reantaso MG (Eds.), Diseases in Asian Aquaculture V, Fish Health Section, Asian Fisheries Society, Manila. pp. 483-494.

Gudding, R., 2012. Disease prevention as a basis for sustainable aquaculture. In: Bondad-Reantaso, M.G., Arthur, J.R., Subasinghe, R.P. (Eds). Improving biosecurity through prudent and responsible use of veterinary medicines in aquatic food production. FAO Fisheries and Aquaculture Technical Paper No. 547. Rome, FAO. pp 207.

Gullberg, E., Cao, S., Berg, O. G., Ilbäck, C., Sandegren, L., Hughes, D., Andersson, D.I. 2011. Selection of resistant bacteria at very low antibiotic concentrations. PLoS Pathogens 7, 9.

Ha, T.T.T., Bush, S.R., Van Dijk, H. 2013. The cluster panacea?: Questioning the role of cooperative shrimp aquaculture in Vietnam. Aquaculture 388-391, 89-98.

Habenbuch, I.M., Pinckney, J.L. 2000. Toxic effect of the combined antibiotics ciprofloxacin, lincomycin, and tylosin on two species of marine diatoms. Water Research 46, 5028-5036.

Halling-Sörensen, B. 2000. Algal toxicity of antibacterial agents used in intensive farming. Chemosphere 40, 731-739.

Halling-Sørensen, B., Holten Lützhøft, H.-C., Andersen, H.R., Ingerslev, F. 2000. Environmental risk assessment of antibiotics: comparison of mecillinam, trimethoprim and ciprofloxacin. Journal of Antimicrobial Chemotherapy 46, 53-58.

Halling-Sörensen, B., Nielsen, N.S., Lanzky, P.F., Ingerslev, F., Holten Lützhøft, H.C., Jørgensen, S.E. 1998. Occurrence, fate and effects of pharmaceutical substances in the environment- A review. Chemosphere 36, 357-393.

Hastie, T.J., Tibshirani, R.J. 1990. Generalised Additive Models. Chapman and Hall, London, UK.

Haya, K., Burridge, L.E., Davies, I.M., Ervik, A. 2005. A review and assessment of environmental risk of chemicals used for the treatment of sea lice infestations of cultured salmon. In: Hargrave, B. (Eds). Handbook of Environmental Chemistry, Water Pollution, Part M, Volume 5 (2005). pp 305-341.

Henderson, A., Gamito, S., Karakassis, I., Pederson, P., Smaal, A. 2001. Use of hydrodynamic and benthic models for managing environmental impacts of marine aquaculture. Journal of Applied Ichthyology 17, 163-172.

Hendriks, A.J., Van der Linde, A., Cornelissen, G., Sijm, D.T.H.M. 2001. The power of size. 1. Rate constants and equilibrium ratios for accumulation of organic substances related to octanol-water partition ratio and species weight. Environmental Toxicology and Chemistry 20, 1399-1420.

Herbeck, L.S., Unger, D., Wu, Y., Jennerjahn, T.C. 2013. Effluent, nutrient and organic matter export from shrimp and fish ponds causing eutrophication in coastal and back-reef waters in NE Hainan, tropical China. Continental Shelf Research 57, 91-104.

Hernández Serrano, P. 2005. Responsible use of antibiotics in aquaculture. Food and Agriculture Organization of the United Nations, Rome, Italy. pp 97.

Hernando, M.D., De Vettori, S., Martínez, Bueno, M.J., Fernández-Alba, A.R. 2007. Toxicity evaluation with *Vibrio fischeri* test of organic chemicals used in aquaculture. Chemosphere 68, 724-730.

Heuer, O.E., Kruse, H., Grave, K., Collignon, P., Karunasagar, I., Angulo, F.J. 2009. Human health consequences of use of antimicrobial agents in aquaculture. Food Safety 29, 1248-1253.

Ho, B.T., Paul, D.R. 2009. Fatty acid profile of Tra Catfish (*Pangasius hypophthalmus*) compared to Atlantic Salmon (*Salmo solar*) and Asian Seabass (*Lates calcarifer*). International Food Research Journal 16, 501-506.

Hoang, T.T.T., Tu, L.T.C., Le, N.P., Dao, Q.P. 2013. A preliminary study on the phytoremediation of antibiotic contaminated sediment. International Journal of Phytoremediation 15, 65-76.

Hoang, T.T.T., Tu, L.T.C., Le, N.P., Dao, Q.P., Trinh, P.H. 2012. Fate of fluoroquinolone antibiotics in Vietnamese coastal wetland ecosystem. Wetlands Ecological Management 20, 399-408.

Holmström, K., Gräslund, S., Wahlström, A., Poungshompoo, S., Bengtsson, B.E., Kautsky, N. 2003. Antibiotic use in shrimp farming and implications for environmental impacts and human health. International Journal of Food Science and Technology 38, 255-266.

Holten Lützhøft, H.C., Halling-Sørensen, B., Jørgensen, S.E. 1999. Algal toxicity of antibacterial agents applied in Danish fish farming. Archives of Environmental Contamination and Toxicology 36, 1-6.

Hommen, U., Düllmer, U., Vith, D. 1994. A computer program to evaluate plankton data from freshwater field tests. In: Hill, I.R., Heimbach, F., Leeuwangh, P., Matthiesen, P. (Eds). Freshwater field tests for hazard assessment of chemicals. Lewis publishers, Baca Raton, USA. pp 503–513.

Hooper, D.C. 1999. Mode of action of fluoroquinolones. Drugs 58, 6-10.

Hortle, K.G. 2007. Consumption and the yield of fish and other aquatic animals from the Lower Mekong Basin. MRC Technical Paper No. 16, Mekong River Commission, Vientiane. pp 87.

Iman, R.L., Conover, W.J. 1980. Small sample sensitivity analysis techniques for computer models with an application to risk assessment. Communications in Statistics A: theory and methods 9: 1749-1842.

Isidori, M., Lavorgna, M., Nardelli, A., Pascarella, L., Parrella, A. 2005. Toxic and genotoxic evaluation of six antibiotics on non-target organisms. Science of the Total Environment 346, 87-89.

ISO. 1995. ISO 10253. Water quality - Marine algal growth inhibition test with *Skeletonema costatum* and *Phaeodactylum tricornutum*. International Organization for Standardisation, Geneva, Switzerland.

ISO. 1999. Water quality - Determination of acute lethal toxicity to marine copepods (Copepoda, Crustacea). International Organization for Standardisation, Geneva, Switzerland.

Jager, T., Albert, C., Preuss, T.G., Ashauer, R. 2011. General unified threshold model of survival- a toxicokinetic-toxicodynamic framework for ecotoxicology. Environmental Science and Technology 45, 2529-2540.

Jansen, M.J.W. ,Withagen J.C.M., Thissen J.T.N.M. 2005. USAGE: uncertainty and sensitivity analysis in a GenStat environment. Manual. Version 2.0. Wageningen University: Biometris report. Wageningen, The Netherlands. pp 45.

Jansen, M.J.W., Rossing, W.A.H., Daamen, R.A. 1994. Monte Carlo estimation of uncertainty contributions from several independent multivariate sources. In: Grasman, J., van Straten, G (Eds). Predictability and Nonlinear Modelling in Natural Sciences and Economics. Kluwer, Dordrecht. pp 334–343.

Jones, A.D., Bruland, G.L., Agrawal, S.G., Vasudevan, D. 2005. Factors influencing the sorption of oxytetracycline to soils. Environmental Toxicology and Chemistry 24, 761-770.

Jones, O.A.H., Voulvoulis, N., Lester, J.N. 2002. Aquatic environmental assessment of the top 25 English prescription pharmaceuticals. Water Research 36, 5013-5022.

Kahlmeter, G., Brown, D.F.J., Goldstein, F.W., MacGowan, A.P., Mouton, J.W., Österlund, A., Rodloff, A., Steinbakk, M., Urbaskovas, P., Vatopoulos, A. 2003. European harmonization of MIC breakpoints for antimicrobial susceptibility testing of bacteria. Journal of Antimicrobial Chemotherapy 52, 145–148.

Kapaun, E., Reisser, W. 1995. A chitin-like glycan in the cell wall of a *Chlorella* sp. (Chlorococcales, Chlorophyceae). Planta 197, 577-582.

Kim, H.J., Lee, H.J., Lee, D.S., Kwon, J.H. 2009. Modelling the fate of priorty pharmaceuticals in Korea in a conventional sewage treatment plant. Environmental Engineering Research 14, 186-194.

Kim, J., Park, J, Kim, P.G., Lee, C., Choi, K., Choi, K. 2010. Implication of global environmental changes on chemical toxicity – effect of water temperature, pH, and ultraviolet B irradiation on acute toxicity of several pharmaceuticals in *Daphnia magna*. Ecotoxicology 19, 662-669.

Klaver, A.L., Matthews, R.A. 1994. Effects of oxytetracycline on nitrification in a model aquatic system. Aquaculture 123, 237-247.

Knapp, C.W., Cardoza, L.A., Hawes, J.N., Wellington, E.M.H., Larive, C.K., Graham, D.W. 2005. Fate and effects of enrofloxacin in aquatic systems under different light conditions. Environmental Science and Technology 39, 9140-9146.

Koelmans, A.A., Van de Heijde, A., Knijff, L.M., Aalderink, R.H. 2001. Integrated modelling of eutrophication and organic contaminant fate and effects in aquatic ecosystems. A review. Water Research 35, 3517-3536.

Kołodziejska, M., Maszkowska, J., Białk-Bielińska, A., Steudte, S., Kumirska, J., Stepnowski, P., Stolte, S. 2013. Aquatic toxicity of four veterinary drugs commonly applied in fish farming and animal husbandry. Chemosphere 92, 1253-1259.

Koschorreck, J., Koch, C., Rönnefahrt, I. 2002. Environmental risk assessment of veterinary medicinal products in the EU - a regulatory perspective. Toxicology Letters 131, 117-124.

Kreuzinger, N., Fuerhacker, M., Scharf, S., Uhlc, M., Gans, O., Grillitsch, B. 2007. Methodological approach towards the environmental significance of uncharacterized substances - quaternaryammonium compounds as an example. Desalination 215, 209-222.

Kumar, K., Gupta, S.C., Baidoo, S.K., Chander, Y., Rosen, C.J. 2005. Antibiotic uptake by plants from soil fertilized with animal manure. Journal of Environmental Quality 34, 2082-2085.

Kümerer, K. 2009. Antibiotics in the aquatic environment – A review – Part I. Chemosphere 75, 417-432.

Lai, H.T., Hou, J.H., Su, C.I., Chen, C.L. 2009a. Effects of chloramphenicol, florfenicol, and thiamphenicol on growth of algae *Chlorella pyrenoidosa*, *Isochrysis galbana*, and *Tetraselmis chui*. Ecotoxicology and Environmental Safety 72, 329-334.

Lai, H.T., Lin, J.J.2009b. Degradation of oxolinic acid and flumequine in aquaculture pond waters and sediments. *Chemosphere* 75, 462-468.

Lalumera, G.M., Calamari, D., Galli, P., Castiglioni, S., Crosa, G., Fanelly, R. 2004. Preliminary investigation on the environmental occurrence and effects of antibiotics used in aquaculture in Italy. Chemosphere 54, 661-668.

Lam, M.W., Young, C., Brain, R., Johnson, D., Hanson, M., Wilson, C., Richards, S., Solomon, K., Mabury, S. 2004. Aquatic persistence of eight pharmaceuticals in a microcosm study. Environmental Toxicology and Chemistry 23, 1431-1440.

Lampang, K.N., Chongsuvivatwong, V., Kitikoon, V. 2007. Pattern and determinant of antibiotics used on broiler farms in Songkhla province, southern Thailand. Tropical Animal Health and Production 39, 355-361.

Le, T.X., Munekage, Y. 2004. Residues of selected antibiotics in water and mud from shrimp ponds in mangrove areas in Viet Nam. Marine Pollution Bulletin 49, 922–929.

Le, T.X., Munekage, Y., Kato, S. 2005. Antibiotic resistance in bacteria from shrimp farming in mangrove areas. Science of the Total Environment 349, 95-105.

Lebel, L., Mungkung, R., Gheewala, S.H., Lebel, P. 2010. Innovation cycles, niches and sustainability in the shrimp aquaculture industry in Thailand. Environmental Science and Policy 13, 291-302.

Lee, Y.J., Lee, S.E., Lee, D.S., Kim, Y.H. 2008. Risk assessment of human antibiotics in Korean aquatic environment. Environmental Toxicology and Pharmacology 26, 216-221.

Li, N., Zhao, Y.L., Yang, J. 2005. Accumulation, distribution, and toxicology of copper sulfate in juvenile giant freshwater prawns, *Macrobrachium rosenbergii*. Bulletin Environmental Contamination and Toxicology 75, 497-504.

Lin, C.K., Yi, Y. 2003. Minimizing environmental impacts of freshwater aquaculture and reuse of pond effluents and mud. Aquaculture 226, 57-68.

Lin, R., Buijse, L., Dimitrov, M.R., Dohmen, P., Kosol, S., Maltby, L., Roessing, I., Sinkeldam, J.A., Smidt, H., Van Wijngaarden, R.P.A., Brock, T.C.M. 2012. Effects of the fungicide metiram in outdoor freshwater microcosms: responses of invertebrates, primary producers and microbes. Ecotoxicology 21, 1550-1569.

