Margot ANDRIEU
September 2012
Report number: 019/2012

Master thesis

Assessment of the fate and effects of antibiotics on freshwater ecosystems surrounding catfish farms in the Mekong delta (Vietnam)

Can tho university
College of aquaculture and fisheries
Campus II, 3/2 street
Can tho city (Vietnam)

Supervisors: Nguyen Thanh Phuong
Do Thi Thanh Huong
Tran Minh Phu

Wageningen University
Aquatic Ecology and Water
Quality Management group
P.O. Box 47
6700 AA Wageningen (The Netherlands)

Supervisors: Paul J. Van den Brink
Andreu Rico

Students: Nguyen Le Nhat Khoa and Nguyen Tran Phuong Thao

Abstract

In this project, the fate of the fluoroquinolone antibiotics enrofloxacin and ciprofloxacin was examined in water samples collected during and after the application of enrofloxacin in the effluent discharge point of a *Pangasius* catfish farm in the Mekong River delta (Vietnam). Then, the effects of these antibiotics were studied on non-target aquatic organisms which might be potentially exposed to these compounds in the surroundings of catfish farms. Finally, the concentrations in the water were compared with the results of toxicity tests to assess the risks posed by the use of these antibiotics in catfish farms. The acute effects of these pharmaceuticals were evaluated for three types of organisms: the green-algae *Chlorella* sp., the cladocera *Moina macrocopa*, and the Nile tilapia (O*reochromis niloticus*). To do so, acute toxicity tests were performed with *Chlorella sp*. (72h-growth inhibition test) and *Moina macrocopa* (48h-acute immobilization test). The toxic effects of these antibiotics were also tested on Nile tilapia using a non-standard test design. In the fish test, the studied fluoroquinolones were applied via oral administration and bath treatment in separate experiments during an exposure period of 5 days. Cholinesterase (ChE) and Catalase (CAT) activity were assessed in the fish brain and muscles, respectively, during the exposure period (day 3 and day 5) and at the end of the experiment (9 days after the last antibiotic administration). The results of the toxicity experiments showed that *Chlorella sp.* was more sensitive to ciprofloxacin (EC_{50}-72h = 31.1 mg/L, which corresponds to 93.8 x 10^{-6} mol/L after 72h) than to enrofloxacin (EC_{50}-72h = 401 mg/L, which corresponds to 1117 x 10^{-6} mol/L after 72h). However, enrofloxacin was more toxic to *Moina macrocopa* than ciprofloxacin (respectively EC_{50}-48h = 70 mg/L, which corresponds to 195 x 10^{-6} mol/L after 48h and EC_{50}-48h = 96.5 mg/L, which corresponds to 291 x 10^{-6} mol/L after 48h). Regarding the biomarker experiments, it was found that fish exposed to high concentrations of the studied antibiotics (oral treatment: 5 g/kg of feed for both fluoroquinolones, bath-treatment: 0.4 mg of ciprofloxacin/L and 0.8 mg of enrofloxacin/L) showed an increase in ChE activity three days after the beginning of the exposure period. However, the enzymatic activity returned to normal levels, five days after the beginning of the experiment, which indicates that there is a physiological adaptability of the fish to antibiotic stress. For all the tests except the bath treatment test with ciprofloxacin, the ChE activity was not found to be significantly different to controls after a post-stress period of 9 days. After 5 days of exposure, the CAT activity decreased significantly for the bath-treatment experiment with ciprofloxacin (0.4 and 50 mg/L) and the in-feed treatment with enrofloxacin (1 and 10 mg/kg of feed). Moreover, this study suggests that when the antibiotic concentration in the environment is in the range of µg/L, ChE and CAT biomarker analysis is probably not the most suitable tool to monitor the effects of antibiotic residues to non-target organisms. However, ChE and CAT activities are interesting to assess the potential mode of action of the tested antibiotics on the fish metabolism from a toxicological point of view. Besides this, fish died in the highest treatment level (100 mg of enrofloxacin/L and 50 mg of ciprofloxacin/L) of the bath-treatment tests. The potential causes of the induced biomarker effects and mortality observed in this study are discussed in light of the published literature, discussing the mode of action of these antibiotics in higher organisms. Finally, the concentrations of enrofloxacin and ciprofloxacin in the effluent discharge point of the catfish pond were found to be approximately 1000 times lower than the concentrations at which acute effects have been observed on the tested organisms. Therefore, the environmental pollution caused by the use of enrofloxacin and its main metabolite ciprofloxacin in aquaculture is not likely to result in acute toxic effects on non-target aquatic organisms in the aquatic ecosystems surrounding *Pangasius* catfish farms.

Table of contents

Introduction

In the last 20 years, the global demand of seafood products has risen all over the world and their consumption has more than doubled compared to 1973 (Washington and Ababouch, 2011). The increase of the world's population and the change in eating habits are the main reasons explaining this sharp enlargement. As capture fisheries cannot meet this demand, the aquaculture production has markedly increased. Less than one million tons of seafood products were produced from aquaculture in 1950's, compared to 52.5 million tons by 2008 (FAO, 2010). Nowadays, most of the seafood products are produced in developing countries such as China and other south-east Asian countries. The main produced species are fast growing catfish (*Pangansaiidae*), carps, tilapias and shrimps (FAO, 2006). The majority of this production is traded to Western countries out of which the European Union is the largest single regional importer, taking up to 42% of the total amount of internationally traded seafood products in 2008 (FAO, 2010). Because the aquaculture sector is developing quickly, the intensification of aquaculture practices has raised questions regarding its sustainability.

In Vietnam the aquaculture production has increased tremendously, becoming the 6th largest seafood exporting country (European External Action Service (EEAS), 2011). This success is mainly explained by the development of catfish farms in the Mekong River Delta. Initially, catfish species were very difficult to reproduce in captivity. In 2000, artificial ovulation of female catfish was successfully stimulated to produce mass scale fry. Then, the culture and feeding techniques developed and the processing industries as well as export markets expanded (Phuong and Oanh, 2010). The amount of exported catfish raised from less than 500 tons in 1997 to around 650,000 tons in 2010 (Cuyvers and Van Binh, 2008; Vietfish international, 2011). From the total amount of catfish produced in the farms, more than 90% is exported to around 100 countries all over the world (Phan *et al.*, 2009), with Europe being the main destination.

Over the years, the catfish culture in ponds developed rapidly because of the low infrastructure required, the short culture period and the high economic profit (Phuong and Oanh, 2010; Phan *et al.*, 2009). The productivity in ponds has improved significantly because of the increasing water depth (3.5 to 4.5 m), the high water exchange for ponds (up to twice a day, from 30 to 100% replenishment) (Phan *et al.*, 2009) and the exceptionally high stocking densities in the ponds (52.8 fish/m^2) (Liem 2009). Moreover, the intensification of aquaculture practices has led to the introduction of a wide array of drugs and chemicals for preventing and treating infectious diseases (Danyi *et al.*., 2010). According to the survey conducted by Phuong (2010) in the Mekong delta, all catfish farmers use chemicals and drugs during the production cycle. Farmers reported to use as much as 17 types of chemicals for water treatment and pond preparation (to kill parasites and other fish). Enrofloxacin is the most widely used antibiotic in the Tra catfish (*Pangasianodon hypothalamus*) farms, due to its broad antibacterial spectrum and efficiency to treat diseases (Tu *et al.*., 2006).

During the last years, concerns have raised regarding the environmental release and potential ecological effects of antibiotics. As enrofloxacin is extensively used in catfish farms (i.e., applied mixed with feed) (Shao, 2001), it is expected to reach the aquatic environment in the surrounding of the farms by untreated effluent discharges and may cause adverse effects on wild organisms (Ferreira *et al.*, 2006). However, the potential toxic effects of enrofloxacin on non-target aquatic organisms will depend on the residual concentration entering the environment as well as the

exposure duration. The environmental concentration of enrofloxacin will be influenced by the pharmacokinetics of the compound and biotransformation processes in the cultured organisms, the physico-chemical properties of the antibiotic and the aquaculture practices (e.g. applied dosage, amount and frequency of water exchange and fish densities).

The physico-chemical properties of enrofloxacin are shown in Table 1.

Table 1: Physico-chemical properties of enrofloxacin

	Value	Reference
Octanol-water partition coefficient: Log K_{ow}	0.7 - 1.1	Ašperger *et al.*, 2009; National library of medicine, 2011; Picó and Andreu, 2007
Organic carbon normalized sorption coefficient : K_{oc}	16 506 – 768 740 L/kg	Picó and Andreu, 2007; Pfizer Inc., 2002; Veterinary substance database., 2011
Solubility in water: S_w	0.3 mg/L (pH 7) – 10.4 mg/L (pH 5)	Ebert *et al.*, 2011

From the values in Table 1, enrofloxacin can be considered as a lipophilic compound which will strongly absorb and bound to organic matter in sediments when released into aquaculture ponds and aquatic ecosystems. Like most antibiotics, the main degradation process for enrofloxacin is photodegradation. In fact, several studies have shown that its half-life in water is greatly reduced with increasing light intensity (Gagliano and Mc Namara, 1996; Knapp *et al.*, 2005; Sukul and Spiteller, 2007). This process is not the only one observed in the environment; biodegradation in the sediments and within the catfish (before its release), as well as dilution and dispersion are also important processes to take into account when studying the fate of this antibiotic in the water phase. After its degradation, most enrofloxacin remains in its original form, but a large fraction is converted into ciprofloxacin, its main metabolite. The amount of metabolized enrofloxacin will vary greatly between all aquatic organisms and with pH, light, temperature, initial dose of enrofloxacin and time of exposure. Xu *et al.* (2006) tested the residues of enrofloxacin on Nile tilapia (oral dose: 1g/kg of feed, treatment duration: 7 days, temperature: 27±2°C). Under these particular experimental conditions, they found that 25% to 43% of enrofloxacin was converted into ciprofloxacin 7 days after the beginning of the experiment. Danyi *et al.* (2010) studied the effects of enrofloxacin on Tra catfish and giant freshwater prawns (oral dose: 1 and 0.7g/kg of feed respectively, treatment duration: 7 days, temperature: 29°C to 32°C). As a result of this experiment, they concluded that only 3-4% of enrofloxacin was transformed into ciprofloxacin three days after the beginning of the treatment.

The main physico-chemical properties of ciprofloxacin are presented in Table 2.

Table 2: Physico-chemical properties of ciprofloxacin

	Value	reference
Octanol-water partition coefficient: Log K_{ow}	0.23 – 1.3	Huang, 2002; National library of medicine, 2011; Veterinary substance database., 2011
Organic carbon normalized sorption coefficient : K_{oc}	13 900 – 134 465 L/kg	Veterinary substance database., 2011; National library of medicine, 2011; Cardoza, 2004
Solubility in water: S_w	1.1 mg/L	Ebert *et al.*, 2011; Said *et al.*, 1995

Comparing Table 1 and Table 2, it is notorious that the physical and chemical properties of ciprofloxacin and enrofloxacin are very similar. Therefore, when these antibiotics enter aquatic systems, it is likely that they will tightly bind to suspended solids and to sediments, where they will

remain rather stable. Lin *et al.* (2010) found half-lives (DT_{50}) with non-sterile treatment of 0.01 and 18.4 days for enrofloxacin and 0.04 and 17.3 days for ciprofloxacin in water and sediment slurry respectively under laboratory conditions. However, half-lives of these antibiotics vary greatly among different studies depending on the pH, light intensity, fluoroquinolone dosage and phosphorous levels (Gagliano and Mc Namara, 1996; Knapp *et al.*, 2005).