Ling, N. 2003. Rotenone – a review of its toxicity and use for fisheries management. New Zealand Department of Conservation. Science for conservation report no. 211. ISSN 1173-2946

Liu, F., Ying, G.G., Yang, J.F., Zhou, L.J., Tao, R., Wang, L., Zhang, L.J., Peng, P.A. 2010. Dissipation of sulfamethoxazole, trimethoprim, and tylosin in a soil under aerobic and anoxic conditions. Environmental Chemistry 7, 370-376.

Love, D.C., Rodman, S., Neff, R.A., Nachman, K.E. 2011. Veterinary drug residues in seafood inspected by the European Union, United States, Canada, and Japan from 2000 to 2009. Environmental Science and Technology 45, 7232-7240.

Lowry, O.H., Rosenbruogh, N.J., Tarr, A.I., Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. The Journal of Biological Chemistry 193, 265–275.

Ludwig, G.M. 1993. Effects of trichlorfon, fenthion, and diflubenzuron on the zooplankton community and on production of reciprocal-cross hybrid striped bass in culture ponds. Aquaculture 110, 301-319.

Lyle-Fritch, L.P., Romero-Beltrán, E., Páez-Osuna F. 2006. A survey on use of the chemical and biological products for shrimp farming in Sinaloa (NW Mexico). Aquacultural Engineering 35, 135-146.

Mackay, D., Leinonen, P.J. 1975. Rate of evaporation of low-solubility contaminants from water bodies to atmosphere. Environmental Science and Technology 9, 1178-1180.

MacRae, I.H., Chapman, G., Nabi, S.M.N., Dhar, G.C. 2002. A survey of health issues in carp/Macrobrachium culture in rice fields in Bangladesh. In: Arthur, J.R., Phillips, M.J., Subasinghe, R.P., Reantaso, M.B., MacRae, I.H. (Eds). Primary aquatic animal health care in rural, small-scale and aquaculture development. FAO Fisheries Technical Paper No. 406. pp 95-112.

Madigan, M.T., Martinko, J.M., Parker, J. 2003. Brock biology of microorganisms (10th Edition). Pearson Education, Inc. New Jersey, USA. pp 707-716.

Maki, T., Hasegawa, H., Kitami, H., Fumoto, K., Munekage, Y., Ueda, K. 2006. Bacterial degradation of antibiotic residues in marine fish farm sediments of Uranouchi Bay and phylogenetic analysis of antibiotic-degrading bacteria using 16S rDNA sequences. Fisheries Science 72, 811-820.

Maltby, L., Blake, N., Brock, T.C.M., Van Den Brink, P.J. 2005. Insecticide species sensitivity distributions: Importance of test species selection and relevance to aquatic ecosystems. Environmental Toxicology and Chemistry 24, 379-388.

Maltby, L., Brock, T.C.M., Van den Brink, P.J. 2009. Fungicide risk assessment for aquatic ecosystems: importance of interspecific variation, toxic mode of action, and exposure regime. Environmental Science and Technology 43: 7556-7563.

Managaki, S., Murata, A., Takada, H., Tuyen, B.C., Chiem, N.H. 2007. Distribution of macrolides, sulfonamides and trimethoprim in tropical waters: ubiquitous occurrence of veterinary antibiotics in the Mekong delta. Environmental Science and Technology 41, 8004-8010.

Martinez, J.L. 2008. Antibiotics and antibiotic resistance genes in natural environments. Science 321, 365-367.

Martins, N., Pereira, R., Abrantes, N., Pereira, J., Gonçalves, F., Marques, C.R. 2012. Ecotoxicological effects of ciprofloxacin on freshwater species: data integration and derivation of toxicity thresholds for risk assessment. Ecotoxicology 21, 1167-1176.

Massana, R., Jürgens, K., 2003. Composition and population dynamics of planktonic bacteria and bacterivorous flagellates in seawater chemostat cultures. Aquatic Microbial Ecology 32, 11-22.

Maul, J.D., Schuler, L.J., Belden, J.B., Whiles, M.R., Lydy, M.J. 2006. Effects of the antibiotic ciprofloxacin on stream microbial communities and detritivorous macroinvertebrates. Environmental Toxicology and Chemistry 2006, 1598-1606.

Mente, E., Pierce, G.J., Santos, M.B., Neofitou, C. 2006. Effect of feed and feeding in the culture of salmonids on the marine aquatic environment: a synthesis for European aquaculture. Aquaculture International 14, 499-522.

Meredith-Williams, M., Carter, L.J., Fussell, R., Raffaelli, D., Ashauer, R., Boxall, A.B.A. 2012. Uptake and depuration of pharmaceuticals in aquatic invertebrates. Environmental Pollution 165, 250-258.

Metcalfe, C., Boxall, A., Fenner, K., Kolpin, D., Servos, M., Silverhorn, E., Staveley, J. 2009. Exposure assessment of veterinary medicines in aquatic systems. In: Crane, M., Boxall, A.B.A., Barret, K. (Eds). Veterinary medicines in the environment. SETAC Publications. Pensacola, FL, pp. 57-96.

Milan, D.J., Jones, I.L., Ellinor, P.T., MacRae, C.A. 2006. In vivo recording of adult zebrafish electrocardiogram and assessment of drug-induced QT prolongation. American Journal of Physiology: Heart and Circulatory Physiology 291, H269-H273.

Mincer, T.J., Fenical, W., Jensen, P.R. 2005. Culture-dependent and culture-independent diversity within the obligate marine actinomycete genus *Salinispora*. Applied Environmental Microbiology 71, 7019-7028.

Miranda, C., Tello, A., Keen, P. 2013. Mechanisms of antimicrobial resistance in finfish aquaculture environments. Frontiers in Microbiology 4, 233.

Montforts, M.H.M.M. 2005. The Trigger Values in the Environmental Risk Assessment for (Veterinary) Medicines in the European Union: A Critical Appraisal. RIVM Report 601500002/2005. Bilthoven, the Netherlands: RIVM.

Murray, F.J., Clausen, J., Dalsgaard, A., Karunasagar, I., Morris, D. 2012. The EU Rapid Alert System for Foods and Feeds (RASFF): an indicator of non-compliance in seafood trade and challenges to producer countries. Proc. of the World Aquaculture Society Conference, 1-5th of September of 2012. Prague, Czech Republic.

Murray, F.J., Haque, M.M., Zhang, W., Thanh, L.P., Nietes Satapornvanit, A., Little, D.C. 2013. Defining boundaries towards understanding sustainable ethical aquaculture trade between Asia and Europe. SEAT Project Report 2.8. University of Stirling, Stirling, UK.

Muyzer, G., De Waal, E.C., Uitterlinden, A.G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Applied Environmental Microbiology 59, 695-700.

Näslund, J., Hedman, J.E., Agestrand, C. 2008. Effects of the antibiotic ciprofloxacin on the bacterial community structure and degradation of pyrene in marine sediment. Aquatic Toxicology 90, 223-227.

Naylor, R.L., Goldburg, R.J., Primavera, J.H., Kautsky, N., Beveridge, M.C.M., Clay, J., Folke, C., Lubchenco, J., Mooney, H., Troell, M. 2000. Effect of aquaculture on world fish supplies. Nature 405, 1017-1024.

Nga, T.T.P. 2004. Survey on the market channels and usages of drugs and chemicals in shrimp farming in Soc Trang, Bac Lieu and Ca Mau provinces, Vietnam (in Vietnamese). Master Thesis Report. College of Aquaculture and Fisheries, Can Tho University, Vietnam. pp 151.

Nhan, D.K., Verdegem, M.C.J., Milstein, A., Verreth, J.A.V. 2008. Water and nutrient budgets of ponds in integrated agriculture–aquaculture systems in the Mekong Delta, Vietnam. Aquaculture Research 39, 1216-1228.

Nie, X., Wang, X., Chen, J., Zitko, V., An, T. 2007. Response of the freshwater alga Chlorella vulgaris to trichloroisocyanuric acid and ciprofloxacin. Environmental Toxicology and Chemistry 27, 168-173.

NIPH, Norwegian Institute of Public Health. 2009. Pharmaceutical use in Norwegian fish farming in 2001–2008. Electronic citation. Accessed January 2013: (http://www.fhi.no/eway/default.aspx?pid=233&trg=Area_5774&MainLeft_5669=5774:0:&Area_5774=5544:73848::0:5776:1:::0:0).

Noga, E.J. 1996. Fish Disease. Diagnosis and Treatment. Mosby-Year Book, Inc. Missoure. US. pp 367.

Nowara, A., Burhenne, J., Spiteller, M. 1997. Binding of fluoroquinolone carboxylic acid derivatives to clay minerals. Journal of Agricultural and Food Chemistry 45, 1459-1463.

Nymenya, H., Delaunois, A., Duong, D.L., Bloden, S., Defour, J., Nicks, B., Ansay, M. 1999. Short-term toxicity of various pharmacological agents on the *in vitro* nitrification process in a simple closed aquatic system. Alternatives to Laboratory Animals 27, 121-135.

OECD, Organization for Economic Cooperation and Development. 1992. Guidance document for aquatic effects assessment. OECD Environment Monograph 92. Environment Directorate, *OECD, Paris.*

OECD, Organization for Economic Cooperation and Development. 2004. OECD Guidelines for the testing of chemicals. *Daphnia* sp., Acute immobilisation test. pp 12.

OECD, Organization for Economic Cooperation and Development. 2006. OECD Guidelines for the testing of chemicals. Freshwater alga and cyanobacteria, growth inhibition test. pp 25.

Oliveira, R., McDonough, S., Ladewig, J.C.L., Soares, A.M.V.M., Nogueira, A.J.A., Domingues, I. 2013. Effects of oxytetracycline and amoxicillin on development and biomarkers activities of zebrafish (*Danio rerio*). Environmental Toxicology and Pharmacology 36, 903-912.

Olson, A. B., Silverman, M., Boyd, D. A., McGeer, A., Willey, B. M., Pong-Porter, V., Daneman, N., Mulvey, M. R. 2005. Identification of a Progenitor of the CTX-M-9 Group of Extended-Spectrum β-Lactamases from *Kluyvera georgiana* Isolated in Guyana. Antimicrobial Agents and Chemotherapy 49, 2112-2115.

Park, R.A., Clough, J.S., Wellman, M.C. 2008. AQUATOX: Modelling environmental fate and ecological effects in aquatic ecosystems. Ecological Modelling 213, 1-15.

Park, S., Choi, K. 2008. Hazard assessment of commonly used agricultural antibiotics on aquatic ecosystems. *Ecotoxicology* 17, 526-538.

Pathak, S.C., Ghosh, S.K., Palanisamy, K. 2000. The use of chemicals in aquaculture in India. In: Arthur JR, Lavilla-Pitogo CR, Subasinghe RP (Eds). Proceedings of the meeting on the use of chemicals in aquaculture in Asia. Tigbauan, Philippines: Southeast Asian Fisheries Development Centre. pp 87-112.

PEIAR Database (2012). Pharmaceuticals in the Environment (PEIAR). Available at: http://www.chbr.noaa.gov/peiar/default.aspx

Pereira, S.P.P., Oliveira, R., Coelho, S., Musso, C., Soares, A.M.V.M., Domingues, I., Nogeuira, A.J.A. 2014. From sub cellular to community level: toxicity of glutaraldehyde to several aquatic organisms. Science of the Total Environment 470/471, 147-158.

Pérez-Estrada, L.A., Agüera, A., Hernando, M.D., Malato, S., Fernández-Alba, A.R. 2008. Photodegradation of malachite green under natural sunlight irradiation: Kinetic and toxicity of the transformation products. Chemosphere 70, 2068-2075.

Peters, K., Bundschuh, M., Schäfer, R.B. 2013. Review on the effects of toxicants on freshwater functions. Environmental Pollution 180, 324-329.

Petersen, A., Dalsgaard, A. 2003. Species composition and antimicrobial resistance genes of *Enterococcus spp*., isolated from integrated and traditional fish farms in Thailand. Environmental Microbiology 5, 395-402.

Phan, L.T., Bui, T.M., Nguyen, T.T.T., Gooley, G.J., Ingram, B.A., Nguyen, H.V., Nguyen, P.T., De Silva, S. 2009. Current status of farming practices of striped catfish, *Pangasianodon hypophthalmus*, in the Mekong Delta, Vietnam. Aquaculture 296, 227-236.

Phillips, M. 2000. The use of chemicals in carp and shrimp aquaculture in Bangladesh, Cambodia, Lao PDR, Nepal, Pakistan, Sri Lanka and Viet Nam. In: Arthur, J.R., Lavilla-Pitogo, C.R., Subasinghe, R.P. (Eds). Proceedings of the meeting on the use of chemicals in aquaculture in Asia. Tigbauan, Philippines: Southeast Asian Fisheries Development Centre. pp 75-86.

Phong, T.K., Nhung, D.T.T., Hiramatsu, K., Watanabe, H. 2009. Prediction of the fate of oxytetracycline and oxolinic acid in a fish pond using simulation model - A preliminary study. Journal of the Faculty of Agriculture of Kyushu University 54, 513-521.

Phuong, N.T. 2010. Analytical and biological methods in support of sustainable aquaculture practices in Vietnam. A joint Vietnamese-Belgian project FUNDED BY SPO. *Final report (May 2007-April 2009).*

Phuong, N.T., Oanh, D.T.H. 2010. Striped catfish (*Pangasianodon hypophthalmus*) aquaculture in Viet Nam: an unprecedented development within a decade. In: De Silva, S.S., Davy, F.B. (Eds). Success Stories in Asian Aquaculture. Springer, NACA and IDRC, Dordrecht, Bangkok and Ottawa. pp 133-149.