The effects of enrofloxacin have already been investigated on cultured animals such as fish and shrimps (Kim *et al.*, 2006; Intorre *et al.*, 2000; Della Rocca *et al.*, 2004; Vaccaro *et al.*, 2003). The use of biomarkers is a sensitive, low-cost and specific way of studying their effects on cultured animals (Van der Oost *et al.*, 2003). Enrofloxacin exposure has been demonstrated to cause a decrease in the cholinesterase activity in Tra catfish (*Pangasianodon hypophthalmus*) (Wang *et al.*, 2009). However, this value remained relatively constant in black tiger shrimps (*Penaeus monodon*) (Tu *et al.*, 2008). In the gills, Wang *et al.* (2009) found that catalase activity increased during the exposure period with enrofloxacin and stabilized afterwards. Moreover, it has been shown that higher stress conditions (e.g. high cultured species density) will often increase its toxicity (Wang *et al.*., 2009; Tu *et al.*., 2008). Regarding the results of these studies, acetylcholinesterase activity has been demonstrated to be the most suitable biomarker to test the effects of antibiotics on cultured fish (Tu *et al.*., 2009). Dose-response relationships using biomarkers of exposure on non-cultured species have not been investigated so far; however, they are considered as a useful tool to assess the exposure and effects of environmental pollutants on aquatic ecosystems (Van der Oost *et al.*., 2003). These results, combined with other toxicity tests, could be used in environmental risk assessments to determine potential effects on ecosystem's health (Crane *et al.*., 2006) and for the investigation of the metabolic effects and mode(s) of action of toxicants in the studied organisms.

Robinson *et al.* (2005) showed that the algae *Pseudokirchneriella subcapitata* is more sensitive to enrofloxacin (EC_{50} = 3 100 µg/L, which corresponds to 8.62 x 10^{-6} mol/L after 72h) than to ciprofloxacin (EC_{50} = 18 700 µg/L, which corresponds to 56.46 x 10^{-6} mol/L, after 72h). Moreover, Qin *et al.* (2011) found that *Pseudokirchneriella subcapitata* is more sensitive to enrofloxacin than *Scenedesmus obliquus* (EC_{50} = 45 100 µg/L, after 72h of exposure in similar conditions).

Regarding acute tests performed on invertebrates, the toxicity of enrofloxacin on *Daphnia magna* is lower compared to ciprofloxacin (Kim *et al.*., 2010). Furthermore, Park and Choi (2008) showed that *Daphnia magna* is more sensitive to enrofloxacin than *Moina macrocopa* with an EC_{50}-48h of 56 700 µg/L and > 200 000 µg/L, respectively.

This literature review emphasizes the fact that the toxic effects of enrofloxacin and ciprofloxacin on non-standard test aquatic organisms (e.g. algae, micro-invertebrates or non-cultured fish) have received little attention to date. Also, even if similar protocols are used in different studies, the toxicity values are discrepant due to different testing conditions (i.e., temperature, pH) and test species (Kim *et al.*., 2010). Furthermore, the potential acute toxicity of these compounds on non-target aquatic organisms surrounding the aquaculture farms in tropical regions has not been investigated so far. This information is of crucial importance for assessing the risks of these pharmaceuticals to tropical freshwater ecosystems and the sustainability of current aquaculture practices in regions like the Mekong delta.

Aim of the project

The current study was conducted as a part of the EU funded SEAT "Sustaining Ethical Aquaculture Trade" project (SEAT, 2011).

In this project, the sensitivity of freshwater organisms was studied when exposed to enrofloxacin and ciprofloxacin in the surrounding of catfish farms. First, the fate of these antibiotics was determined in water samples collected during and after the application of enrofloxacin in the effluent discharge point of one catfish farm. Then, the acute effects of these pharmaceuticals were tested on three types of organisms: algae (*Chlorella* sp.), invertebrate (*Moina macrocopa*), and the Nile tilapia (O*reochromis niloticus*). The results were expressed for *Chlorella sp.* and *Moina macrocopa* as EC_{50} (Concentration for which 50% of the exposed organisms show effects for the studied endpoint) and NOEC (No Observed Effect Concentration). The toxic effects of these fluoroquinolones on Nile tilapia was studied by assessing the effects on cholinesterase and catalase enzymatic activity. Finally, the concentrations from the effluent discharge point were compared with the results found in the toxicity tests in order to assess the potential ecological risks posed by the use of these antibiotics in catfish farms.

Research questions

- What are the acute effects of enrofloxacin and ciprofloxacin on the algae *Chlorella* sp., the invertebrate *Moina macocopa*, and the Nile tilapia (O*reochromis niloticus*)?
- What are the residual environmental concentrations found after antibiotic treatment in catfish ponds?
- What are the potential ecological risks posed by the use of enrofloxacin and ciprofloxacin in *Pangasius* catfish farms of the Mekong Delta?

Hypothesis

Acute effects of enrofloxacin and ciprofloxacin on the algae *Chlorella* sp., the invertebrate *Moina macrocopa*, and the Nile tilapia (O*reochromis niloticus*) are not expected to occur at concentrations that can be found in the environment after use in catfish farms, given their moderate sensitivity to the studied compounds reported in previous studies.

Materials and methods

I. Antibiotic fate assessment

I.1. Experimental set up

Enrofloxacin was applied once a day for a period of 5 days at a dose of 6.28 mg/kg body weight (mixed with feed representing 0.21 g/kg of feed) in a *Pangasius* catfish pond. The concentration of enrofloxacin in water samples was monitored for a period of 31 days.

Samples of water were taken after de first application (day 1), after the third application (day 3) after the last application (day 5) and on day 3, 7, 14 and 21 after the last application (figure 1). To do so, one meter depth water samples were taken in 1L plastic bottles and further kept in an ice box until storage in a fridge (4°C) in Can Tho University.

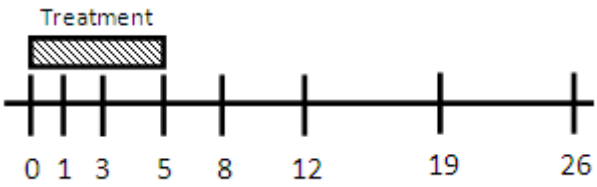


Figure 1: Experiment time-line

In the pond, water samples were taken in nine sampling points at each sampling time, including the water supply and the two water discharge points (figure 3), in order to test potential in-pond enrofloxacin concentrations and environmental pollution respectively (figure 2).

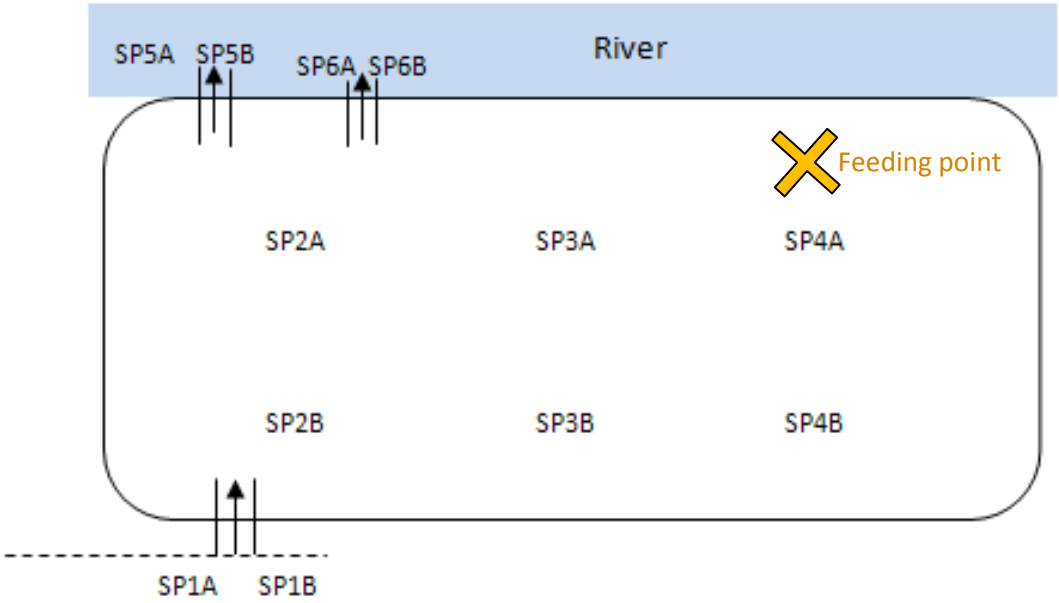


Figure 2: Scheme of the catfish pond with the selected water sampling points in the pond inlet, pond and pond outlet.

Figure 3: Localisation of the two water discharge points where water was sampled (SP5 and SP6)

Moreover, the water samples of SP A and B were mixed before analysis in order to reduce the number of samples.

Only the concentrations of fluoroquinolones in water samples SP5 and SP6 will be presented in the results as they are the most interesting ones for this study. In fact, the concentration of enrofloxacin and ciprofloxacin in the water coming out from the catfish ponds were compared with the results found in the toxicity tests. From this evaluation, it became possible to assess the risks posed by the use of these antibiotics in catfish farms.

I.2. Antibiotic analysis in the water samples

Water samples collected from the ponds were stored in the fridge (4°C) for approximately 24 h until extraction. Prior the beginning of this step, the water sample was filtered through a 0.45 µm microfiber membrane filter (Whatman GF/C). Then, a known volume of water (approximately 300 mL) was passed through Oasis HLB solid phase extraction (SPE) cartridges of 3 cc. SPE columns were preconditioned with 5 mL methanol and 5 mL of distilled water. The water sample was passed through the SPE columns at a speed of 5 mL/min and stored at -20 °C until elution. Elution was performed by passing 5 batches of 1 mL of a solution of NaOH (0.1 M) in ACN (high purity) (75:25 v/v). Then, 1 mL of the eluent was introduced in plastic vials and 100 µL of internal standard (lomefloxacin) was added.

The antibiotic analysis was made by LC-MS/MS. Prior to injection dilution of the extract was required for the toxicity test samples. 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-C_{18} 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 until min 10, then moved to 80% and held for 4 mins, and then moved to 20% and held for 6 mins. The mass spectrometry analysis was conducted with a triple quadrupole mass spectrometer 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. The calculated recoveries of enrofloxacin and ciprofloxacin in water following the extraction method used with the environmental samples and using a nominal concentration of 10 µg/L were 111.8±12.8 % (n=4) and 90.7±9.6 % (n=5), respectively. The limit of detection (LOD) was 0.01 for enrofloxacin and 0.03 for ciprofloxacin. The limit of quantification (LOQ) of the antibiotics was calculated: 0.05 for enrofloxacin and 0.09 for ciprofloxacin.

II. Toxicity experiments

The effects of enrofloxacin and ciprofloxacin were tested on three types of aquatic organisms: an alga (*Chlorella* sp.), an invertebrate (*Moina macrocopa*), and the Nile tilapia (O*reochromis niloticus*).

II.1. Chemicals

The formulated products of enrofloxacin and ciprofloxacin were used as the dilution of the pure antibiotics with water alone was impossible for the concentrations wanted (low solubility of the compounds). The Vimenro 200® formulation (Vemedim company) was purchased from a chemical outlet specialized in veterinary products in Can Tho city (Vietnam). This liquid preparation contained 20% of active ingredient (a.i.) and 80% of an unknown solvent. Regarding the formulated product of ciprofloxacin, the farmers could only buy it as a powder form. Therefore, no information was provided on the product except that it contained 50% of a.i.

II.2. Test organisms

Chlorella sp. was provided by the Algae Department of Can Tho University. Similarly to the other algae, they were cultured in a laboratory under constant light and temperature (26±1°C, typical water conditions in the Mekong delta).

Moina macrocopa organisms were purchased in the campus 2 of Can Tho University. These test organisms were then kept in a small tank with water coming from the shallow pond in which they were cultured (water temperature = 31±1°C).

For the fish experiment, 1 500 Nile tilapias (*Oreochromis niloticus*; 10.5 g ± 2g) were bought from a nearby hatchery and reared in a big tank of oxygenated water. The fish were acclimatized to the experimental conditions for one month prior to the start of the experiment and fed with commercial feed in form of pellets (30% protein, 5% fat), administrated twice a day at 8 am and at 5 pm.

II.3. Toxicity tests with *Chlorella sp.*

Toxicity tests with enrofloxacin and ciprofloxacin were performed using *Chlorella sp*. following the "OECD 201" (Freshwater Alga and Cyanobacteria, Growth Inhibition Test) protocol (OECD, 2006). The cultured algae were exposed to the two fluoroquinolones independently for a period of 72h. The following concentrations of antibiotics were used in the toxicity experiments:

- ✓ [Enrofloxacin] = 0, 21, 42, 75, 150, 300, 600 mg/L
- ✓ [Ciprofloxacin] = 0, 3.15, 6.3, 12,5, 25, 50, 100 mg/L

These concentrations were selected based on the results of toxic effect range-finding tests performed prior to the final experiments.

During three days before the start of the experiment, the number of cells in the algae culture (further used as inoculum) was counted to guarantee its exponential growth. Moreover, the Walne medium was prepared and further used as the algae growth medium (for the composition of the Walne growth medium, see Annex 1). In addition, 5L of tap water were filtrated and placed in an autoclave for 1h at 120°C for sterilization.

At the beginning of the test, all the equipment and mixtures were placed under a laminar flow cabinet and further sterilized using a UV light for 20 minutes. This was done in order to produce sterile conditions. Then, a mixture of filtrated tap water (mechanic filtration), exponentially growing *Chlorella* sp. (0.6×10^6 algae cells per mL), 200 µL of growth medium and 3 drops of vitamins was added to 21 Erlenmeyers. Then, the 6 concentrations of antibiotic were spiked in the first 18 flasks (in 3 replicates). In the last three, no antibiotic was added (untreated controls). The total volume for each Erlenmeyer was 100 mL. Subsequently, the test vessels were closed with cotton, placed in the laboratory (T = 26 ± 1°C; constant illumination with light intensity selected to suit *Chlorella sp.*) and shaken 3 times a day during 72h (figure 4).

Figure 4: Experimental set-up for the algae toxicity test

At 0h (right after the antibiotic application) and 72h, 1 mL of two replicates (for each of the 6 initial concentrations and the untreated controls) was sampled using an Eppendorf tube. All samples were further placed in the freezer (-20°C) before the chemical analysis (explained below).

After 0h, 36h and 72h, the temperature and pH were measured in the remaining Erlenmeyers (portable pH meter SevenGo, Mettler Toledo).

After 0h, 36h and 72h, 2 mL of algae media was sampled from each test unit and mixed with 100 µL of formalin (4% v/v) in a small test tube. Afterwards, the algae cells in each sample were counted using a counting chamber (Bürker CE-Marienfeld Germany; Tiefe depth profondeur = 0.100mm, 0.0025mm^2, 0.004mm^2) and a powerful microscope (OLYMPUS CX 21; magnification: x400). From these measurements, the average specific growth rate (equation 1) and the percentage inhibition in average specific growth rate were quantified as a function of time for each treatment group including the controls (equation 2) (OECD, 2006).

Equation 1: Average specific growth rate

$$\mu_{i-j} = \frac{lnX_j - lnX_i}{t_j - t_i}$$

With: μ_{i-j} = average specific growth rate from time i to j

X_i = biomass at time i

X_j = biomass at time j

Equation 2: Percentage of inhibition of the average specific growth rate

$$\%I_r = \frac{\mu_C - \mu_T}{\mu_C} \times 100$$

With: $\%I_r$ = Percentage inhibition of the average specific growth rate

μ_C = mean value for the average specific growth rate in the control group

μ_T = average specific growth rate for treatment replicates

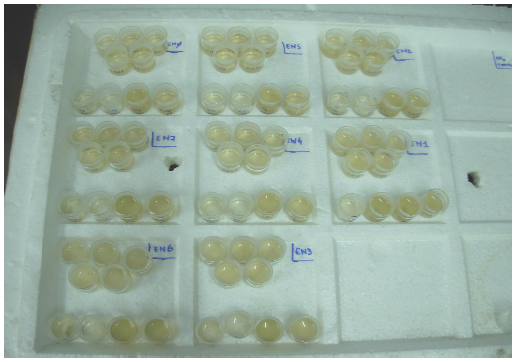
II.4. Toxicity test with *Moina macrocopa*

Toxicity tests with enrofloxacin and ciprofloxacin on *Moina macrocopa* were performed based to the available protocol for *Daphnia sp*.: "OECD 202 - *Daphnia* sp. Acute immobilization test" (OECD, 2004). However, adaptation of the temperature was necessary to reproduce the conditions seen in the tropical ecosystems. The following concentrations of antibiotics were used based on toxic effect range-finding tests performed with *Moina macrocopa*:

- ✓ [Enrofloxacin] = 0, 40, 80, 160, 320, 640, 1280 mg/L
- ✓ [Ciprofloxacin] = 0, 20, 40, 80, 160, 320, 640 mg/L

First, 6 stock solutions of enrofloxacin were prepared in 150 mL of filtered pond water. For each of the 6 concentrations, 3 mL of the stock solution was added to 9 small cuvettes (capacity of 5mL). In 9 extra small cuvettes, no antibiotic was added (untreated controls). Five small cuvettes per treatment level three neonates of *Moina macrocopa* (<24h old) were placed in each cuvette (blank included) in order to perform the toxicity test. The three remaining cuvettes per concentration were used for the chemical analysis and the ultimate one to record the water quality parameters. Then, the cuvettes

were placed on a platter in the laboratory (T = 31°C ±1°C; 12h light - 12h dark regime) during 48h (figure 5).



mental set-up for the toxicity test on *Moina macrocopa*

At 0h (right after the antibiotic application), 24h and 48h, 1 mL of each of the 6 initial concentrations and the untreated controls was sampled using an Eppendorf tube. All samples were further placed in the freezer (-20°C) before determining the concentration of enrofloxacin (cf. chemical analysis).

After 0h, 24h and 48h, the temperature and pH were measured in the cuvettes prepared for this matter (portable pH meter SevenGo, Mettler Toledo).

At 0h, 24h and 48h, the number of immobilized Daphnids was recorded for each cuvette. The *Moina macrocopa* were considered immobile when no movement was detected for 15 seconds after gentle agitation of the test vessel.

II.5. Toxicity tests with Nile tilapia (*Oreochromis niloticus*)

The toxicity of enrofloxacin and ciprofloxacin for Nile tilapia was studied by assessing the effects on the Catalase (CAT) and Cholinesterase (ChE) enzymatic activities in the muscles and brain of the fish, respectively. Experiments were performed for both antibiotics using two different methods of drug administration: mixed with feed (oral administration) and added directly to water (bath treatment). In both cases, four concentrations were chosen based on the commonly used dose of enrofloxacin and ciprofloxacin in catfish farms: 5g/kg of feed (Tu *et al.*., 2009). All experiments lasted for 14 days, and were separated in two phases: the exposure period (day 1 to 5) and the recovery period (day 6 to 14). Then, the biomarkers response were compared for the two antibiotic administration methods in order to determine which one causes the highest stress in the studied organisms and is more suitable as biomarker of exposure for the environmental monitoring of antibiotic residues.

II.5.a. Preparation of the tests

Two weeks prior the start of each experiment (and for both antibiotics), 15 tanks were prepared with 60 L of tap water and one oxygen tube to ensure the constant aeration of the containers. After 3 days, 24 Nile tilapia of similar size were weighted and further placed in each of the 15 tanks (figure 6 and 7).

Figure 6: Tanks used for the fish experiments

Figure 7: Nile tilapia in a tank

The amount of food to give daily during the experimental period was calculated using the equation 3 (annex 2).

Equation 3: weight of food given twice on day d

$$W(d) = \frac{F(d) \times \hat{w} \times BM(d)}{2}$$

With : $W(d)$ = weight of food given twice per day on day d (in g)

$F(d)$ = number of fish in the tank on day d (dimensionless)

$\hat{w}$ = mean fish weight in the tank (in g)

$BM(d)$ = percentage of the fish body mass (dimensionless)

- From day 1 to 5, $BM(d)$ was set at 3%. This value was chosen to make sure that the fish would eat all the food given during the exposure period.
- From day 6 to 14, $BM(d)$ was set at 5%. Generally, the fish eat an amount of food corresponding to 5% of their body mass.

II.5.b. Drug administration regime

II.5.b.i. Oral administration experiments

For the experiments performed with orally administered enrofloxacin and ciprofloxacin, the following 4 concentrations of antibiotics were chosen to be tested:

- ✓ [Enrofloxacin] = 0, 1, 2.5, 5, 10 g a.i./kg of feed (representing respectively 0, 18.8, 37.5, 75, 150, 300 mg a.i./kg of fish body weight)
- ✓ [Ciprofloxacin] = 0, 0.5, 1.12, 2.5, 5 g a.i./kg of feed

Prior the start of these experiments, small bags of "contaminated" and "uncontaminated" food had to be prepared for the entire test periods. The total amount of food needed (for all tanks during the whole experimental period) was calculated for the test of enrofloxacin (W_e) and ciprofloxacin (W_c),

using the results of equation 3. As four concentrations were decided to be tested for both antibiotics, the total amount of food for each tests W_e and W_c was divided into 4 batches. Stock solutions of enrofloxacin and ciprofloxacin were prepared with water and sprayed on the pellets in order to reach the feed concentrations reported above. Then, the food was mixed with a spoon (from the lowest to the highest concentration) and covered with aluminium paper. Once the pellets were dry, small bags of "contaminated" food were prepared for every tank and every day of the exposure periods. "Uncontaminated" food pellets were weighted and further placed in small bags for every tank during the recovery periods. Subsequently, all the food was placed into a freezer (-20°C) to avoid antibiotic degradation on the pellets.