Poirel, L., Kämpfer, P., Nordmann, P. 2002. Chromosome-Encoded Ambler Class A β-Lactamase of Kluyvera georgiana, a Probable Progenitor of a Subgroup of CTX-M Extended-Spectrum β-Lactamases. Antimicrobial Agents and Chemotherapy 46, 4038-4040.

Poirel, L., Lartigue, M.-F., Decousser, J.-W., Nordmann, P. 2005. ISEcp1B-Mediated Transposition of bla CTX-M in Escherichia coli. Antimicrobial Agents and Chemotherapy 49, 1-4.

Posthuma, L., Suter, G.W., Traas, T.P. 2002. Species-sensitivity distributions in ecotoxicology. Lewis, Boca Raton.

Primavera, J.H. 2006. Overcoming the impacts of aquaculture on the coastal zone. Ocean Coastal Management 49, 531-545.

Pro, J., Ortiz, J.A., Boleas, S., Fernández, C., Carbonell, G., Tarazona, J.V., 2003. Effect assessment of antimicrobial pharmaceuticals on the aquatic plant *Lemna minnor*. Bulletin of Environmental Contamination and Toxicology 70, 290-295.

Pruden, A., Larsson, J.D.G., Amézquita, A., Collignon, P., Brandt, K.K., Graham, D.W., Lazorchak, J.M., Suzuki, S., Silley, P., Snape, J.R., Topp, E., Zhang, T., Zhu, Y.-G. 2013. Management options for reducing the release of antibiotics and antibiotic resistance genes to the environment. Environ Health Perspectives 121, 878-885.

Pumart, P., Phodha, T., Thamlikitkul, V., Riewpaiboon, A., Prakongsai, P., Limwattananon, S., 2012. Health and economic impacts of antimicrobial resistance in Thailand. Journal Health Systems Research 6, 352-60.

Qi, Z., Zhang, X., Boon, N., Bossier, P. 2009. Probiotics in aquaculture in China - Current state, problems and prospect. Aquaculture 290, 15-21.

Qin, H., Chen, L., Lu, N., Zhao, Y., Yuan, X. 2011. Toxic effects of enrofloxacin on *Scenedesmus obliquus*. Frontiers in Environmental Science and Engineering in China. 1/2007-5/2011, 1-10.

Qin, J.G., Dong, P. 2004. Acute toxicity of trichlorfon to juvenile yabby *Cherax destructor* (Clark) and selected zooplankton species. Aquaculture Research 35, 1104-1107.

Quinn, B., Gange, F., Blaise, C. 2008. An investigation into the acute and chronic toxicity of eleven pharmaceuticals (and their solvents) found in wastewater effluent on the cnidarian, *Hydra attenuata.* Science of the Total Environment 389, 306-314.

Rawi, S.M., Arafa, M.S., En-Hazmi, M.M. 2011. Evaluation of the effects of ciprofloxacin or gatifloxacin on neurotransmitters levels in rat cortex and hippocampus. Journal of Pharmacology 5, 993-1005.

Redshaw, C.J. 1995. Ecotoxicological risk assessment of chemicals used in aquaculture: a regulatory viewpoint. Aquaculture Research 26, 629-637.

Reimschuessel, R., Stewart, L., Squibb, E., Hirokawa, K., Brady, T., Brooks, D., Shaikh, B., Hosdon, C. 2005. Fish Drug Analysis – Phish-Pharm: a searchable database of pharmacokinetics data in fish. Journal of Applied Sciences 7, E288-E327.

Rendal, C., Kusk, K.O., Trapp, S. 2011. Optimal choice of pH for toxicity and bioaccumulation studies of ionizing organic chemicals. Environmental Toxicology and Chemistry 30, 2395-2406.

Rico, A., Waichman, A.V., Geber-Corrêa, R., Van den Brink, P.J. 2011. Effect of malathion and carbendazim on Amazonian freshwater organisms: comparison of tropical and temperate species sensitivity distributions. Ecotoxicology 20: 625-634.

Rico, A., Satapornvanit, K., Haque, M.M., Min, J., Nguyen, P.T., Telfer, T.C., Van den Brink, P.J. 2012a. Use of chemicals and biological products in Asian aquaculture and their potential environmental risks: a critical review. Reviews in Aquaculture 4, 75-93.

Rico, A., Geng, Y., Focks, A., Van den Brink, P.J. 2012b. ERA-AQUA version 2.0, technical description and manual. A decision support system for the Environmental Risk Assessment of veterinary medicines applied in pond AQUAculture. Alterra report 2320. Wageningen, The Netherlands: Alterra. pp 62.

Rico, A., Phu, T.M., Satapornvanit, K., Min, J., Shahabuddin, A.M., Henriksson, P.J.G., Murray, F., Little, D.C., Dalsgaard, A., Van den Brink, P.J. 2013a. Use of veterinary medicines, feed additives and probiotics in four major internationally traded aquaculture species farmed in Asia. Aquaculture 412/413, 231-243.

Rico, A., Geng, Y., Focks, A., Van den Brink, P.J. 2013b. Modeling environmental and human health risks of veterinary medicinal products applied in pond aquaculture. Environmental Toxicology and Chemistry 32, 1196-207.

Rico, A., Van den Brink, P.J. 2014. Probabilistic risk assessment of veterinary medicines applied to four major aquaculture species produced in Asia. Science of the Total Environment 468/469, 630-641.

Rico, A., Dimitrov, M.R., Van Wijngaarden, R.P.A., Satapornvanit, K., Smidt, H., Van den Brink, P.J. 2014. Effects of the antibiotic enrofloxacin on the ecology of tropical eutrophic freshwater microcosms. Aquatic Toxicology 147, 92-104.

Rico, A., Oliveira, R., McDonough, S., Matser, A., Khatikarn, J., Satapornvanit, K., Nogueira, A.J.A., Soares, A.M.V.M., Domingues, I., Van den Brink, P.J. Submitted. Use, fate and ecological risk of antibiotics applied in tilapia cage farming in Thailand.

Rigos, G., Alexis, M., Nengas, I. 1999. Leaching, palatability and digestibility of oxytetracycline and oxolinic acid included in diets fed to seabass *Decentrarchus labrax* L. Aquaculture Research 30, 841-847.

Rivera-Ferre, M.G. 2009. Can export-oriented aquaculture in developing countries be sustainable and promote sustainable development? The shrimp case. Journal of Agricultural and Environmental Ethics 22, 301-321.

Robinson, A.A., Belden, J.B., Lydy, M.J. 2005. Toxicity of fluoroquinolone antibiotics to aquatic organisms. Environmental Toxicology and Chemistry 24, 423-430.

Rösch, C., Mergel, A., Bothe, H. 2002. Biodiversity of denitrifying and nitrogen-fixing bacteria in an acid forest soil. Applied Environmental Microbiology 68, 3818-3829.

Rose, P.E., Pedersen, J.A. 2005. Fate of oxytetracycline in streams receiving aquaculture discharges: model simulations. Environmental Toxicology and Chemistry 24, 40-50.

Rotthauwe, J.H., Witzel, K.P., Liesack, W. 1997. The ammonia monooxygenase structural gene amoA as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Applied Environmental Microbiology 63, 4704-4712.

Saltelli A, Chan K, Scott EM. 2000. Sensitivity analysis. Wiley, Chichester, UK.

Samuelsen, O.B., Torsvik, V., Ervik, A. 1992. Low-range changes in oxytetracycline concentration and bacterial resistance towards oxytetracycline in a fish farm sediment after medication. Science of the Total Environment 114, 25-36.

Sánchez-Bayo, F., Hyne, R.V. 2011. Comparison of environmental risks of pesticides between tropical and non-tropical regions. Integrated Environmental Assessment and Management 7, 1-10.

Sanderson, H., Ingerslev, F., Brain, R.A., Halling-Sørensen, B., Bestari, J.K., Wilson, C.J., Johnson, D.J., Solomon, K.R. 2005. Dissipation of oxytetracycline, chlortetracycline, tetracycline and doxycycline using HPLC-UV and LC/MS/MS under aquatic semi-field microcosm conditions. Chemosphere 60, 619-629.

Sanderson, H., Laird, B., Pope, L., Brain, R., Wilson, C., Johnson, D., Bryning, G., Peregrine, A.S., Boxall, A., Solomon, K. 2007. Assessment of the environmental fate and effects of ivermectin in aquatic mesocosms. Aquatic toxicology 85, 229-240.

Sanguinetti, C.J., Dias Neto, E., Simpson, A.J. 1994. Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. Biotechniques 17, 914–921.

Santos, L.H.M.L.M., Araújo, A.N., Fachini, A., Pena, A., Delerue-Matos, C., Montenegro, M.C.B.S.M. 2010. Ecotoxicological aspects related to the presence of pharmaceuticals in the aquatic environment. Journal of Hazardous Materials 175, 45-95.

Sapkota, A., Sapkota, A.R., Kucharski, M., Burke, J., McKenzie, S., Walker, P., Lawrence, R. 2008. Aquaculture practices and potential human health risks: current knowledge and future priorities. Environment International 34, 1215-1226.

Sarmah, A.K., Meyer, M.T., Boxall, A.B.A. 2006. A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. Chemosphere 65, 725-759.

Sarter, S., Nguyen, H.N.K., Hung, L.T., Lazard, J., Montet, D. 2007. Antibiotic resistance in Gram-negative bacteria isolated from farmed catfish. Food Control 17, 1391-1396.

Satapornvanit, K., Baird, D.J., Little, D.C. 2009. Laboratory toxicity test and post-exposure feeding inhibition using the giant freshwater prawn *Macrobrachium rosenbergii*. *Chemosphere* 74, 1209-1215.

Schanné, C., Van der Kolk, J. 2001. [14C]-Deltamethrin formulated as emulsifiable concentrate (25 g/L Deltamethrin): Outdoor aquatic microcosm study of the ecological effects and environmental fate. Report No. C015510. Springborn Laboratories, Horn, Switzerland.

Schmidt, A.S., Bruun, M.S., Larsen, J.L., Dalsgaard, I. 2001. Characterization of class 1 integrons associated with R-plasmids in clinical *Aeromonas salmonicida* isolates from various geographical areas. Journal of Antimicrobial Chemotherapy 47, 735-743.

Schmidt, W., O'Shea, T., Quinn, B. 2012. The effect of shore location on biomarker expression in wild *Mytilus spp.* and its comparison with long line cultivated mussels. Marine Environmental Research 80, 70-76.

SEPA, Scottish Environmental Protection Agency. 2003. Regulatory and monitoring of marine cage fish farming in Scotland: a manual of procedures. Available at: www.sepa.org.uk/guidance/fishfarmmanual/manual.asp

Shimizu, A., Takada, H., Koike, T., Takeshita, A., Saha, M., Rinawati, Nakada, N., Murata, A., Suzuki, T., Suzuki, S., Chiem, N.H., Tuyen, B.C., Viet, P.H., Siringam, M.A., Kwan, C., Zakaria, M.P., Reungsang, A. 2013. Ubiquitous occurrence of sulfonamides in tropical Asian waters. Science of the Total Environment 452-453, 108-115.

Smith, P. 2012. Antimicrobial resistance: complexities and difficulties of determination. In: Bondad-Reantaso, M.G., Arthur, J.R., Subasinghe, R.P. (Eds). Improving biosecurity through prudent and responsible use of veterinary medicines in aquatic food production, FAO Fisheries and Aquaculture Technical Paper No. 547. Rome, FAO. pp 207.

Solomon, K., Giesy, J., Jones, P. 2000. Probabilistic risk assessment of agrochemicals in the environment. Crop Protection 19, 649-655.

Solomon, K.R., Brock, T.C.M., De Zwart, D., Dyer, S.D., Posthuma, L., Richards, S.M., Sanderson, H., Sibley P.K., Van den Brink P.J. (Eds). 2008. Extrapolation practice for ecotoxicological effect characterization of chemicals. SETAC Press & CRC Press, Boca Raton, USA.

Somga, S.S., Somga, J.R. and Regidor, S.E. 2012. Use of veterinary medicines in Phillippine aquaculture: current status. In: Bondad-Reantaso, M.G., Arthur, J.R., Subasinghe, R.P. (Eds). Improving biosecurity through prudent and responsible use of veterinary medicines in aquatic food production, pp 69-82. FAO Fisheries and Aquaculture Technical Paper No. 547. Rome, FAO. pp 207.

Soto, D., Aguilar-Manjarrez, J., Brugère, C., Angel, D., Bailey, C., Black, K., Edwards, P., Costa-Pierce, B., Chopin, T., Deudero, S., Freeman, S., Hambrey, J., Hishamunda, N., Knowler, D., Silvert, W., Marba, N., Mathe, S., Norambuena, R., Simard, F., Tett, P., Troell, M., Wainberg, A. 2008. Applying an ecosystem-based approach to aquaculture: principles, scales and some management measures. In: D. Soto, J. Aguilar-Manjarrez and N. Hishamunda (eds). Building an ecosystem approach to aquaculture. FAO/Universitat de les Illes Balears Expert Workshop. 7-11 May 2007, Palma de Mallorca, Spain. FAO Fisheries and Aquaculture Proceedings. No. 14. Rome, FAO. pp 15-35.

SPAH, Schering-Plough Animal Health, 2004. Environmental assessment of AQUAFLOR@50 type a medicated article for catfish. INAD 8519. 70 pp.