During the exposure period (day 1 to 5), the fish of 12 tanks out of the first 15 tanks were fed with food mixed with antibiotic, previously prepared. The three remaining tanks were used as controls such that the fish were fed with uncontaminated pellets during the entire experiment. During the recovery period (day 6 to 14), the fish of all tanks were fed with uncontaminated pellets already separated into small bags. The food was given two times per day at 8 am and 5 pm.

II.5.b.ii. Bath treatment experiments

For the bath-treatment tests, the following 4 concentrations of antibiotics were chosen to be tested:

- ✓ [Enrofloxacin] = 0, 0.1, 0.8*, 10, 100 mg a.i./L
- ✓ [Ciprofloxacin] = 0, 0.05, 0.4*, 5, 50 mg a.i./L

  * The concentration of 0.8 mg enrofloxacin/L is comparable to the 5g/kg of food, commonly used dose of enrofloxacin in catfish farms. The concentration of 0.4 mg ciprofloxacin/L is comparable to half of its recommended dose (Tu *et al.*., 2009).

On day 1 to 5, four stock solutions of antibiotic were prepared in triplicate, based on the 4 concentrations chosen to be tested. This volume was then added to the water of the corresponding 12 tanks. Three other ones were used as controls without antibiotic application into the water.

As it was done for the oral administration experiments, the fish of all tanks were fed two times per day (8 am and 5 pm) from the small bags of pellets previously prepared (using equation 3).

II.5.c. Common methodology for both drug administrations

Every day of the test, 20 L of test media was removed from each tank and replaced by new tap water to avoid excessive water quality deterioration.

On day 1, 3, 5, 7, 10 and 14, water and fish were sampled and water quality parameters were recorded (figure 8).

Figure 8: Fish experiment time-line

Water from each tank was sampled using an Eppendorf tube. This volume was further placed into a freezer (-20°C) prior the determination of the antibiotics' concentration (cf. chemical analysis).

Moreover, temperature and pH were recorded for each tank (waterproof digital pH meter, Hanna). As the tests were run outdoors, the temperature was recorded but could not be controlled precisely.

Finally, three fish per tank were sampled, killed by a cold shock in ice and directly dissected to extract the brains and a part of the muscles (figure 9). These samples were then stored at -80 °C prior the biomarker analysis.

Figure 9: Fish dissection

II.5.d. Biomarker analysis

The biomarker analysis was performed in triplicates as each antibiotic concentration was tested in three tanks. Moreover, the samples of days 3, 5 and 14 were the only ones investigated due to financial reasons. Those days were chosen as they represent the exposure (middle and end) as well as the recovery.

II.5.d.i. Sample preparation

To assess CAT and ChE activities, the fish muscles and brains had to be first homogenized. To do so, the samples were mixed with phosphate buffer (KH_2PO_4/K_2HPO_4 50mM; pH = 7.5) using a sample mill (T18 basic Ultra Turrax, United States) and 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. In this way, all the biomarkers could be analysed at once. Furthermore, the total protein content was assessed in each supernatant mixture according to the method of Lowry *et al.* (1951) in order to normalize the CAT and ChE levels to the protein content.

II.5.d.ii. CAT analysis

CAT activity was assessed in the muscles, based on the method of Baudhuin *et al.* (1964). To do so, 25 µL of homogenate was added to 25 µL Triton X-100 2%- and 1 250 µL of substrate solution (composed by bovine serum albumin, H_2O_2 and imidazol) in an Eppendorf cuvette. After incubation at 0°C for 6 min, 750 µL of $TiSO_4$ was added. After 5 to 10 min, absorbance was read at 420 nm using

a Varian – Cary 50 UV / Visible spectrophotometer. One unit of CAT activity is 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 is expressed as Baudhuin units (BU)/min/mg protein.

II.5.d.iii. ChE analysis

ChE activity was determined in the brains, according to Ellman *et al.* (1961). To do so, 50 µL of homogenate was mixed with 50 µL of acetylthiocholine (ATC) and 900 µL DTNB in an Eppendorf cuvette. After 10 min of incubation at ambient temperature, absorbance was measured at 412 nm using a Varian – Cary 50 UV / Visible spectrophotometer. ChE was expressed in nmol of hydrolysed ATC per min and per milligram of protein.

II.6. Chemical analysis

High-Performance Liquid Chromatography (HPLC) was used in order to determine the concentrations of antibiotics in the water samples.

In all experiments, the concentration of antibiotics was measured in one replicate per treatment level due to financial reasons. For the tests with *Chlorella sp*. and *Moina macrocopa*, one replicate was decided to be analyzed at the beginning and the end of the tests due to financial reasons. For the oral administration experiments, the chemical analysis was not extensive as the pharmaceutical's concentration was expected to remain very small. Out of all, 4 samples were chosen for investigation: 2 samples for the controls and 2 others from the highest nominal concentration, on day 1 and 5. For the bath treatment experiments, only one replicate of each concentration was analyzed on day 1 (after application), 2, 5, 7 and 14, as well as 2 controls on day 1 (before application) and on day 5.

First, all samples were diluted for the antibiotics' concentration to approximate 0.05 mg/L. HPLC water was used for the first dilution(s) and phosphate buffer (80: K_2HPO_4 0.02M and 20: CAN; pH = 3) for the final dilution. Further, all samples were placed into a syringe 13mm and pushed through a 0.2µm filter prior the injection into the HPLC (volume of injection = 20µL). The HPLC was a Shimadzu HPLC system including LC-10ATvp Pump, SCL-10A VP System controller, DGU- 12A Degasser, RF-10A XL Fluorescence Detector. Compounds were separated on a reversed phase analytical column (Gemini 3 µm C6-Phenyl 110 Å, 150-3 mm i.d., 3 micron particle, from Phenomenex; temperature ambient). The isocratic mobile phase (phosphate buffer described previously) lasted for 20 min and was performed at a constant flow rate of 0.3 mL/min. The fluorescence excitation/emission wavelengths was 280/450 nm.

The recovery of the method was determined using enrofloxacin (ENR>98%) and ciprofloxacin (CIP>98%), purchased from Sigma Aldrich (St Louis, MO, USA). The individual standard stock solutions (0.1 mg/mL) were made in methanol. This solution was then diluted with the mobile phase to prepare the mixed working standards (1000 ng/mL). The recovery of the method was 98.0 ± 3.54 % for enrofloxacin and 100 ± 2.57 for ciprofloxacin (mean ± SD; n=3).

II.7. Statistical analysis

For the tests with *Chlorella sp*. and *Moina macrocopa*, calculations were performed based on the measured concentrations of the antibiotics in the culture media at the start of the experimental period.

II.7.a. Toxicity tests with *Chlorella sp*.

The concentration resulting in 10% and 50% inhibition of the calculated growth rate were determined (EC_{10} and EC_{50}) after 72h, as well as the no observed effect concentration (NOEC), using the software ToxRat Professional Version 2.01 (Toxrat, 2003). The EC_{10} and EC_{50} values as well as their 95% confidence intervals were calculated by probit analysis using linear maximum likelihood regression. The associated dose-response curve was built using the equation 4 (Rubach *et al*., 2011).

Equation 4: Equation used to calculate the dose-response curve

$$y(conc) = c + \frac{1 - c}{1 + e^{-b(\ln conc - a)}}$$

With: *y* = growth inhibition fraction (dimensionless)

conc = applied dose in µg/L on basis of the measured concentrations at t = 0

a = ln (EC_{50})

b = slope in L/µg

c = fraction of background effect

To determinate the NOEC, the Kolmogorov-Smirnov test was chosen to investigate whether the data was normally distributed, followed by a Cochran's test to assess the variance homogeneity. If the normal distribution and variance homogeneity prerequisites were met, the one way ANOVA test was performed. Next, the Dunnett's multiple t-test procedure was applied to compare the treatments with the untreated controls and determine the NOEC value.

II.7.b. Toxicity tests with *Moina macrocopa*

The percentages of immobilized *Moina macrocopa* at 48h were plotted against the tested concentrations. The concentration resulting in 10% and 50% inhibition of the calculated growth rate were determined (EC_{10} and EC_{50}) as well as their confidence intervals by using the software ToxRat Professional (Version 2.01). To do so, the probit analysis using linear maximum likelihood regression was applied. The associated dose-response curve was built using equation 4, with *y* being the fraction of dead or affected *Moina macrocopa* (dimensionless) (Rubach *et al*.., 2011). For these tests, the no observed effect concentration (NOEC) could not be calculated by the program since the variance homogeneity and the normality conditions of the data were not met.

II.7.c. Toxicity tests with Nile tilapia (*Oreochromis niloticus*)

For both biomarkers experiments, enzymatic activity was compared between the controls and the different treatment levels separately for each sampling date. The data from controls and the

different treatment levels were compared by a one-way analysis of variance (ANOVA) followed by a post-hoc analysis performed by using the Dunnett's test. Independently, the Kolmogorov-Smirnov and Levene's test were run to verify the normality and the variance homogeneity of the data, respectively. For the datasets for which these two criterions were not met, a Kruskal-Wallis test was used followed by a Mann-Whitney U test to determine the differences between the treatments and the controls. All differences were considered significant at $p<0.05$. Statistical analyses were performed using the SPSS statistical package (ver. 19.0, SPSS Company, Chicago, IL, USA).

Results

I. Antibiotic fate assessment

As determined by LC-MS/MS, the water samples collected in the effluent mixing point of the drainage canal of the studied Pangasius catfish farm had very low concentration of antibiotics. As a result, a precise quantification of the antibiotics was not possible. However, enrofloxacin and ciprofloxacin were detected in most samples in the range of µg/L (+ signs in table 3).

Table 3: Detection of enrofloxacin and ciprofloxacin in the outlet of the *Pangasius* catfish pond

Day after antibiotic administration in the catfish pond	Enrofloxacin detection	Ciprofloxacin detection
1	+	+
3	+	+
5	+	<LOD
8	+	<LOD
12	+	+
19	+	+
26	+	<LOD

II. Toxicity experiments

II.1. Toxicity test with *Chlorella sp.*

II.1.a. Chemical analysis

The measured concentrations of enrofloxacin and ciprofloxacin in the culture media are presented in tables 4 and 5 respectively.

Table 4 : Concentrations of enrofloxacin measured with HPLC (Algae experiment)

nominal concentration of enrofloxacin (in mg/L)	measured concentration of enrofloxacin (in mg/L)	
	0h	72h
0	0	0
21	21.3	16.3
42	40.2	27.8
75	97.1	83.3
150	209	203
300	384	266
600	728	517

Table 5: Concentrations of ciprofloxacin measured with HPLC (Algae experiment)

nominal concentration of ciprofloxacin (in mg/L)	measured concentration of ciprofloxacin (mg/L)	
	0h	72h
0	0	0
3.15	3.81	2.44
6.25	6.69	5.03
12.5	17.2	12.2
25	26.8	22.7
50	57.7	39.5
100	103	64.7

The concentration of antibiotics in the medium decreased during the experimental period: 22% on average for enrofloxacin and 29% on average for ciprofloxacin. Dissipation of the compound in the test medium is probably caused by hydrolysis, photohydrolysis, and uptake by the algae cells.