Srivastava, S., Sinha, R., Roy, D., 2004. Toxicological effects of malachite green. Aquatic Toxicology 66, 319-329.

Stepanauskas, R., Glenn, T.C., Jagoe, C.H., Tuckfield, R.C., Lindell, A.H., King, C.J., McArthur, J.V. 2006. Co-selection for microbial resistance to metals and antibiotics in freshwater microcosms. Environmental Microbiology 8, 1510-1514.

Sukul, P., Spiteller, M. 2007. Fluoroquinolone antibiotics in the environment. Reviews in Environmental Contamination and Toxicology 191, 131-162.

Sumpradit, N., Chongtrakul, P., Anuwong, K., Pumtong, S., Kongsomboon, K., Butdeemee, P., Khonglormyati, J., Chomyong, S., Tongyoung, P., Losiriwat, S., Seesuk, P., Suwanwaree, P., Tangcharoensathien, V. 2012. Antibiotics Smart Use: a workable model for promoting the rational use of medicines in Thailand. Bulletin of the World Health Organization 90, 905-913.

Supriyadi, H., Rukyani, A. 2000. The use of chemicals in aquaculture in Indonesia. In: Arthur, J.R., Lavilla-Pitogo, C.R., Subasinghe, R.P. (Eds). Proceedings of the meeting on the use of chemicals in aquaculture in Asia. Tigbauan, Philippines: Southeast Asian Fisheries Development Centre. pp 113-118.

Suzuki, M.T., Taylor, L.T., DeLong, E.F. 2000. Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5'-nuclease assays. Applied Environmental Microbiology 66, 4605-4614.

Suzuki, S., Hoa, P.T.P. 2012. Distribution of quinolone, sulfonamides, tetracyclines in aquatic environment and antibiotic resistance in Indochina. Frontiers in Microbiology 3, 67.

Tai, M.V. 2012. Use of veterinary medicines in Vietnamese aquaculture: current status. In Bondad Reantaso, M.G., Arthur, J.R., Subasinghe, R.P. (Eds). Improving biosecurity through prudent and responsible use of veterinary medicines in aquatic food production. pp 91-98. FAO Fisheries and Aquaculture Technical Paper No. 547. Rome, FAO. pp 207.

Takasu, H., Suzuki, S., Reungsang, A., Viet, P.H. 2011. Fluoroquinolone (FQ) contamination does not correlate with occurrence of FQ-resistant bacteria in aquatic environments of Vietnam and Thailand. Microbes and Environments 26, 135-143.

Telfer, T.C., Baird, D.J., McHenery, J.G., Stone, J., Sutherland, I., Wislocki, P. 2006. Environmental effects of the anti-sea lice (Copepoda: Caligidae) therapeutant emamectin benzoate under commercial use conditions in the marine environment. Aquaculture 260, 163–180.

Tello, A., Austin, B., and Telfer, T.C. 2012. Selective pressure of antibiotic pollution on bacteria of importance to public health. Environmental Health Perspectives 120, 1100-1106.

Tello, A., Corner, R.A., Telfer, T.C. 2010. How do land-based salmonid farms affect stream ecology? Environmental Pollution 158, 1147-1158.

Tendencia, E.A., De la Peña, L.D. 2001. Antibiotic resistance of bacteria from shrimp ponds. Aquaculture 195, 193-204.

Ter Braak, C.J.F., Šmilauer, P. 2012. Canoco reference manual and user's guide: software for ordination (version 5.0). Microcomputer Power (Ithaca, NY, USA). pp 496.

Ternes, T.A., Joss, A., Siegrist, H. 2004. Scrutinizing pharmaceuticals and personal care products in wastewater treatment. Environmental Science and Technology 38, 392A-399A.

Thaipichitburapa, P., Meksumpun, C., Meksumpun, S. 2010. Province-based self-remediation efficiency of the Tha Chin river basin, Thailand. Water Science and Technology 62, 594-602.

Thiele-Bruhn, S. 2003. Pharmaceutical antibiotic compounds in soils - a review. Journal of Plant Nutrition and Soil Science 166, 145-167.

Thinh, N.H., Kuo, T.Y., Hung, L.T., Loc, T.H., Chen, S.C., Evensen, O., Schuurman, H.J. 2009. Combined immersion and oral vaccination of Vietnamese catfish (*Pangasianodon hypophthalmus*) confers protection against mortality caused by *Edwardsiella ictaluri.* Fish and Shellfish Immunonology 27, 773-776.

Thurman, E.M., Dietze, J.E., Scribner, E.A. 2002. Occurrence of antibiotics in water from fish hatcheries. USGS Fact Sheet 120–02. U.S. Geological Survey.

Thuy, H.T.T., Nga, L.P., Loan, T.T.C. 2011. Antibiotic contaminants in coastal wetlands from Vietnamese shrimp farming. Environmental Science and Pollution Research 18, 835-841.

Tišler, T., Zagorc-Končan, J. 1997. Comparative assessment of toxicity of phenol, formaldehyde, and industrial wastewater to aquatic organisms. Water, Air, and Soil Pollution 97, 315-322.

Tonguthai, K. 2000. The use of chemicals in aquaculture in Thailand. In: Arthur JR, Lavilla-Pitogo CR, Subasinghe RP (Eds.), Proceedings of the meeting on the use of chemicals in aquaculture in Asia. Tigbauan, Philippines: Southeast Asian Fisheries Development Centre. pp 207-220.

Tu, H.T. 2009. Biomarkers in black tiger shrimp (*Penaeus monodon*) exposed to antibiotics and pesticides. PhD Thesis. University of Namur, Belgium.

Tu, H.T., Phuong, N.T., Silvestre, F., Douny, C., Tao, C.T., Maghuin-Rogister, G., Kestemont, P. 2006. Investigation on the use of drugs and chemicals in shrimp farming and the kinetics of enrofloxacin and furazolidone in black tiger shrimp (*Penaeus monodon*). Scientific Journal of Can Tho University 1, 70-78 (In Vietnamese).

Tu, H.T., Silvestre, F., Bernard, A., Douny, C., Phuong, N.T., Tao, C.T., Maghuin-Rigister, G. 2008. Oxidative stress response of black tiger shrimp (*Penaeus monodon*) to enrofloxacin and to culture system. Aquaculture 285, 244-248.

Tu, H.T., Silvestre, F., Phuong, N.T., Kestemont, P. 2009a. Effects of pesticides and antibiotics on penaeid shrimp with special emphases on behavioral and biomarker responses. Environmental Toxicology and Chemistry 4, 929-938.

Tu, H.T., Silvestre, F., Scippo, M.-L., Thome, J.-P., Phuong, N.T., Kestemont, P. 2009b. Acetylcholinesterase activity as a biomarker of exposure to antibiotics and pesticides in the black tiger shrimp (*Penaeus monodon*). Ecotoxicology and Environmental Safety 72, 1463-1470.

Tukwinas, S. 2002. Auditing System for Quality Cultured Shrimp by the Department of Fisheries. Thai Fisheries Gazette, Volume 55, No. 3, May-June 2002. pp 227-243 (In Thai).

Tuševljak, N., Dutil, L., Rajić, A., Uhland, F.C., McClure, C., St-Hilaire, S., Reid-Smith, R.J., McEwen, S.A. 2013. Antimicrobial use and resistance in aquaculture: findings of a globally administered survey of aquaculture-allied professionals. Zoonoses Public Health 60, 426-436.

Tzeneva, V.A., Heilig, H.G., Van Vliet, W.A., Akkermans, A.D., De Vos, W.M., Smidt, H. 2008. 16S rRNA targeted DGGE fingerprinting of microbial communities. Methods in Molecular Biology 410, 335-449.

US EPA. 2006. Re-registration Eligibility Decision for Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC). United States Environmental Protection Agency. Prevention, Pesticides and Toxic Substances Unit. EPA739-R-06-009. pp 126.

US EPA. 2011. Estimation Programs Interface Suite™ for Microsoft® Windows, v 4.10. United States Environmental Protection Agency, Washington, DC, USA. Available at: http://www.epa.gov/oppt/exposure/pubs/episuite.htm

US EPA. 2012. Estimation Programs Interface Suite™ for Microsoft® Windows, v 4.10. United States Environmental Protection Agency, Washington, D.C., US. Available at: http://www.epa.gov/oppt/exposure/pubs/episuite.htm

Van den Brink, P.J., Blake, N., Brock, T.C.M, Maltby L. 2006. Predictive value of Species Sensitivity Distributions for effects of herbicides in freshwater ecosystems. Journal of Human and Ecological Risk Assessment 12, 645-674.

Van den Brink, P.J., Hattink, J., Bransen, F., Van Donk, E., Brock, T.C.M. 2000. Impact of the fungicide carbedazim in freshwater microcosms. II. Zooplankton, primary producers and final conclusions. Aquatic Toxicology 48, 251–264.

Van den Brink, P.J., Roelsma, J., Van Nes, E.H., Scheffer, M., Brock, T.C.M. 2002. Perpest model, a case-based reasoning approach to predict ecological risks of pesticides. Environmental Toxicology and Chemistry 21: 2500-2506.

Van den Brink, P.J., Tarazona, J.V., Solomon, K.R., Knacker, T., Van den Brink, N.W., Brock, T.C.M., Hoogland, J.P.H. 2005. The use of terrestrial and aquatic microcosms and mesocosms for the ecological risk assessment of veterinary medicinal products. Environmental Toxicology and Chemistry 24, 820-829.

Van den Brink, P.J., Ter Braak, C.J.F. 1999. Principal response curves: analysis of time-dependent multivariate responses of a biological community to stress. Environmental Toxicology and Chemistry 18, 138-148.

Van den Brink, P.J., Van Wijngaarden, R.P.A., Lucassen, W.G.H, Brock, T.C.M., Leeuwangh, P. 1996. Effects of the insecticide Dursban® 4E (a.i. chlorpyrifos) in outdoor experimental ditches. II. Invertebrate community responses. Environmental Toxicology and Chemistry 15, 1143-1153.

Van der Grinten, E., Pikkemaat, M.G., Van den Brandhof, E.J., Stroomberg, G.J., Kraak, M.H.S. 2010. Comparing the sensitivity of algal, cyanobacterial and bacterial bioassays to different groups of antibiotics. Chemosphere 80, 1-6.

Van der Linde, A., Hendriks, A.J., Sijm, D.T.H.M. 2001. Estimating biotransformation rate constants by substracting predicted physico-chemical elimination from experimentally derived elimination. Chemosphere 44, 423-435.

Van Houtte, A. 2000. Preliminary review of the legal framework governing the use of chemicals in aquaculture in Asia. In: Arthur, J.R., Lavilla-Pitogo, C.R., Subasinghe, R.P. (Eds). Proceedings of the meeting on the use of chemicals in aquaculture in Asia. Tigbauan, Philippines: Southeast Asian Fisheries Development Centre. pp 61-74.

Van Leeuwen, C.J., Vermeire, T.G. 2007. Risk assessment of chemicals: an introduction. 2nd Ed. Springer, Dordrecht, The Netherlands.

Van Vlaardingen, P.L.A., Traas, T.P., Wintersen, A.M., Aldenberg, T. 2004. ETX 2.0. A program to calculate hazardous concentrations and fraction affected, based on normally distributed toxicity data. Bilthoven, the Netherlands: National Institute for Public Health and the Environment (RIVM). Report no. 601501028/2004. p 68

Van Wijngaarden, R.P.A., Brock, T.C.M., Van den Brink, P.J. 2005. Threshold levels for effects of insecticides in freshwater ecosystems: a review. Ecotoxicology 14, 355-380.

Vass, M., Hruska, K., Franek, M. 2008. Nitrofuran antibiotics: a review on the application, prohibition and residual analysis. Veterinary Medicine 53, 469-500.

VICH. 2000. International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Products. Topic GL6: Environmental Impact Assessments (EIAs) for Veterinary Medicinal Products (VMPs) – Phase I. London (UK): VICH. CVMP/VICH/592/98.

VICH. 2004. International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Products. Topic GL38: Environmental Impact Assessment (EIAs) for Veterinary Medicinal Products (VMPs) - Phase II. London (UK): VICH. CVMP/VICH/790/03.

VMARD. 2009. Vietnamese Ministry of Agriculture and Rural Development. Circular No 15/2009/TT-BNN, dated 17/3/2009, on the list of drugs, chemicals and antibiotics banned or restricted to use in Vietnamese aquaculture (In Vietnamese).

VMARD. 2012. Ministry of Agriculture and Rural Development of Vietnam. Cicular No.03/2012/ TT-BNN dated January 16, 2012 on removing Cypermethrin, Deltamethrin and Enrofloxacin from the list of limited veterinary drugs and products use in aquaculture and adding Cypermethrin, Deltamethrin and Enrofloxacin to the list of banned veterinary drugs and products use in aquaculture (In Vietnamese).

Von Bertalanffy, L. 1938. A quantitative theory of organic growth. Human Biology 10, 181-213.

Walne, P.R. 1970. Studies on the food value of nineteen genera of algae to juvenile bivalves of the genera *Ostrea*, *Crassostrea*, *Mercenaria*, and *Mytilis*. Fishery Investigation. Series 2, vol. 26, no. 5. 62 pp.

Wang, N., Nkejabega, N., Hien, N.N., Huynh, T.T., Silvestre, F., Phuong, N.T., Danyi, S., Widart, J., Douny, C., Scippo, M.L., Kestemont, P., Huong, D.T.T. 2009. Adverse effects of enrofloxacin when associated with environmental stress in Tra catfish (*Pangasianodon hypophthalmus*). Chemosphere 77, 1577-1584.