II.1.b. Validity of the experiment

When the OECD guideline 201 is used, three criterions must be tested for the experiment to be valid (table 6). The present experiments are not valid according to the OECD guideline 201 because the biomass of the controls increased by a factor lower than 16. This could be due to a lack of light, which is an essential feature for the growth of algae. In this experiment, the algae were not constantly shaken as they were only mixed manually three times per day. Therefore, the external layer received more light than the other cells, such that not all algae could grow and develop as fast. A rotating platter combined with lights around it would have been useful to ensure a better algae growth. In fact, similar conditions were used to produce the inoculum culture and run the toxicity tests. The only difference remained in the oxygen supply in the inoculums culture. Small pipes were added into the medium to enrich it with oxygen and in the same time, the bubbles ensured a continuous movement of the algae. However, the laboratory conditions were such that it was not physically possible to add one oxygen supply to all Erlenmeyers of the toxicity tests.

Even though the toxicity tests presented here are not valid regarding the OECD guideline 201 criteria, the statistical analysis was performed since the test still provides an indication of toxicity.

Table 6: Validity criterions of the test according to the OECD guideline 201

	Value from the test with enrofloxacin	Value from the test with ciprofloxacin	Indispensable value for the test to be valid (OECD guideline 201)
Factor of biomass increase in the control cultures	10.9	8.3	> 16
Mean coefficient of variation for the section by section specific growth rate in the controls	5.5%	6.5%	< 35%
Coefficient of variation of the average specific growth rate during the whole test period	6.2%	7.2%	< 10%

II.1.c. Algae growth inhibition

Results of the algae cell counts for enrofloxacin and ciprofloxacin are shown in figures 10 through 14 respectively, as well as in Annex 3. In both cases, it is clear that the algae population is growing over time in the control treatment (figures 10 and 12). Moreover, the growth of the algae population was totally inhibited in the highest treatment level. This is also clear looking at the color variation between the different Erlenmeyers (figures 11 and 13). With increasing concentration of both antibiotics, the color of the medium becomes lighter; which reflects the expected dose-growth inhibition effect.

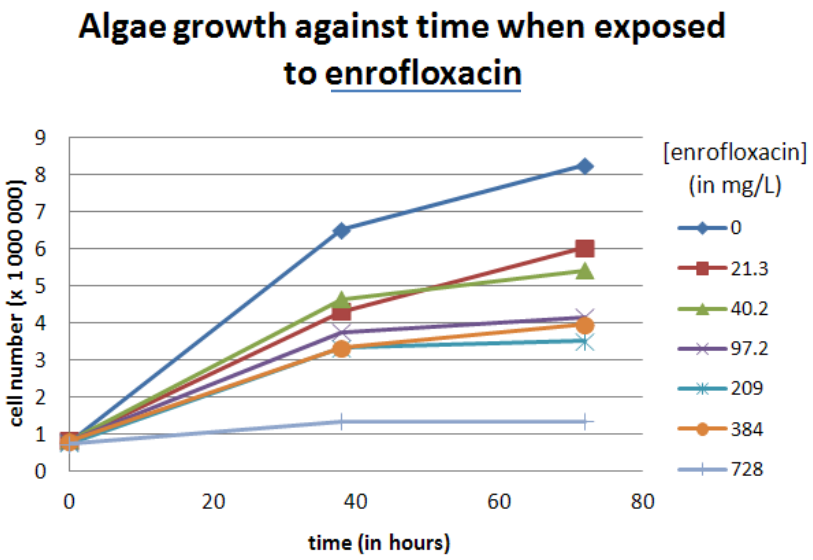


Figure 10: Algae growth against time when exposed to different concentration of enrofloxacin

Figure 11: Test vessels after being in contact with enrofloxacin for 72h

the highest concentrations are closer to the reader

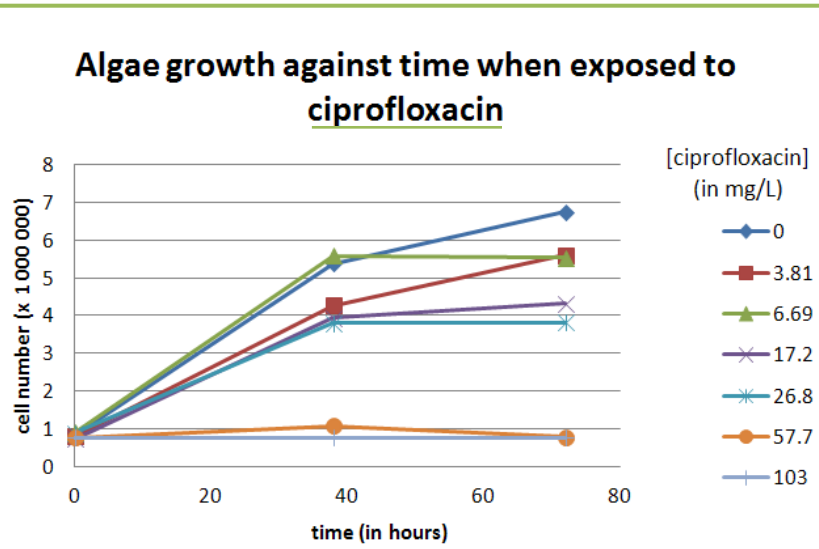


Figure 12: Algae growth against time when exposed to different concentration of ciprofloxacin

Figure 13: Test vessels after being in contact with ciprofloxacin for 72h

the highest concentrations are closer to the reader

II.1.d. Statistical analysis

The growth inhibition dose-response curves for enrofloxacin or ciprofloxacin concentration (after 72h) are shown in figures 14 and 15. The curve slopes for enrofloxacin and ciprofloxacin were 0.93 and 4.64 respectively. The calculated EC_{50}-72h values were 406 mg/L for enrofloxacin and 31.1 mg/L for ciprofloxacin (table 7).

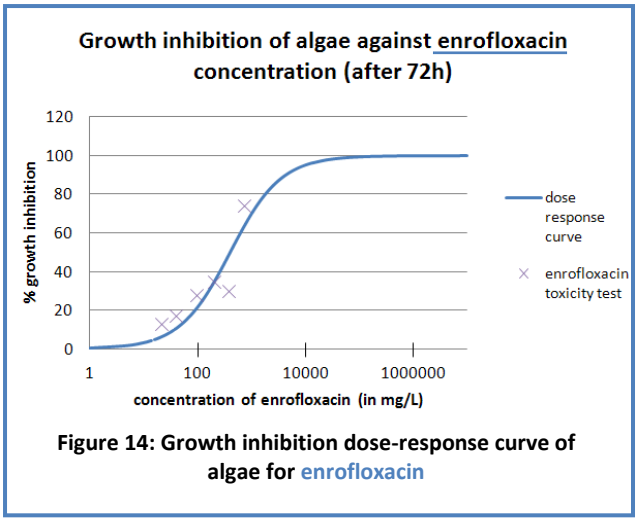


Figure 14: Growth inhibition dose-response curve of algae for enrofloxacin

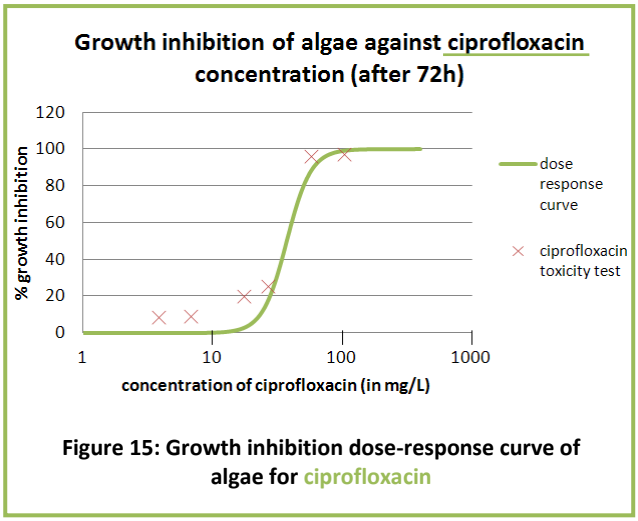


Figure 15: Growth inhibition dose-response curve of algae for ciprofloxacin

II.1.e. Water parameters

In general the temperature and pH values remained rather constant during the experimental period (table 7). However, the pH seems to increase a little in time, probably due to the release of basic solvent present in the formulated compounds.

Table 7: Results of the acute toxicity tests on *Chlorella sp*. after 72h and water parameters

Test	NOEC (in mg/L)	EC_{10} (in mg/L) (95% confidence limits)	EC_{50} (in mg/L) (95% confidence limits)	Temperature (°C) Mean ± SD	pH Mean ± SD
Enrofloxacin	< 21.3	16.8 (n.d. - 69.3)	406 (144 – 9083538)	26.2 ± 1.8	9.08 ± 1.11
Ciprofloxacin	3.8	18.8 (n.d.*)	31.1 (n.d.*)	25.3 ± 1.5	8.70 ± 1.16

*n.d.: not determined by the software due to mathematical reasons

II.2. Toxicity tests with *Moina macrocopa*

II.2.a. Chemical analysis

The concentrations of enrofloxacin and ciprofloxacin measured with the HPLC are presented in tables 8 and 9. The concentration of ciprofloxacin in the medium decreased of 42% on average during the experimental period (if the highest tested concentration is not taken into account). However, the concentration of enrofloxacin is only slightly declining (2% on average) in the 48 h period. Conversely to what was observed in the algae test, ciprofloxacin seemed to degrade faster under the test conditions used for *Moina macrocopa* (e.g. higher temperature).

Table 8: Concentrations of enrofloxacin measured with HPLC (*Moina macrocopa* experiment)

nominal concentration of enrofloxacin in mg/L	measured concentration of enrofloxacin (mg/L)	
	0h	72h
0	0	0
40	35.6	34.8
80	63.7	62.2
160	126	123
320	289	283
640	599	586
1280	1115	1101

Table 9: Concentrations of ciprofloxacin measured with HPLC (*Moina macrocopa* experiment)

nominal concentration of ciprofloxacin in mg/L	measured concentration of ciprofloxacin (mg/L)	
	0h	72h
0	0	0
20	13.2	8.06
40	45.0	21.5
80	90.0	42.9
160	146	69.7
320	455	378
640	475	474

II.2.b. Validity of the experiment

When the OECD guideline 202 is used, two criteria must be tested for the experiment to be valid. In the controls, no more than 10% of daphnids should be immobilized and the concentration of dissolved oxygen at the end of the test should be higher or equal to 3 mg/L. The first criterion is met as all individuals were still moving after 72h (Annex 4). However, the dissolved oxygen concentration could not be measured due to the failure of the apparatus.

II.2.c. Immobilized daphnids

After 0h (following the application), 24h and 48h, the number of immobilized daphnids per cuvette was recorded (Annex 4). In general, the immobility of *Moina macrocopa* is enhanced when the antibiotic concentration increases.

II.2.d. Statistical analysis

The dose-response curves for the toxicity experiments performed with *Moina macrocopa* are shown in figures 16 and 17. The slopes were 5.59 for enrofloxacin and 8.34 for ciprofloxacin. The calculated EC_{50}-48h values were 70.0 mg/L for enrofloxacin and 96.5 mg/L for ciprofloxacin (table 10).

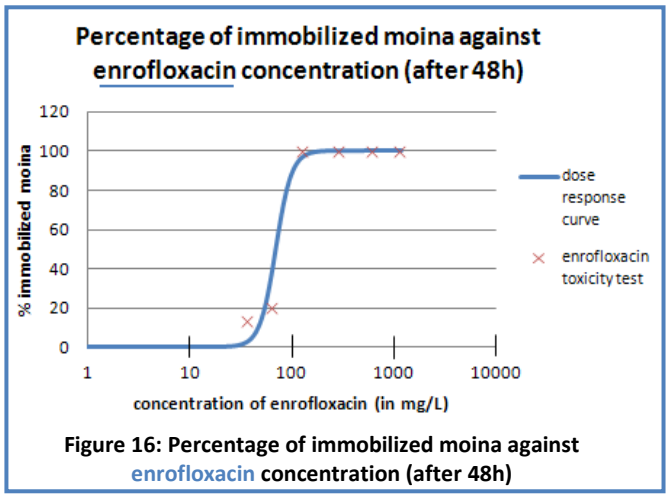


Figure 16: Percentage of immobilized moina against enrofloxacin concentration (after 48h)

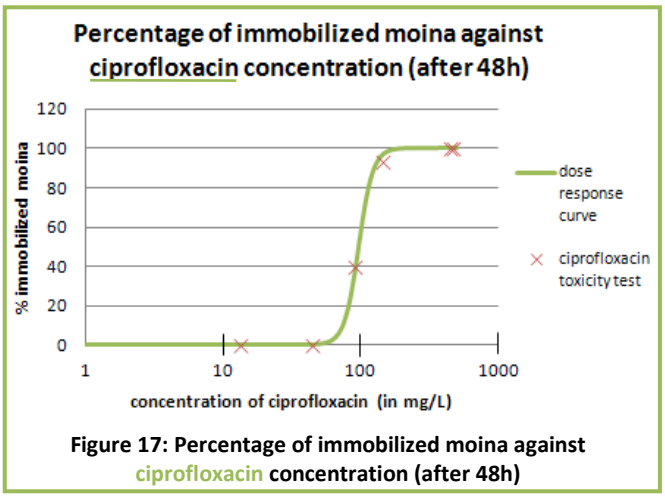


Figure 17: Percentage of immobilized moina against ciprofloxacin concentration (after 48h)

II.2.e. Water parameters

In general the temperature and pH values remained rather constant during the experimental period (table 10). However, the pH seems to increase a little in time, probably due to the release of basic solvent present in the formulated compounds or decomposition of organic matter.

Table 10: Results of the acute toxicity tests on *Moina macrocopa* after 48h and water parameters

Test	NOEC (in mg/L)	EC_{10} (in mg/L) (95% confidence limits)	EC50 (in mg/L) 95% confidence limits)	Temperature (°C) Mean ± SD	pH Mean ± SD
Enrofloxacin	/	41.3 (30.7 - 55.7)	70.0 (57.6 - 85.1)	31.6 ± 1.0	8.54 ± 0.20
Ciprofloxacin	/	67.8 (49.1 - 93.5)	96.5 (82.5 – 113)	32.7 ±2.4	8.41 ± 0.30

II.3. Toxicity tests with *Oreochromis niloticus*

II.3.a. First test: in-feed treatment

II.3.a.i. Chemical analysis

The concentrations of enrofloxacin and ciprofloxacin measured with the HPLC are presented in tables 11 and 12. In general, the pharmaceutical's concentrations in the water remained very small. This was expected because most of the antibiotics are taken up through the digestive system, such that very little concentrations are released in the aquatic environment. However, a non-negligible amount of pharmaceuticals is probably found in the feces, but this was not measured in the current experiment. These results also confirm that in this first test, the Nile tilapias are in contact mainly with the antibiotics through the contaminated food (the concentrations in the water are considered negligible in comparison to the bath treatment experiments).

Table 11: Concentrations of enrofloxacin measured with HPLC (in-feed treatment)

nominal concentration of enrofloxacin in the food (in g/kg of food)	measured concentration of enrofloxacin in the water (in mg/L)	
	day 1	day 5
0	0	0
10	0	0.45

Table 12: Concentrations of ciprofloxacin measured with HPLC (in-feed treatment)

nominal concentration of ciprofloxacin in the food (in g/kg of food)	measured concentration of ciprofloxacin in the water (in mg/L)	
	day 1	day 5
0	0	0
5	0	0.29

II.3.a.ii. Validity of the tests

In total, 4 and 9 fish died during the experiment with enrofloxacin and ciprofloxacin respectively (Annex 5). However, the tests are considered valid because the mortality in the control groups remained below 10%.

II.3.a.iii. Biomarker analysis

- *CAT activity*

The activities of CAT during the experimental periods are presented for enrofloxacin and ciprofloxacin in figure 18 and 19, respectively.

For all tested concentrations of ciprofloxacin, the CAT activity was not significantly different from the controls during the entire experiment. Regarding the test with enrofloxacin, the CAT activity decreased significantly on day 5 for almost all tested concentrations.

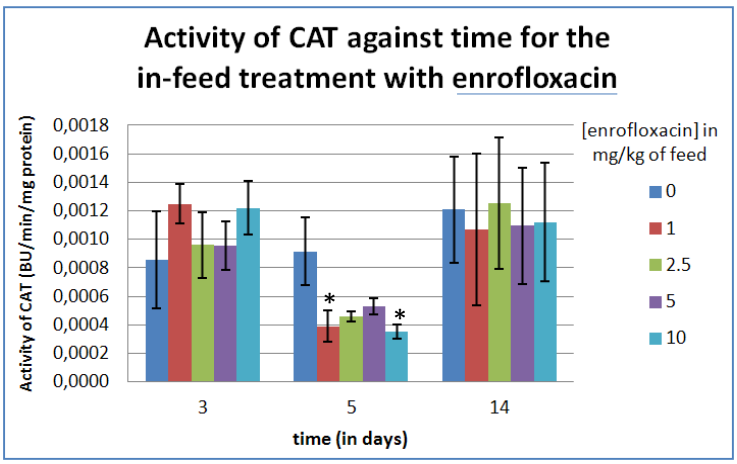


Figure 18: Activity of CAT against time for the in-feed treatment with enrofloxacin

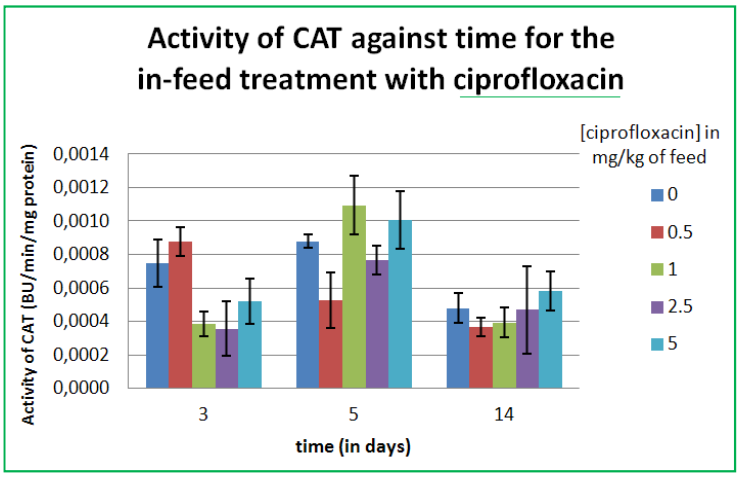


Figure 19: Activity of CAT against time for the in-feed treatment with ciprofloxacin

- *ChE activity*

The activities of ChE during the experimental periods are presented for enrofloxacin and ciprofloxacin in figure 20 and 21, respectively.

An increase in the ChE activity was observed for the concentrations of 5 and 10 g of enrofloxacin/kg of feed and 5 g of ciprofloxacin/kg of feed on day 3 and 5 when compared with the controls. Moreover, it is clear that this activity was higher on day 3, decreased on day 5 (end of the exposure period) to finally show no significant difference with the controls on day 14.

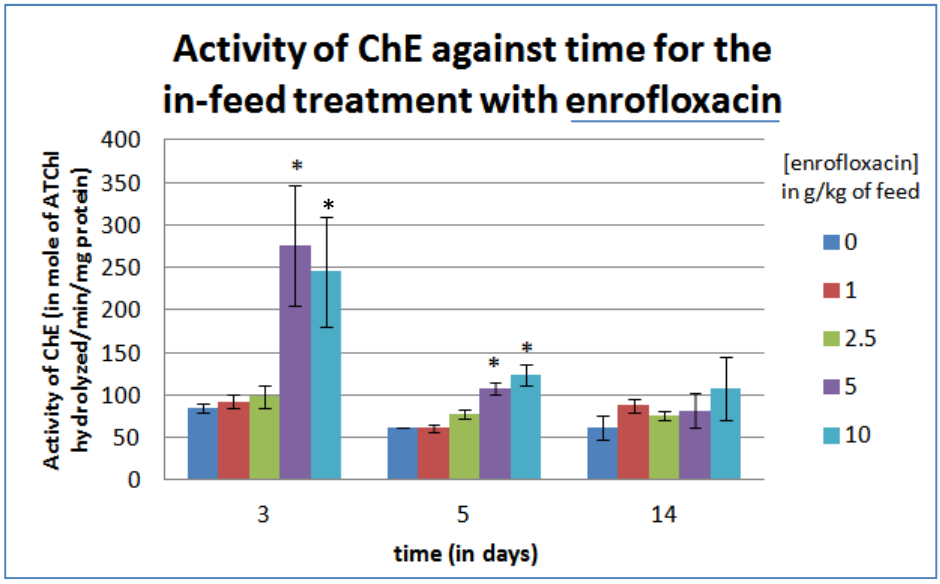


Figure 20: Activity of ChE against time for the in-feed treatment with enrofloxacin

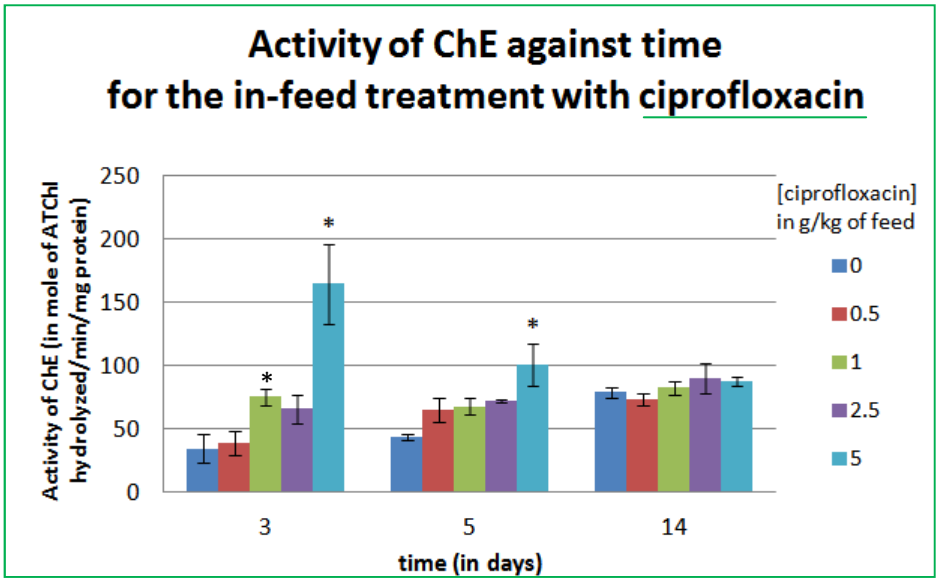


Figure 21: Activity of ChE against time for the in-feed treatment with ciprofloxacin

II.3.b. Second test: bath treatment

II.3.b.i. Chemical analysis

The evaluation of enrofloxacin and ciprofloxacin concentrations measured with the HPLC is presented in figures 22 and 23 as well as in Annex 6. The highest tested concentration of enrofloxacin is not shown in figure 20, due to the fact that the test corresponding to 100 mg/L was stopped after

three days (more explanations are given below). In general, the pharmaceutical's concentrations increased in the water during the exposure period (from day 1 to day 5). On day 5, a peak concentration is reached for all tests. Quickly after the beginning of the recovery period, the concentration of antibiotics in the water decreased to almost zero on day 14.