Wang, Y.B., Li, J.R., Lin, J. 2008. Probiotics in aquaculture: Challenges and outlook. Aquaculture 281, 1-4.

Wei, R., Ge, F., Chen, M., Wang, R. 2012. Occurrence of ciprofloxacin, enrofloxacin, and florfenicol in animal wastewater and water resources. Journal of Environmental Quality 41, 1481-1486.

Wheeler, J.R., Leung, K.M.Y., Morritt, D., Sorokin, N., Rodgers, H., Toy, R., Holt, M., Whitehouse, P., Crane, M. 2002. Freshwater to saltwater toxicity extrapolations using species sensitivity distributions. Environmental Toxicology and Chemistry 21, 2459-2467.

WHO. 2006. Antimicrobial use in aquaculture and antimicrobial resistance. Report of a joint FAO/OIE/WHO expert consultation on antimicrobial use in aquaculture and antimicrobial resistance. Seoul, Republic of Korea, 13-16 June 2006. Department of food safety, zoonoses and foodborne diseases. World Health Organization, Geneva, Switzerland. pp 97.

WHO. 2012. Critically important antimicrobials for human medicine, 3rd Revision. Available at: http://apps.who.int/iris/bitstream/10665/77376/1/9789241504485_eng.pdf

Williams, D.A. 1972. The comparison of several dose levels with zero dose control. Biometrics 28, 519-531.

Williams, R.R., Bell, T.A., Lighter, D.V. 1992. Shrimp antimicrobial testing II. Toxicity testing and safety determination for twelve antimicrobials with penaied shrimp larvae. Journal of Aquatic Animal Health 4, 262-270.

Willis, K.J., Gillibrand, P.A., Cromey, C.J., Balck, K.D. 2005. Sea lice treatments on salmon farms have no adverse effects on zooplankton communities: a case study. Marine Pollution Bulletin 50, 806-816.

Wilson, C., Brain, R.A., Sanderson, H., Johnson, D.J., Bestari, K.T., Sibley, P.K., Solomon, K.R. 2004. Structural and functional responses of plankton to a mixture of four tetracyclines in aquatic microcosms. Environmental Science and Technology 38, 6430-6439.

WMO. 2013. World Meteorological Organization. Data retrieved on 27-3-2013 from: www.wmo.int.

Wollenberger, L., Halling-Sörensen, B., Kusk, K.O. 2000. Acute and chronic toxicity of veterinary antibiotics to *Daphnia magna*. Chemosphere 40, 723-730.

Wongrat, L. 2000. Zooplankton. Kasetsart University Press, Bangkok, Thailand. 2nd Printing. pp 787.

Wunder, D.B., Tan, D.T., LaPara, T.M., Hozalski, R.M. 2013. The effects of antibiotic cocktails at environmentally relevant concentrations on the community composition and acetate biodegradation kinetics of bacterial biofilms. Chemosphere 90, 2261-2266.

Xue, B., Zhang, R., Wang, Y., Liu, Z., Li, J., Zhang, G. 2013. Antibiotic contamination in a typical developing city in south China: occurrence and ecological risks in the Yongjiang River impacted by tributary discharge and anthropogenic activities. Ecotoxicology and Environmental Safety 92, 229-236.

Yan, C., Dinh, Q.T., Chevreuil, M., Garnier, J., Roose-Amsaleg, C., Labadie, P., Laverman, A.M. 2013. The effect of environmental and therapeutic concentrations of antibiotics on nitrate reduction rates in river sediment. Water Research 47, 3654-3662.

Yang, J.-F., Ying, G., Zhao, J.-L., Tao, R., Su, H.-C., Chen, F. 2010. Simultaneous determination of four classes of antibiotics in sediments of the Pearl Rivers using RRLC-MS/MS. Science of the Total Environment 408, 3424-3432.

Yang, M.C., Fang, J.M., Kuo, T.F., Wang, D.M., Huang, Y.L., Liu, L.Y., Chen, P.H., Chang, T.H. 2007. Production of antibodies for selective detection of malachite green and the related triphenylmethane dyes in fish and fish pond water. Journal of Agriculture Food Chemistry 55, 8851-8856.

Yang, X.L., Zheng, Z.L. 2007. The application status and strategy of fishery medicine in China (in Chinese). Journal of Shanghai Fisheries University 16, 374-380.

Yergeau, E., Sanschagrin, S., Waiser, M.J., Lawrence, J.R., Greer, C.W. 2012. Sub-inhibitory concentrations of different pharmaceutical products affect the meta-transcriptome of river biofilm communities cultivated in rotating annular reactors. Environmental Microbiology Reports 4, 350-359.

Yuan, X., Chen, W. 2012. Use of veterinary medicines in Chinese aquaculture: current status. In: Bondad-Reantaso, M.G., Arthur, J.R., Subasinghe, R.P., (Eds). Improving biosecurity through prudent and responsible use of veterinary medicines in aquatic food production. pp 51-67. FAO Fisheries and Aquaculture Technical Paper No. 547. Rome, FAO. pp 207.

Yulin, J. 2000. The use of chemicals in aquaculture in the People's Republic of China. In: Arthur, J.R., Lavilla-Pitogo, C.R., Subasinghe, R.P. (Eds). Proceedings of the meeting on the use of chemicals in aquaculture in Asia. Tigbauan, Philippines: Southeast Asian Fisheries Development Centre. pp 141-153.

Zhang, T., Li, B. 2011. Occurrence, transformation, and fate of antibiotics in municipal wastewater treatment plants. Critical Reviews in Environmental Science and Technology 41, 951-998

Zheng, Z.L., Xiang, L. 2002. Status of drug use in fisheries in China (in Chinese). Aquaculture (Chinese Journal) 4, 36-39.

Zhou, L.-J., Ying, G.-G., Zhao, J.-L., Yang, J.-F., Wang, L., Yang, B., Liu, S. 2011. Trends in the occurrence of human and veterinary antibiotics in the sediments of the Yellow River, Hai River and Liao River in northern China. Environmental Pollution 159, 1877-1885.

Zou, S., Xu, W., Zhang, R., Tang, J., Chen, Y., Zhang, G. 2011. Occurrence and distribution of antibiotics in coastal water of the Bohai Bay, China: impacts of river discharge and aquaculture activities. Environmental Pollution 159, 2913-2920.

Zounková, R., Klimešova, Z., Nepejchalová, L., Hilscherová, K., Bláha, L. 2011. Complex evaluation of ecotoxicity and genotoxicity of antimicrobials oxytetracycline and flumequine used in aquaculture. Environmental Toxicology and Chemistry 30, 1184-1198.

Summary

Asian aquaculture accounts for nearly 90% of the global aquaculture production and has undergone decades of unprecedented growth. One of the major constraints for the development and expansion of the Asian aquaculture industry has been the proliferation of disease outbreaks. To overcome this issue, a wide range of veterinary medicines including antibiotics, parasiticides and medical disinfectants have recently been introduced in Asian aquaculture. Residual concentrations of veterinary medicines applied to aquaculture ponds or cages may enter the environment by several routes, including effluent discharges, leaching from medicated feeds and excretion by treated animals. Environmental contamination with veterinary medicines has raised concerns on its potential implications for biodiversity loss in surrounding aquatic ecosystems and for the development of antimicrobial resistance. In addition, the deterioration of aquatic ecosystems could lead to increased mortalities and dramatic economic consequences for the sector, since aquatic ecosystems provide the water resources needed for the farming operations. Therefore, research into the Environmental Risk Assessment (ERA) of aquaculture medicines is urgently needed to support the long-term sustainability of the Asian aquaculture production sector.

The aims of this thesis were: (1) to assess the current use of veterinary medicines in Asian aquaculture production; (2) to develop modelling tools to support the risk assessment of aquaculture medicines; (3) to identify compounds and Asian aquaculture production scenarios that may pose high environmental risks; and (4) to monitor the environmental fate of aquaculture antibiotics and to assess their risks for tropical aquatic ecosystems.

This thesis begins with a literature review on the use of chemicals and biological products in the major aquaculture producing countries of Asia (**Chapter 2**). This review discusses the state-of-the-art on the risk assessment of aquaculture chemicals and emphasizes the need to include the ERA paradigm into the registration and evaluation of aquaculture medicines in Asia, as already done in most developed countries. In addition, the review shows that the information available on all aspects related to the risk assessment of aquaculture medicines is very limited and highlights the need (1) to collect up-to-date data on chemical use practices, (2) to develop risk assessment modelling tools tailored to the characteristics of the most important aquaculture production systems in Asia, and (3) to monitor the exposure and effects of aquaculture medicines on tropical aquatic ecosystems impacted by aquaculture pollution.

Chapter 3 shows the outcomes of the most extensive chemical use survey performed in Asian aquaculture, which includes four aquaculture commodities (Penaeid shrimps, *Macrobrachium* prawn, tilapia, and *Pangasius* catfish) produced in four major aquaculture production nations of Asia: China, Thailand, Vietnam and Bangladesh. During this survey, sixty different veterinary medicinal ingredients were recorded, together with their mode of application, reported dosages, and treatment durations. The prevalence of the use of antimicrobial treatments is significantly higher in the Vietnamese *Pangasius* farms than in farms of the other investigated species. The survey also suggested that the use of antibiotic treatments in semi-intensive and intensive shrimp farms in China, Thailand and Vietnam, was lower than was reported in previous surveys. Farmers generally do not exceed recommended dosages of veterinary medicines, and chemical use practices in the investigated farms are generally in accordance with national and international regulations. Factors underlying the observed differences in chemical use patterns were also investigated. Geographical location is the most important factor influencing chemical application patterns, and stocking density and production intensity play a major role for the chemical use in

shrimp farming. Moreover, the adoption of certification schemes does not always correlate with lower antimicrobial use.

Chapter 4 describes the scientific background and potential applications of the ERA-AQUA model, a risk assessment model that was developed to perform environmental and human health risk assessments of veterinary medicines applied in pond aquaculture. This chapter defines the input parameters, sub-models and underlying equations used by the ERA-AQUA model, and shows the outcomes of a sensitivity analysis that identifies the most important parameters to take into consideration when parameterizing the model (i.e., fraction of organic matter in sediment, temperature, organism weight, and the organic carbon and octanol-water partition coefficients characteristics of the substance). In this chapter, the applications of the model are shown by performing a risk assessment for two antimicrobials (oxytetracycline and benzalkonium chloride) applied to a *Pangasius* catfish scenario.

In **Chapter 5,** risk assessments were performed to identify chemicals and Asian aquaculture production scenarios that pose a major environmental hazard. The risk calculations were performed by using the ERA-AQUA model and the dataset generated in Chapter 3, together with up-to-date information on aquaculture production practices and physico-chemical and toxicological properties of the evaluated compounds. This chapter demonstrates that production intensity and water exchange positively correlates to the environmental discharge of veterinary medicines and their environmental risk potential. Of all the studied compounds, the highest risks are posed by the application of some antiparasitic compounds, due to their high toxic potential for non-target invertebrate communities. Of all evaluated scenarios, the highest environmental risks are posed by the *Pangasius* catfish production in Vietnam, followed by the shrimp production in China. The risk-based ranking of compounds and scenarios performed in this study offers a prioritisation list of chemicals to be evaluated in further chemical and biological field and laboratory monitoring research for the combination of species and countries investigated.

In **Chapter 6,** the environmental fate and ecological risks posed by the use of the antibiotics oxytetracycline and enrofloxacin in tilapia cage farming was investigated. Monitoring of water and sediment was performed for two important rivers of Thailand (Tha Chin River and Mun River). Furthermore, the aquatic toxicity of the selected antibiotics was assessed for five species of tropical freshwater invertebrates. **Chapter 7** shows the results of a study investigating the environmental discharge of the antibiotic enrofloxacin applied to a *Pangasius* catfish pond in Vietnam, and an ecological risk assessment based on toxicity data for green algae, invertebrates and fish. In both studies (Chapters 6 and 7) antibiotics were found to accumulate to relatively high concentrations in sediments next to or down-stream of the aquaculture facilities. Also, both studies concluded that aquaculture antibiotics pose limited ecotoxicological risks for green-algae, invertebrate and fish populations, but highlight the need to perform further assessments with benthic organisms and to test the potential toxic effects of chronic antibiotic exposures on the structure of microbial communities and their mediated ecological functions.

In **Chapter 8,** the effects of the antibiotic enrofloxacin were investigated on several structural and functional endpoints of tropical aquatic ecosystems by using freshwater microcosms. This study confirmed the high tolerance of planktonic and macroinvertebrate communities to the concentrations that have been measured in the environment. This study also revealed that the abundance of certain microbial functional groups, particularly ammonia oxidizing bacteria and archaea, is likely to be reduced by environmentally relevant concentrations, although their ecological function does not seem to be significantly affected. Based on the environmental monitoring performed within this thesis and the ERAs performed with different tropical species and levels of biological organization (molecular, individual, population, community), it can be concluded that the short-term risks of aquaculture antibiotics for tropical aquatic ecosystems

appear to be relatively low, and effects on the structure of microbial communities are expected to be transient.

Chapter 9 presents a modelling approach for assessing the risks of bacterial resistance development in aquaculture production systems and their surrounding environments. The approach is grounded in the theory of probabilistic risk assessment and relies on the use of exposure concentration distributions and available antibiotic susceptibility data for clinically relevant bacteria. In this chapter, the proposed modelling approach is used to predict the antibiotic resistance development risks for 12 antibiotics applied in intensive *Pangasius* catfish aquaculture. This study shows that most antibiotics, even when used according to recommendations, may increase the prevalence of antibiotic resistance genes in bacteria associated to sediments of intensive aquaculture ponds. The presented modelling approach requires further field evaluations but sets the stage for the inclusion of relevant resistance endpoints in the prospective, screening-level risk assessment of aquaculture antibiotics.

Finally, in **Chapter 10** the overall results of the various studies that compose this thesis are discussed and recommendations are provided (1) to improve the regulatory control on the use of aquaculture medicines in Asian countries, (2) to reduce the environmental discharge of aquaculture medicines, and (3) to further improve the knowledge and tools that underpin the ERA of veterinary medicines used in Asian aquaculture.

Samenvatting

De aquacultuur in Azië is goed voor bijna 90 % van de wereldwijde aquacultuur productie en heeft tientallen jaren van ongekende groei doorgemaakt. Een van de belangrijkste belemmeringen voor de ontwikkeling en uitbreiding van de aquacultuur sector in Azië wordt gevormd door de verspreiding van ziekten. Om dit probleem op te lossen is recent een breed assortiment diergeneesmiddelen, waaronder antibiotica, parasiticiden en medische ontsmettingsmiddelen, in de sector geïntroduceerd. Diergeneesmiddelen toegepast in vijvers of kooien kunnen in het milieu terechtkomen via verschillende routes, onder meer door lozingen, uitloging van met medicijnen behandelde diervoeders en de uitscheiding door behandelde dieren. Verontreiniging van het milieu met diergeneesmiddelen heeft geleid tot bezorgdheid over een mogelijk resulterend verlies aan biodiversiteit in de omliggende aquatische ecosystemen en voor de ontwikkeling van microbiële resistentie tegen antibiotica. Daarnaast zou de achteruitgang van aquatische ecosystemen kunnen leiden tot verhoogde sterfte en dramatische economische gevolgen voor de aquacultuur sector kunnen hebben, doordat aquatische ecosystemen deels de watervoorraden vormen die nodig zijn voor landbouwbedrijven. Voor de ondersteuning van de duurzaamheid van de aquacultuur sector in Azië op lange termijn is daarom onderzoek naar de inschatting van milieurisico's (Environmental Risk Assessment, ERA) als gevolg van medicijngebruik in de aquacultuur dringend nodig.

De doelstellingen van dit proefschrift waren: (1) beschrijven en beoordelen van het huidige gebruik van diergeneesmiddelen in de Aziatische aquacultuur productie, (2) onderzoek naar modelmatige hulpmiddelen ter ondersteuning van de risicobeoordeling van de geneesmiddelen die in de Aziatische aquacultuur worden gebruikt, (3) identificatie van verbindingen en productie scenario's die in de Aziatische aquacultuur mogelijk hoge milieurisico's opleveren, en (4) onderzoek naar de lotgevallen van de in de aquacultuur gebruikte antibiotica en beoordeling van de risico's van deze verbindingen voor tropische aquatische ecosystemen.

Dit proefschrift begint met een literatuurstudie over het gebruik van chemische en biologische producten in de belangrijkste aquacultuur producerende landen van Azië (**Hoofdstuk 2**). De studie behandelt tevens de huidige stand van zaken op het gebied van de risicobeoordeling van chemische stoffen in de aquacultuur. Hierbij wordt ingegaan op de noodzaak van het hanteren van het risico-beoordelings paradigma zoals dit in de meeste ontwikkelde landen in de registratie en evaluatie van deze stoffen reeds gebeurt. Daarnaast blijkt dat de beschikbare informatie over alle aspecten met betrekking tot de risicobeoordeling van de in de aquacultuur gebruikte medicijnen zeer beperkt is, en dat het noodzakelijk is om (1) recente gegevens te verzamelen over het gebruik van deze chemicaliën in de praktijk, (2) om modelmatige instrumenten voor de risicobeoordeling te ontwikkelen, toegesneden op de kenmerken van de belangrijkste aquacultuur productiesystemen in Azië, en (3) om de blootstelling en de effecten van in de aquacultuur gebruikte geneesmiddelen op tropische aquatische ecosystemen te meten.

Hoofdstuk 3 geeft de resultaten van de meest uitgebreide enquête naar chemisch middelengebruik tot dusverre uitgevoerd in de aquacultuur sector in Azië. De enquête bestreek vier onderdelen van de aquacultuur (peneïde garnalen, *Macrobrachium* garnalen, Tilapia en *Pangasius* meerval) en omvatte vier (qua omvang van de aquacultuur productie) grote naties van Azië: China, Thailand, Vietnam en Bangladesh. Tijdens dit onderzoek werd het gebruik van zestig verschillende diergeneesmiddelen geïnventariseerd, samen met hun wijze van toepassing, de gerapporteerde doseringen en de duur van de behandeling. De gangbaarheid van het gebruik van antimicrobiële behandelingen is in de Vietnamese *Pangasius* bedrijven significant hoger dan in bedrijven van de andere onderzochte soorten. Het onderzoek lijkt te bevestigen dat het gebruik van antibiotica in de semi-intensieve en intensieve garnalen boerderijen in China, Thailand en

Vietnam lager was dan werd gemeld in eerdere enquêtes. Boeren gebruiken in het algemeen niet meer dan de aanbevolen doseringen van diergeneeskundige middelen, en het praktische gebruik was in de onderzochte bedrijven over het algemeen in overeenstemming met de nationale en internationale regelgeving. Ook werd onderzocht welke factoren mogelijk de waargenomen verschillen in gebruikspatronen kunnen verklaren. Geografische ligging is de belangrijkste factor die van invloed was op toepassingspatronen, en de dichtheid waarmee dieren worden gehouden en de productie-intensiteit spelen een belangrijke rol bij het gebruik van chemicaliën in de teelt van garnalen. Bovendien is de goedkeuring van certificatieschema's niet altijd gecorreleerd met een lager gebruik van antimicrobiële middelen .

Hoofdstuk 4 beschrijft de wetenschappelijke achtergrond en de mogelijke toepassingen van ERA-AQUA, een risico-evaluatie model dat werd ontwikkeld om risico's (voor het milieu en de menselijke gezondheid) die mogelijk optreden als gevolg van het gebruik van diergeneesmiddelen in de aquacultuur in vijvers te beoordelen. Dit hoofdstuk beschrijft de invoerparameters, sub-modellen en onderliggende vergelijkingen die door het ERA-AQUA model worden gebruikt. Tevens worden de resultaten getoond van een gevoeligheidsanalyse, en worden de belangrijkste parameters geïdentificeerd waarmee bij de parameterisering rekening dient te worden gehouden (d.w.z. fractie van organische stof in sediment, temperatuur, gewicht van het organisme, en de organische koolstof- en octanol-water verdelingscoëfficiënten van de stof). In dit hoofdstuk wordt gebruik van het model gedemonstreerd door een risicobeoordeling voor twee antimicrobiële middelen (oxytetracycline en benzalkoniumchloride) uit te voeren voor een *Pangasius* meerval scenario.

In **Hoofdstuk 5** worden risicoanalyses uitgevoerd met als doel de identificatie van chemicaliën en Aziatische aquacultuur productiescenario's die een groot gevaar voor het milieu opleveren. De berekeningen werden uitgevoerd met behulp van het ERA-AQUA model en de dataset gegenereerd in hoofdstuk 3, in combinatie met recente informatie over gangbare praktijken op aquacultuur productiebedrijven, en fysisch-chemische en toxicologische eigenschappen van de geëvalueerde verbindingen. Dit hoofdstuk laat zien dat de productie-intensiteit en water uitwisseling positief gecorreleerd is aan de lozing van diergeneesmiddelen naar het milieu en hun potentiële milieurisico's. Van alle onderzochte verbindingen worden de hoogste risico's gevonden voor de toepassing van een aantal anti-parasitaire verbindingen, als gevolg van hun hoge toxische potentieel voor gemeenschappen van niet-doelwit ongewervelden. Van alle onderzochte scenario's worden de hoogste milieu-risico's gevonden voor de *Pangasius* meerval productie in Vietnam, gevolgd door de garnalen productie in China. De op risico gebaseerde rangsschikking van verbindingen en scenario's biedt de mogelijkheid tot prioriteren van verder onderzoek naar chemische stoffen in zowel chemisch als biologisch veld- en laboratorium-onderzoek voor de onderzochte combinaties van soorten en landen.

In **Hoofdstuk 6** worden de lotgevallen in het milieu en de ecologische risico's als gevolg van het gebruik van de antibiotica oxytetracycline en enrofloxacin in de teelt van Tilapia in kooien onderzocht. Metingen in water en sediment werden uitgevoerd in twee belangrijke rivieren van Thailand (de Tha Chin rivier en de Mun rivier). Bovendien werd de aquatische toxiciteit van de geselecteerde antibiotica bepaald voor vijf soorten tropische zoetwater ongewervelden. **Hoofdstuk 7** geeft de resultaten van een onderzoek naar de milieu-afvoer van het antibioticum enrofloxacine na toepassing in een *Pangasius* meerval vijver in Vietnam, en een ecologische risicobeoordeling op basis van gegevens over de toxiciteit voor groene algen, ongewervelden en vissen. In beide studies (hoofdstukken 6 en 7) bleken antibiotica in relatief hoge concentraties op te hopen in sedimenten naast of stroomafwaarts van de aquacultuur faciliteiten. Ook werd in beide studies geconcludeerd dat de in aquacultuur gebruikte antibiotica slechts een beperkt ecotoxicologische risico vormen voor groene algen, ongewervelden en vispopulaties. Beide studies tonen echter tevens de noodzaak om verdere evaluaties uit te voeren met sediment organismen, en verder onderzoek uit te voeren naar de mogelijke toxische effecten van

chronische blootstellingen aan antibiotica op de structuur van microbiële gemeenschappen en de hiermee gerelateerde ecologische functies.

Hoofdstuk 8 beschrijft de effecten van het antibioticum enrofloxacine op verschillende structurele en functionele eindpunten van tropische ecosystemen, onderzocht in zoetwater microcosms. Deze studie bevestigt de hoge tolerantie van levensgemeenschappen van plankton en macro-ongewervelden voor de concentraties die zijn gemeten in het milieu. Uit deze studie bleek ook dat de overvloed van bepaalde microbiële functionele groepen, met name ammoniak oxiderende bacteriën en archaea, waarschijnlijk zal worden verminderd door milieu-relevante concentraties, hoewel hun ecologische functie niet significant lijkt te worden aangetast. Op basis van de binnen dit proefschrift uitgevoerde en beschreven metingen in het milieu en de risico-beoordelingen uitgevoerd met verschillende soorten en diverse niveaus van biologische organisatie (moleculair en individueel, populatie, gemeenschap) kan worden geconcludeerd dat de korte termijn risico's van de aquacultuur antibiotica voor tropische aquatische ecosystemen relatief laag lijken te zijn, en dat effecten op de structuur van microbiële gemeenschappen naar verwachting van voorbijgaande aard zullen zijn.

Hoofdstuk 9 geeft een modelmatige benadering voor de beoordeling van de risico's van bacteriële resistentie-ontwikkeling in aquacultuur productie systemen en in hun omgeving. De aanpak is gebaseerd op de theorie van probabilistische risicobeoordeling en berust op het gebruik van verwachte verdelingen qua blootstelling en beschikbare gegevens omtrent de gevoeligheid van klinisch relevante bacteriën voor antibiotica. In dit hoofdstuk wordt deze modelmatige benadering gebruikt om het risico voor de ontwikkeling van resistentie tegen antibiotica te voorspellen voor 12 antibiotica die worden toegepast in de intensieve teelt van de *Pangasius* katvis. Deze studie toont aan dat de meeste antibiotica, zelfs bij gebruik volgens de aanbevelingen, kunnen leiden tot een verhoging van de aanwezigheid van antibiotica-resistentie genen in bacteriën die worden geassocieerd met de sedimenten van waterbekkens met intensieve aquacultuur. De gepresenteerde modelmatige benadering behoeft verdere evaluatie in het veld, maar geeft de noodzaak aan voor de toevoeging van relevante resistentie eindpunten in de risicobeoordeling van antibiotica die worden gebruikt in de aquacultuur.

Tenslotte worden in **Hoofdstuk 10** de resultaten van de verschillende studies in dit proefschrift besproken en worden aanbevelingen gegeven (1) om de regelgeving omtrent het gebruik van geneesmiddelen in aquacultuur in Aziatische landen te verbeteren, (2) om de lozingen naar het milieu van aquacultuur geneesmiddelen te verlagen, en (3) om tot een verdere verbetering te komen van de kennis en hulpmiddelen die nodig zijn voor de onderbouwing van de milieu-risicobeoordeling van diergeneesmiddelen die in de Aziatische aquacultuur worden gebruikt.