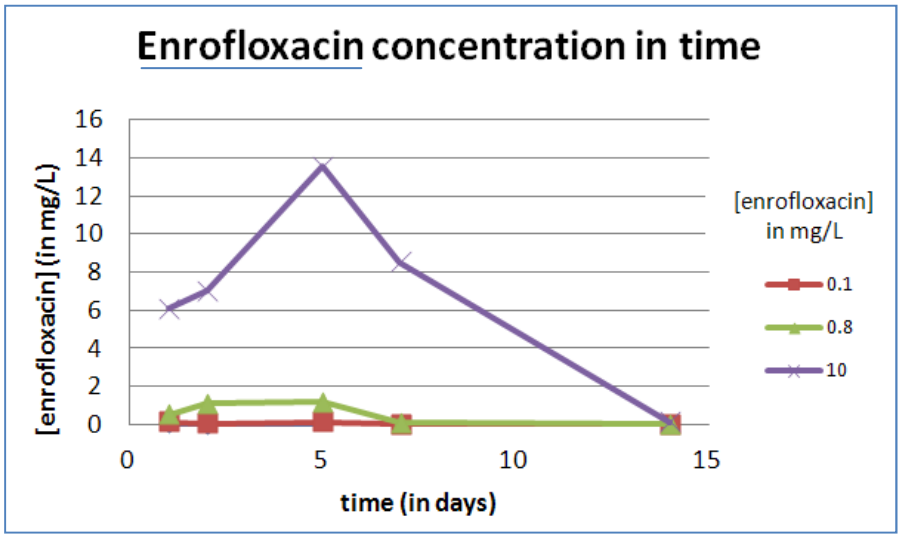


Figure 22: Enrofloxacin **concentration in time (bath treatment)**

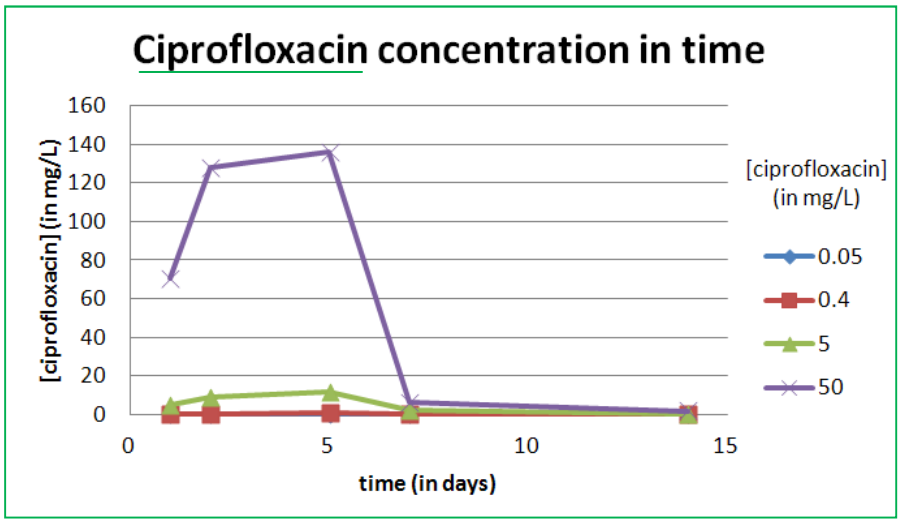


Figure 23: Ciprofloxacin **concentration in time (bath treatment)**

II.3.b.ii. Validity of the tests

As it was the case with the in-feed treatment tests, 13 and 3 fish died during the experiment with enrofloxacin and ciprofloxacin respectively (Annex 5). However, the tests are considered valid because the mortality in the control groups remained below 10%.

II.3.b.iii. Unexpected death and behavior of Nile tilapias

This bath-treatment test was more harmful to Nile tilapia than expected. In fact, after two days, all fish exposed to 100 mg/L of enrofloxacin died in all three replicates (63 fishes). This is the reason why this treatment (corresponding to 100 mg/L) was stopped after three days. Moreover, mortality was also seen in the bath treatment test with ciprofloxacin. After 6 days, all fish exposed with 50 mg/L died in two replicates out of three (the results from remaining tank are presented in this report without standard deviation) (Annex 5).

Furthermore, the fish mobility was compared on day 2 for the tanks treated with 0.1 and 10 mg/L of enrofloxacin during feeding time. As soon as the pellets touched the water, all fish tested with the lowest concentration were swimming very quickly to the surface to eat, such that all the food was eaten within 25 to 30 seconds. The fish tested with the concentration of 10 mg/L had no or very slow movements. After 30 seconds, the first individual started to eat followed by few others. It took them more than 1h to eat everything. However, this unexpected fish mobility return to normal at the beginning of the recovery period (day 6).

II.3.b.iv. Biomarker analysis

- *CAT activity*

The activities of CAT during the experimental periods are presented for enrofloxacin and ciprofloxacin in figure 24 and 25, respectively.

The CAT activity decreased on day 5 for all tested concentrations of enrofloxacin and ciprofloxacin. Moreover, this enzymatic activity did not totally recover on day 14 for the highest concentrations tested.

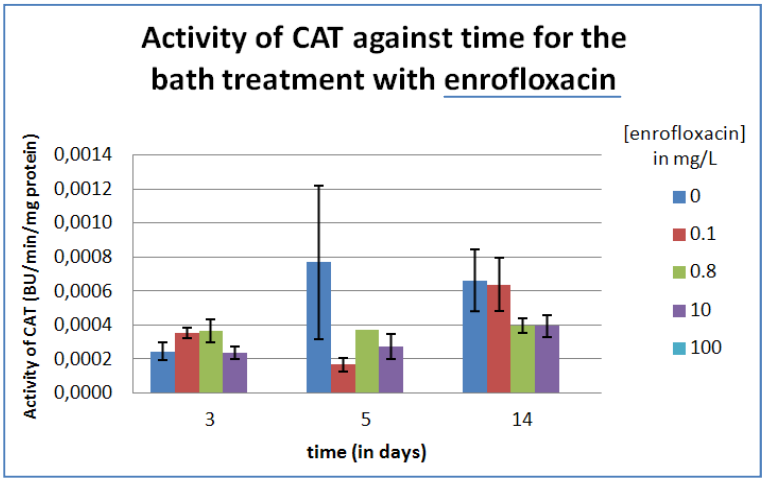


Figure 24: Activity of CAT against time for the bath treatment with enrofloxacin

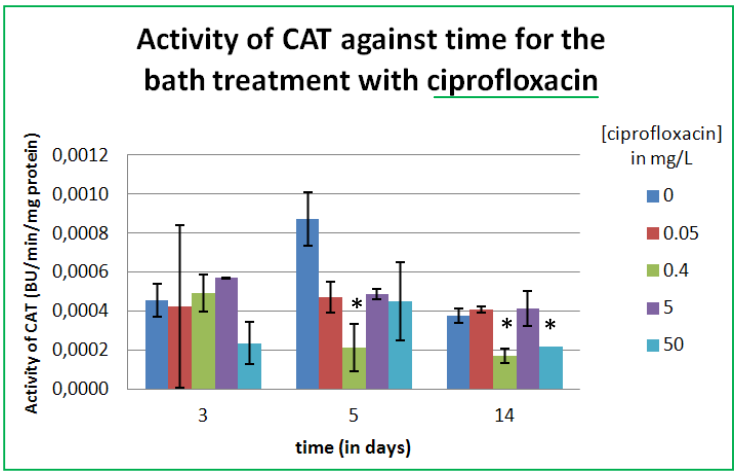


Figure 25: Activity of CAT against time for the bath treatment with ciprofloxacin

- *ChE activity*

The activities of ChE during the experimental periods are presented for enrofloxacin and ciprofloxacin in figure 26 and 27 respectively.

An increase in the ChE activity was observed for the concentration of 10 and 0.8 mg of enrofloxacin/L on day 3 and 14 respectively when compared with the controls. For the second bath treatment test, it seems that ChE activity gradually increased when the concentration of ciprofloxacin also increased on days 3, 5 and 14. However, the overall increase of ChE activity remains smaller compared to what have been observed for the bath treatment test with enrofloxacin.

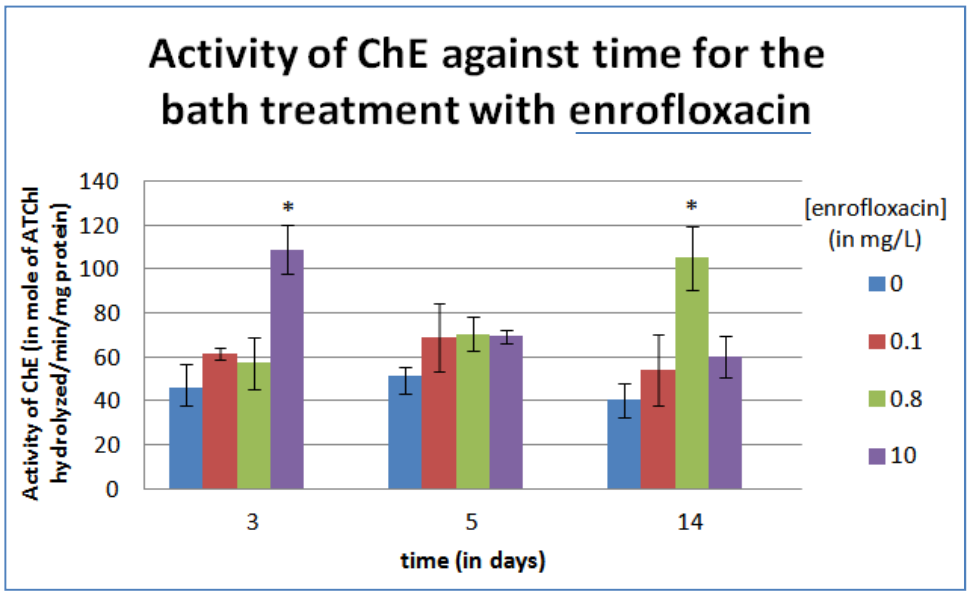


Figure 26: Activity of AChE against time for the bath treatment with enrofloxacin