Resumen

Durante las últimas décadas el sector acuícola de Asia ha experimentado un crecimiento sin precedentes y actualmente representa alrededor del 90% de la producción acuícola mundial. Uno de los principales obstáculos para el desarrollo y la expansión de dicho sector ha sido el brote y la proliferación de ciertas patologías y enfermedades acuícolas que afectan a la productividad del sector. Con el objetivo de abordar dicha problemática, una amplia gama de productos veterinarios, incluyendo antibióticos, antiparasitarios y desinfectantes médicos, han sido recientemente utilizados. Residuos de dichos medicamentos veterinarios pueden ser liberados al medio ambiente a través de la descarga de efluentes procedentes de estanques acuícolas o a través de la entrada directa de excretas producidos por los animales tratados en jaulas flotantes. La contaminación ambiental producida por medicamentos veterinarios puede afectar a la biodiversidad de los ecosistemas acuáticos que rodean las granjas acuícolas y puede contribuir al desarrollo de resistencia bacteriana en el medio natural, considerándose así como uno de los principales impactos ambientales producidos por la industria acuícola asiática. Además, la contaminación de los ecosistemas acuáticos puede acarrear un aumento de la mortalidad en las granjas acuícolas y drásticas consecuencias económicas, ya que los ecosistemas acuáticos proporcionan los recursos hídricos necesarios para dicha producción. Por todo ello, y con la intención de apoyar el desarrollo sostenible del sector acuícola en Asia, es necesario investigar la trascendencia de dichos impactos ambientales y desarrollar el conocimiento necesario para la Evaluación del Riesgo Ambiental (ERA) de los medicamentos usados en la acuicultura asiática.

Basándonos en dichas premisas, los principales objetivos de esta tesis doctoral fueron: (1) evaluar el uso de medicamentos veterinarios en la producción acuícola en Asia, (2) desarrollar modelos matemáticos para llevar a cabo la evaluación del riesgo ambiental de los medicamentos usados en acuicultura, (3) identificar compuestos y escenarios de producción acuícola que pueden plantear riesgos ambientales elevados, (4) evaluar el destino ambiental de los antibióticos aplicados en acuicultura y evaluar sus posibles riesgos para los ecosistemas acuáticos tropicales.

Esta tesis doctoral comienza con una revisión bibliográfica sobre el uso de sustancias químicas y productos biológicos en los principales países productores de acuicultura de Asia (**Capítulo 2**). Este trabajo describe el conocimiento actual y las técnicas disponibles para la evaluación de los riesgos de los productos químicos usados en acuicultura y hace hincapié en la necesidad de incluir estudios de ERA en el registro y la evaluación de los medicamentos usados en la acuicultura en Asia, como ya se hace en la mayoría de los países desarrollados. Además, esta revisión bibliográfico muestra que la información disponible sobre todos los aspectos relacionados con la evaluación del riesgo de los medicamentos usados en acuicultura es muy limitada y pone de relieve la necesidad (1) de obtener información sobre el uso de productos veterinarios y sobre las prácticas acuícolas actuales, (2) de desarrollar modelos para la evaluación del riesgo que sean adaptables a las características de los sistemas de producción acuícola más importantes de Asia, y (3) de llevar a cabo estudios de monitoreo para evaluar la exposición ambiental y los posibles efectos biológicos sobre los ecosistemas acuáticos tropicales afectados por la contaminación acuícola.

El **Capítulo 3** muestra los resultados de la más amplia encuesta sobre el uso de productos veterinarios realizada hasta la fecha en el sector acuícola asiático. Dicha encuesta se llevó a cabo incluyendo cuatro especies acuícolas diferentes (el langostino jumbo de la familia *Penaeidae*, el langostino de agua dulce del género *Macrobrachium*, la tilapia y la panga) producidas en cuatro países de Asia con importante producción acuícola: China, Tailandia, Vietnam y Bangladés. A través de esta encuesta se registraron sesenta ingredientes activos diferentes, así como su modo de aplicación, e información acerca de la dosis de uso y duración del tratamiento. Los resultados

de este trabajo demuestran que el porcentaje de uso de tratamientos antimicrobianos es significativamente mayor en las granjas de panga de Vietnam en relación a las granjas de las otras especies investigadas. La encuesta también sugiere que el uso de tratamientos con antibióticos en las granjas (semi-)intensivas de langostino jumbo de China, Tailandia y Vietnam, se ha visto reducido en comparación a resultados de encuestas anteriores. Este estudio demuestra que los trabajadores de estas granjas generalmente no exceden las dosis recomendadas para dichos medicamentos veterinarios, y que las prácticas de uso de químicos en las granjas investigadas están generalmente de acuerdo con lo especificado en los reglamentos nacionales e internacionales. También se investigaron los factores que subyacen a las diferencias observadas en los patrones de uso de productos veterinarios. Este análisis demostró que la ubicación geográfica es el factor más importante, y que la intensidad de producción en las granjas de langostinos jumbo está relacionado con la cantidad y el tipo de compuestos utilizados. Por otra parte, este trabajo muestra que la adopción de sistemas de certificación no siempre está directamente relacionada con un menor uso de compuestos antimicrobianos.

El **Capítulo 4** describe la base científica y las aplicaciones potenciales del modelo ERA-AQUA, un modelo matemático que fue desarrollado para llevar a cabo una evaluación integral de los riesgos para la salud humana y ambiental causados por el uso de medicamentos veterinarios en estanques de acuicultura. En este capítulo se definen los parámetros y ecuaciones utilizadas por el modelo, y se muestran los resultados de un análisis de sensibilidad que identifica los parámetros más importantes a tener en cuenta al llevar a cabo la parametrización de éste. Estos fueron: la fracción de materia orgánica en el sedimento, la temperatura, el peso inicial del organismo, y el coeficiente de partición en carbono orgánico y en octanol de la sustancia aplicada. En este capítulo, las aplicaciones del modelo se muestran mediante la realización de una evaluación del riesgo para dos compuestos (oxitetraciclina y cloruro de benzalconio) aplicados a un escenario acuícola para la producción de panga en Vietnam.

En el **Capítulo 5** se llevó a cabo una evaluación de riesgo preliminar con el objetivo de identificar los productos químicos y los escenarios de producción acuícola de Asia que representan un riesgo ambiental importante. Los cálculos de riesgo se realizaron mediante el modelo ERA-AQUA y el conjunto de datos generado en el Capítulo 3, junto con información actualizada sobre las prácticas de producción acuícola y propiedades físico-químicas y toxicológicas de los compuestos evaluados. En este capítulo se demuestra que la intensidad de producción y el intercambio de agua están positivamente relacionados con la descarga ambiental de residuos de los medicamentos veterinarios utilizados y con su riesgo potencial para el medio ambiente. De todos los compuestos estudiados, los mayores riesgos se plantean por la aplicación de algunos compuestos antiparasitarios, debido a su alto potencial tóxico para las comunidades de especies no diana de invertebrados. Los riesgos ambientales más altos fueron calculados para la producción de panga en Vietnam, seguida de la producción de langostino en China. Este estudio proporciona una clasificación basada en el riesgo ambiental para los compuestos y escenarios acuícolas asiáticos, la cual ofrece amplias posibilidades para establecer prioridades y ayudar a la toma de decisiones acerca de los compuestos que deben ser evaluados en posteriores estudios de laboratorio y de campo, y para los que programas de monitoreo ambiental son estrictamente necesarios.

En el **Capítulo 6** se investigó la exposición ambiental y los riesgos ecológicos que plantea el uso de dos antibióticos, la oxitetraciclina y la enrofloxacina, en el cultivo de tilapia en jaulas flotantes distribuidas a lo largo de ríos tropicales. Este capítulo muestra los resultados del análisis de las concentraciones ambientales de antibióticos en muestras de agua y sedimentos tomadas en dos ríos de Tailandia con importante producción acuícola: el río Tha Chin y el río Mun. Por otra parte, en este estudio se evaluó la toxicidad de dichos antibióticos para cinco especies de invertebrados acuáticos tropicales. El **Capítulo 7** muestra los resultados de un estudio que investiga la contaminación ambiental por el uso del antibiótico enrofloxacina en una granja de panga en Vietnam. Además dicho estudio proporciona una evaluación del riesgo ecológico llevado a cabo a

partir de datos de toxicidad para algas, invertebrados y peces. En ambos estudios (Capítulos 6 y 7) se demostró que los antibióticos usados en acuicultura tienden a acumularse en los sedimentos adyacentes a las instalaciones acuícolas en concentraciones relativamente altas. Asimismo, ambos estudios concluyeron que dichos antibióticos suponen riesgos ecotoxicológicos limitados para las poblaciones de algas, invertebrados y peces, pero ponen de relieve la necesidad de realizar nuevas evaluaciones basadas en ensayos de toxicidad con organismos bentónicos, y de investigar los posibles efectos tóxicos de dichos antibióticos sobre la estructura de las comunidades microbianas y sus funciones ecológicas a largo plazo.

En el **Capítulo 8** los efectos toxicológicos del antibiótico enrofloxacina fueron investigados sobre varios parámetros estructurales y funcionales de los ecosistemas acuáticos tropicales a través de la utilización de microcosmos. Este estudio confirma la alta tolerancia de las comunidades planctónicas y de macroinvertebrados a las concentraciones que se han medido en el ambiente. Además, este estudio reveló que la abundancia de ciertos grupos funcionales microbianos, particularmente las bacterias y arqueas encargadas de la oxidación de amoníaco, puede verse reducida a las concentraciones que se han medido en los ecosistemas expuestos, aunque su función ecológica no parece verse afectada de manera significativa. Basándose en los resultados del monitoreo ambiental de antibióticos llevados a cabo en esta tesis y los estudios de ERA realizados con diferentes especies tropicales y sobre diferentes niveles de organización biológica (molecular, individual, poblacional, comunidad), se puede concluir que los riesgos ecológicos de los antibióticos usados en acuicultura para los ecosistemas acuáticos tropicales parecen ser relativamente bajos a corto plazo. Además los posibles efectos sobre la estructura de las comunidades microbianas se espera que sean más bien de carácter transitorio.

El **Capítulo 9** presenta un nuevo método para evaluar el riesgo de aparición de resistencia bacteriana en los sistemas de producción acuícola y sus ambientes circundantes. Dicho método se fundamenta en la teoría de evaluación probabilística del riesgo y hace uso de distribuciones de exposición y de sensibilidad basadas en tests de laboratorio realizados con bacterias con relevancia clínica. En este capítulo, el método propuesto se utiliza para predecir el riesgo potencial para el desarrollo de resistencia bacteriana de 12 antibióticos usados en la producción de panga. Este estudio demuestra que la mayoría de los antibióticos, incluso cuando se usan siguiendo las recomendaciones establecidas, pueden aumentar la prevalencia de genes de resistencia en bacterias asociadas a los sedimentos de los estanques de acuicultura intensiva. Aunque el método propuesto en este estudio requiere una evaluación más exhaustiva bajo condiciones de campo, este estudio sienta las bases para la inclusión de criterios de valoración de resistencia bacteriana relevantes a la evaluación del riesgo preliminar de antibióticos usados en acuicultura.

Por último, en el **Capítulo 10,** se discuten los resultados generales de los diversos estudios que componen esta tesis y se proporcionan recomendaciones (1) para mejorar el control sobre el uso de medicamentos en la acuicultura, (2) para reducir la contaminación ambiental con residuos de medicamentos, y (3) para mejorar el conocimiento científico y las herramientas técnicas que sustentan los estudios de ERA para los compuestos veterinarios utilizados en acuicultura en Asia.

Acknowledgements

People use to say that time flies when you are having fun, but I believe that it also passes quickly when your work becomes challenging enough and when you are accompanied by the best colleagues and friends. Here I would like to thank to some of the people that have contributed to make a success of this thesis and that have taken part in this great journey.

First of all, I would like to express my gratitude to my supervisor Paul van den Brink. Paul, I believe that our cooperation has been extremely fruitful during this time because of several reasons. We both have been efficient co-workers and have managed to keep a fluid communication during this period, even when we were in the antipodes. This has been supported by innumerable emails, Skype meetings and enjoyable moments in some remote spots around the globe, often accompanied by nice distillery products. Our cooperation has also benefited from our efforts to be flexible and to keep a mutual understanding at work, but also in our personal life. Your supervising approach has always been supportive and has offered an ample margin of freedom to carry out my own ideas. This has allowed me to grow as a self-confident and independent scientist. Moreover, you have always encouraged me to attend conferences and to interact with some members of your network. For all this, and for providing your best advices and friendship during this period, I would like to say: many thanks.

Obviously, this thesis is the result of the work done by many people. I am extremely grateful to Andreas Focks for offering his precious time and advices during this period, for being the paranymph on my defence, for his enthusiastic introduction to modelling, and for his great contribution to the development of the ERA-AQUA model. Thanks to Rhaul Oliveira for his endless support and inspiring discussions on our research, and also for the unforgettable moments in Seville, Bangkok, Berlin, Prague, Wageningen. I am really thankful to René van Wijngaarden for teaching me so much about invertebrates and microcosms, for the nice evenings in our balcony in Bangkok, and for our fantastic dives into the Thai coral reefs. Thanks to Alfredo Tello for keeping my feet on the ground and for forcing me to see that the first approach to antibiotic resistance risk assessment should be less complex than I tended to imagine. Thanks to Mauricio Rocha for showing to me so many molecular techniques and for helping me to understand that tiny life matters so much. Thanks to Phu (Tran Minh Phu) and Jugk (Jidapa Khatikarn) for their support and friendship during our field work in Vietnam and Thailand, respectively. Thanks to Key (Kriengkrai Satapornvanit) for his hospitality and for offering the best resources for my experiments and field trips in Thailand. Thanks to Arrienne Matser, for the time spent with me in the lab and for her contribution to the antibiotic analysis. Thanks to the students that participated in this project (Bart de Vreede, Frederieke Knopperts, Margot Andrieu) for their willingness to learn, for their tireless dedication and for the enjoyable times together. I would also like to thank the rest of co-authors of the chapters of this thesis for their helpful comments and discussions: Mohammad Haque (Ripon), Jiang Min, Phuong Nguyen, Trevor Telfer, A. Shahabuddin (Hero), Patrik Henriksson, Francis Murray, David Little, Anders Dalsgaard, Yue Geng (Gary), Sakchai McDonough (Bat), António Nogueira, Amadeu Soares, Inês Domingues, Huong Do, Hauke Smidt.

I am indebted to all my SEAT project colleagues for their support and for offering to me the opportunity to participate in so many interesting discussions, meetings and field visits, and for teaching to me so much about aquaculture. Especially thanks to Dave and Francis, for creating SEAT and keeping up their enthusiasm during the whole period. Also thanks to all SEAT PhDs

with whom I have shared so many stories, ideas and laughs (Patrik, Jesper, Richard, Swan, Doug, Ingrid, Phu, Hanne, Lynne, Lynn, Wenbo, Lam, Jigsz, Likang).

To my beloved stress ecology group, thanks for offering the best support and discussions on my research, and for the greatest fun at work and outside work. Jacqui, thanks for being next to me all the time (often without other possible option), for listening and providing advice in too many occasions, for our PhD-life discussions, for your moral support and for being (with the permission of Andreas) my most beautiful paranymph. Noël, thanks for your end-of-day visits to check whether I was still alive in front of my computer and for your stimulating thoughts. Thanks to my dearest already graduated PhDs: Nika, Mazhar and Mascha. Nika, thanks for being there all the time, for your smart advices, for sharing with me your inspiring work, and for passing to me some of your courage and strength to overcome the difficult parts of the PhD. Mazhar, thanks for our interesting discussions and drawings in the whiteboard in which we almost solved the great risk assessment equation, for sharing your approach to see life, for all the fun, and for all the chips (I still owe you a lot). Mascha, thanks for your guidance and friendship while taking the first steps of my PhD. To the other PhD members of the group (Berhan, Concillia, Nancy, Jugk, Kizar, Mauricio, Michael, Zhang), thanks for the enjoyable moments we have spent together (and for the ones that have to come).

Thanks to the staff members of the Aquatic Ecology and Water Quality Management group of Wageningen University for hosting me during the whole PhD and for providing such a relaxed, dynamic and inspiring environment to conduct my research. Also thanks to my peers for the fun times together (Ariadna, Bastiaan, Els, Ingrid, Jelle, Jochem, Joris, Livia, Marlies, Mohammad), and to the colleagues that already left the group (Andrea, Annelies, Darya, Irene, Kristina, Vasilis). Especially, thanks to Darya for her moral support, friendship and fun, and to Kristina for her spirituality, the English proofreads, and for helping to keep our home-fauna alive while travelling.

I would also like to thank the whole Environmental Risk Assessment team of Alterra for inviting me to join interesting presentations and discussions, for the nice outings, and for the technical support. Thanks to Arrienne, Laura and Steven for the help with the analytical work. Thanks to Ivo for always being willing to help with anything I needed. Thanks to Jan for creating and managing the ERA-AQUA website, to John for the dutch summary translation, and to Theo for his always inspiring presentations.

The Faculty of Fisheries from Kasetsart University (Bangkok, Thailand) has been my second home while performing most of my field work. I would like to thank Key and Jigsz for hosting me there, and to all members of the SEAT office for their company and support. I am also extremely grateful to the staff members of Kasetsart for their help. Especially, to Hatairat Soodta, Sinchai Maneekat, Supachit Thaweevirotkitti for providing so many sampling materials and for their best assistance. Also to Chairat Thammachit, Sordakorn Pimla and Apisit Jiraseve (from Central Lab) for their contribution to the microcosm experiment.

I am grateful to all Asian farmers that have participated in this study for always receiving us with their best smile, for sharing their knowledge, and for allowing us to mess around their farms. Especially thanks to the tilapia farmers of Suphanburi province in Thailand (Mr Tawin and Mr ‘Fuji’) for allowing us to spend several days in their farms and for offering everything we needed.

I want to thank Michiel Daam for always being supportive with my work, for the proofreads, for the advices on how to succed in Thailand, and for the nice moments together in SETAC conferences. Also thanks to Andrea Waichman for encouraging me to start the PhD and for being so positive about my whole work and career.

I would also like to acknowledge my opponents (Johan Verreth, Jonas Gunnarsson, Kees van Gestel and Jeroen Guinée) for critically evaluating this thesis and for their efforts to attend the PhD defence.

Fortunately, during this period I have had the privilege to share lots of enjoyable moments with many amazing people. Thanks to all my Spanish friends for being there and, despite the distance, always receiving me with open arms. Especially thanks to Mireia for her visits to Wageningen and for our discussions about how life should be. Thanks to all my Erasmus friends for their support and for our amusing (and often irrational) meetings. Especial mention goes to Julia, Ewout, Ander and Bette, for being part of my Dutch family. Thanks to all friends (some also house-mates) that have passed by Wageningen during this period and with whom I have had the opportunity to enjoy life. Also thanks to all the people that I have found on the way in Asia and that have offered their kindest company and generous support.

My deepest gratitude goes to my parents and to my sister for believing in me, for teaching to me so many helpful things, for tracking (or at least trying) my steps in different countries, for always supporting my career, and for making their greatest efforts to attend my PhD defence. Papá, gracias por despertar mi pasión por los números y por enseñarme que con esfuerzo y dedicación todo se consigue. Mamá, gracias por añadir una chispa de locura a la vida y por enseñarme a ser crítico conmigo mismo. Nuria, gracias por siempre estar ahí, por entenderme y por colaborar con la parte gráfica de la tesis. Gracias también al resto de mi familia (abuelas, tios, primos) por ser un apoyo tan grade y por entender mi ausencia, y a Inma y Borja por siempre darme ánimos para llevar a cabo cualquier reto que se me proponga.

Finally, I want to dedicate this work to my partner. Neus, I am infinitely grateful to you for accompanying me during this whole journey, for believing in my work and helping so much, for your willingness to discover new places, for your patience to stand the distance and those long working days, for your careless support and last, but obviously not least, for your love and for making me feel a fortunate person every single day. Also thanks to your wonderful family (Ernesto, Amparo, Laia, Carlos, Adri, Carmen, and also Pol, Joan, Ona) for their affection and generosity. Neus, I must admit that I am really excited to see what life brings us next, but I have to say that this will not be nearly as important as being by your side.

Andreu

About the author

Andreu Rico Artero was born in 1983 in Valencia, Spain. He obtained his BSc with honours on Agricultural Engineering with specialization on Animal Production at the Polytechnic University of Valencia in 2004. After completing his BSc studies he moved into the field of environmental research. He got a degree on Environmental Sciences from the Polytechnic University of Valencia in 2007. During the last year of this study he got an ERASMUS scholarship and moved to the Netherlands, where he started his MSc on Aquatic Ecology and Water Quality Management at Wageningen University. During that time he became interested in the environmental risk assessment of agrochemicals and undertook his career as ecotoxicologist. In 2008 he did an internship for the Spanish Ministry of Environment and assessed the ecological status of water bodies according to the methodologies established by the European Water Framework Directive. For his MSc thesis he studied the sensitivity of Amazonian aquatic species to agricultural pesticides and worked for the Federal University of the Amazon (UFAM) and the Brazilian Enterprise for Agricultural Research (EMBRAPA). In the beginning of 2009, after the completion of his MSc, he was awarded with a Leonardo grant and moved to Costa Rica, where he studied the ecological risks posed by the use of pesticides in banana plantations at the National University of Costa Rica (UNA). In November of 2009, he returned to the Netherlands to start his PhD at Wageningen University under the supervision of Prof. Paul van den Brink, which resulted in this thesis. As part of his PhD he co-supervised three MSc students, participated in international workshops, and contributed to the dissemination of his research outcomes through several presentations at international conferences. During the last four years he has collaborated with several research groups in Europe (Portugal, United Kingdom, Denmark) and Asia (China, Thailand, Vietnam, Bangladesh), and the environmental risk assessment group of Alterra (The Netherlands). Andreu has published eight papers in peer-reviewed journals and several scientific reports for the EU, and is member of the Student Advisory Council of the Society of Environmental Toxicology and Chemistry (SETAC). Currently, Andreu's research interests lie on assessing the environmental fate and effects of chemicals used in agriculture production in the tropics, on improving the current higher-tier risk assessment methods for pesticides and veterinary pharmaceuticals, and on assessing the combined effects of chemical pollution and other stressors for the sustainability of aquatic ecosystems.

Contact: *andreu.rico@wur.nl* or *andreu.rico@gmail.com*

List of publications

Rico, A., Oliveira, R., McDonough, S., Matser, A., Khatikarn, J., Satapornvanit, K., Nogueira, A., Soares, A.M.V.M., Domingues, I., Van den Brink, P.J., Accepted. Use, fate and ecological risks of antibiotics applied in tilapia cage farming in Thailand. *Environmental Pollution*.

Rico, A., Dimitrov, M.R., Van Wijngaarden, R.P.A., Satapornvanit, K., Smidt, H., Van den Brink, P.J., 2014. Effects of the antibiotic enrofloxacin on the ecology of tropical eutrophic freshwater microcosms. *Aquatic Toxicology* 147, 92- 104.

Rico, A., Van den Brink, P.J., 2014. Probabilistic risk assessment of veterinary medicines applied to four major aquaculture species produced in Asia. *Science of the Total Environment* 468-469, 630-641.

Rico, A., Phu, T.M., Satapornvanit, K., Min, J., Shahabuddin, A.M., Henriksson, P.J.G., Murray, F., Little, D.C., Dalsgaard, A., Van den Brink, P.J., 2013. Use of veterinary medicines, feed additives and probiotics in four major internationally traded aquaculture species farmed in Asia. *Aquaculture* 412-413, 231-243.

Rico, A., Geng, Y., Focks, A., Van den Brink, P.J., 2013. Modeling human health and environmental risks of veterinary medicines applied in pond aquaculture. *Environmental Toxicology and Chemistry* 32, 1196-1207.

Rico, A., Satapornvanit, K., Mahfujul Haque, M., Min, J., Nguyen, P.T., Telfer, T.C., Van den Brink, P.J., 2012. Use of chemicals and biological products in Asian aquaculture and their potential environmental risks: a critical review. *Reviews in Aquaculture* 4, 75-93.

Rico, A., Waichman, A.V., Geber-Corrêa, R., Van den Brink, P.J., 2011. Effects of malathion and carbendazim on Amazonian freshwater organisms: comparison of tropical and temperate species sensitivity distributions. *Ecotoxicology* 20, 625-634.

Rico, A., Geber-Corrêa, R., Campos, P.S., Garcia, M.V.B., Waichman, A.V., Van den Brink, P.J., 2010. Effect of parathion-methyl on Amazonian fish and invertebrates: A comparison of sensitivity with temperate data. *Archives of Environmental Contamination and Toxicology* 58, 765-771.

Netherlands Research School for the
Socio-Economic and Natural Sciences of the Environment

C E R T I F I C A T E

The Netherlands Research School for the
Socio-Economic and Natural Sciences of the Environment
(SENSE), declares that

Andreu Rico Artero

born on 17 September 1983 in Valencia, Spain

has successfully fulfilled all requirements of the
Educational Programme of SENSE.

Wageningen, 26 May 2014

the Chairman of the SENSE board	the SENSE Director of Education
Prof. dr. Rik Leemans	Dr. Ad van Dommelen

The SENSE Research School has been accredited by the Royal Netherlands Academy of Arts and Sciences (KNAW)

KONINKLIJKE NEDERLANDSE
AKADEMIE VAN WETENSCHAPPEN

The SENSE Research School declares that **Mr. Andreu Rico Artero** has successfully fulfilled all requirements of the Educational PhD Programme of SENSE with a work load of 51 ECTS, including the following activities:

SENSE PhD Courses

- Environmental Research in Context
- Research Context Activity: 'Co-organizing SENSE Symposium: Ecosystems under stress: assessing the impact of chemical and physical disturbances on ecological processes and ecosystem structure', Wageningen, 14 December 2012
- Risk Assessment
- Multivariate Analysis
- Introduction to R for Statistical Analysis

Other PhD Courses

- Exposure Assessment I: Chemical exposure assessment analysis and modelling
- Laboratory Animal Science
- Teaching and Supervising Master Thesis Students

Management and Didactic Skills Training

- Co-organizing the workshop on 'Environmental Monitoring' within the SEAT project
- Member of the SETAC Student Advisory Council
- Supervising three MSc thesis students
- Guest Lecturer, MSc course 'Chemical Stress Ecology and Risk Assessment'

Oral Presentations

- *Modelling environmental risks of veterinary medicines applied in Asian pond aquaculture.* SETAC World Conference, 20-24 May 2012, Berlin, Germany
- *Direct and indirect effects of the antibiotic enrofloxacin in tropical freshwater microcosms.* SETAC YES Meeting, 11-13 February 2013, Krakow, Poland
- *Ecological risk assessment of veterinary medicines applied in Asian aquaculture.* SETAC Europe, 12-16 May 2013, Glasgow, United Kingdom

SENSE Coordinator PhD Education

Dr. ing. Monique Gulickx

The research described in this thesis was conducted under the umbrella of the Sustaining Ethical Aquaculture Trade (SEAT) project (www.seatglobal.eu; contract number: 222889) and was financially supported by the Seventh Framework Programme of the European Commission - Food, agriculture and fisheries, and biotechnology.

Financial support from the Aquatic Ecology and Water Quality Management group of Wageningen University for printing this thesis is gratefully acknowledged.

Cover design by Nuria Rico Artero
Printed by GVO drukkers & vormgevers B.V.