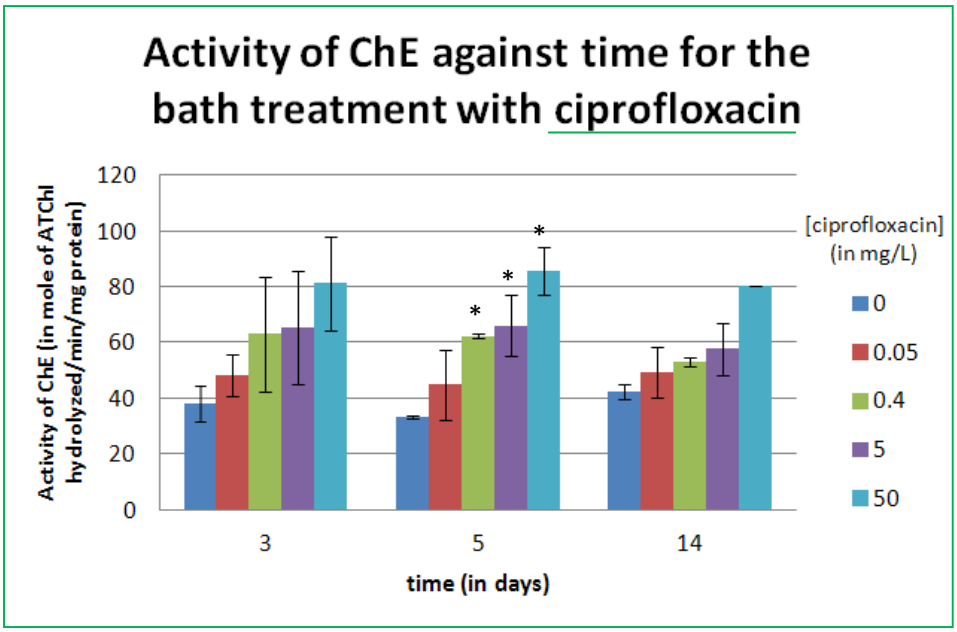


Figure 27: Activity of AChE against time for the bath treatment with ciprofloxacin

II.3.c. Water parameters

The results of the measured water parameters can be found in table 13. In general, the temperature and pH values remain rather constants during the experimental periods. As explained previously, the fish experiments were run outdoors, therefore, the water temperature could not be controlled precisely. It was clear that after a strong episode of rain (up to 3-4 days during the course of the in bath treatment test with enrofloxacin), the water temperature in the tanks decreased by 1 to 2°C.

Table 13: Water parameters values for the fish experiments

Antibiotic	Test	Temperature (°C) Mean ± SD	pH Mean ± SD
Enrofloxacin	in feed treatment	28.1 ± 1.7	8.13 ± 0.34
	bath treatment	27.1 ± 0.5	8.05 ± 0.42
Ciprofloxacin	in feed treatment	28.0 ± 1.2	7.90 ± 0.10
	bath treatment	28.0 ± 1.0	7.80 ± 0.17

Discussion

I. Antibiotic fate assessment

The concentrations of enrofloxacin and ciprofloxacin in the effluent discharge point of the catfish pond were found to be approximately 1000 times lower than the concentrations at which acute effects have been observed on the tested organisms. Therefore, the environmental pollution caused by the use of enrofloxacin and ciprofloxacin in aquaculture (at the particular dosage and environmental conditions of the monitored pond) is not likely to result in acute toxic effects on non-target aquatic organisms inhabiting ecosystems surrounding *Pangasius* catfish farms.

II. Toxicity experiments

II.1. Tests with algae (*Chlorella sp.*)

Table 14 shows a summary of the toxicity studies available for enrofloxacin and ciprofloxacin on algae. In general, the toxicity tests were performed on the species recommended by the OECD guideline 201: *Pseudokirchneriella subcapitata* and *Desmodesmus subspicatus* (OECD, 2006). Moreover, the conditions were typical of more temperate climates such that the temperature was not exceeding 23.5°C. Also, the pure antibiotics were generally applied in these studies.

Table 14: Overview of the toxicity data of enrofloxacin and ciprofloxacin to different species of algae

Antibiotic	Algae specie	Time	EC_{50} (in mg/L) (95% confidence limits)	Difference in the methodology used*	Reference
Enrofloxacin	*Pseudokirchneriella subcapitata*	72h	3.1 (2.6 - 3.6)	- temperature: 20°C - test tubes used as test vessels - growth chamber - no information on the purity of the chemical	Robinson *et al.*, 2005
	Desmodesmus subspicatus	48h	28.369 (23.885 – 35.375)	- temperature: 21-23.5°C - purity of the compound: 98%	Ebert *et al.*, 2011
	Scenedesmus obliquus	72h	45.1 (40.2 – 50.6)	similar conditions - Enrofloxacin HCl	Qin *et al.*, 2011
Ciprofloxacin	*Pseudokirchneriella subcapitata*	72h	18.7 (16.2 – 21.2)	- temperature: 20°C - test tubes used as test vessels - growth chamber - no information on the purity of the chemical	Robinson *et al.*, 2005
	Pseudokirchneriella subcapitata	72h	2.97 (2.41 – 3.66)	No specific information given - Ciprofloxacin - HCl	Halling-Sørensen *et al.*, 2000
	Desmodesmus subspicatus	48h	> 8.042	- temperature: 21-23.5°C - purity of the compound: 98%	Ebert *et al.*, 2011
	Chlorella vulgaris	72h	20.6	- temperature: 22.1 °C - pure compound used	Nie *et al.*, 2007

*In all these studies except the one from Robinson *et al.* (2005), the OECD guideline 201 was used as a basis for the methodology.

In general, it is possible to see that all EC_{50} values are different within and between antibiotics (up to ten fold disparity). This can be explained by the difference in methodology as well as the use of various species of algae with their own sensitivity to antibiotics. *Pseudokirchneriella subcapitata* has been found to be more sensitive to enrofloxacin than to ciprofloxacin (Robinson *et al*., 2005). Moreover, Qin *et al.* (2011) found that *Pseudokirchneriella subcapitata* is more sensitive to enrofloxacin than *Scenedesmus obliquus*. However, the water parameters and the enrofloxacin product used in this study were much different from the ones applied by Robinson *et al.* (2005). In fact, the conditions in the experiment of Qin and al (2011) are more similar to the situations found in tropical regions.

The EC_{50} values calculated in the present study for *Chlorella* sp. are higher than the ones showed in table 11. This could be explained by two main reasons:

- ✓ The first explanation could be that the antibiotics degraded faster in the tests vessels as the illumination was constant during the whole experiment. Like most antibiotics, the main degradation process for enrofloxacin and ciprofloxacin is photodegradation. In fact, few studies have shown that their half-life in water is greatly reduced with increasing light intensity (Gagliano and Mc Namara, 1996; Knapp C. *et al.*, 2005; Sukul and Spiteller, 2007). In all the studies presented in table 11, light-dark regimes were used during the entire experimental periods. Therefore, the dissipation rate in the present experiment is probably higher than in the studies presented above. However, the authors do not mention the possibility of photodegradation process during the laboratory experiments. Thus, more study is necessary to confirm this hypothesis.
  As explained previously, the experiments were run inside with artificial lamps (neons) such that the photodegradation is reduced compared to field situations. Therefore, the dissipation rate of this experiment still remains lower than the one that probably will occur in the field and semi-field experiments. For this reason, the response of algae species in the field could be lower than those observed under laboratory conditions.

- ✓ The second explanation is related to the intrinsic characteristics of the algae species tested in this study. Similarly to this experiment, Nie *et al.* (2007) used a *Chlorella* species in their toxicity tests. They also found a higher EC_{50} value compared to the other studies performed with ciprofloxacin (table 11). Eckardt (2010) as well as Kapaun and Reisser (1995) argued that the cell walls of *Chlorella* sp. contain cellulose and hemicelluloses, usual component of algae cell walls, but also glucosamine polymers such as chitin and chitosan. These materials are harder than cellulose and typical components of the fungi cell walls. This particular composition could act as an extra permeability barrier (Bernard and Latgea, 2001). Therefore, it could be more difficult for the pharmaceuticals to pass through the cell wall of *Chlorella* sp., thus reducing the compound uptake and making them more resistant. However, research on the physico-structural organization cell wall of *Chlorella* sp. received little attention to date. This information is necessary to better understand the antibiotics' permeability through this membrane and with special attention to the molecules of the fluoroquinolone family.

II.2. Tests with invertebrates (*Moina macrocopa*)

A literature review has been conducted to collect the toxicity data available for *Moina macrocopa* for enrofloxacin and ciprofloxacin. The study of Park and Choi (2008) is the only one that considered the effects of some fluoroquinolone antibiotics on micro-invertebrates. This study showed that *Daphnia magna* is more sensitive to enrofloxacin than *Moina macrocopa* with an EC_{50}-48h of 56.7 mg/L and > 200 mg/L, respectively. In order to run their acute toxicity tests, the US EPA guideline was used as a basis for the methodology. Moreover, the water was reconstituted and further used as medium for the micro-invertebrates. The water temperature was set at 25±1°C under a photoperiod of 16h light/8h dark.

So far, the toxicity of ciprofloxacin has not been studied on *Moina macrocopa.* However, Kim *et al.* (2010) demonstrated that ciprofloxacin has more detrimental effects on *Daphnia magna* than enrofloxacin (EC_{50}-48h = 1.2 mg/L, which corresponds to 3.62 x 10^{-6} mol/L after 48h for ciprofloxacin and EC_{50}-48h = 53.3 mg/L, which corresponds to 148 x 10^{-6} mol/L after 48h for enrofloxacin).

Comparing the data found in the present study with those from the previous literature, two points can be discussed. First, the EC_{50}-48h determined for enrofloxacin is lower than the one given by Park and Choi (2008). This can be explained by the fact that the temperature set in the present experiments was higher (>5°C) than the one used in the tests of Park and Choi (2008). In fact, Kim *et al.* (2009) have demonstrated that an increase in water temperature enhanced the acute toxicity of enrofloxacin. This noxious effect could be explained by an alteration in toxicokinetics of the chemicals or an impact on the physiological mechanisms of the micro-invertebrates.

The second point of discussion is related to the results given by Kim *et al.* (2010). They present an EC_{50}-48h which is smaller for ciprofloxacin than enrofloxacin, which is not confirmed by the present results. In the referred studies, the pure compounds (97-99% purity) were used, which is not the case in this experiment. To confirm this, an extra test was performed with “pure” enrofloxacin in order to examine if the solvent in the Vimenro 200 adds an extra toxicity to the antibiotic. The results presented in annex 7 confirm the fact that the toxicity of enrofloxacin added to the one from the solvent leads to an EC_{50}-48h value nearly two times lower. Although, this hypothesis cannot be totally verified since the solvent composition in the Vimenro 200 remains unknown. As the formulated version of ciprofloxacin was bought as a powder form, the solvents of the two formulated compounds are probably different. However, an extra toxicity test could not be performed with pure ciprofloxacin due to its very low solubility in water.

Since only one study evaluating the acute toxicity of fluoroquinolones on *Moina macrocopa* was found, more research needs to be conducted with a larger number of native invertebrate species. This is especially needed to draw conclusions on their sensitivity as a group and to assess the risks posed by the use of these antibiotics in the tropical regions.

II.3. Nile tilapia (*Oreochromis niloticus*)

II.3.a. Threshold and adaptation period of the fish

Looking at the biomarker results, it is clear that the lowest concentrations of antibiotics did not have any effects on ChE or CAT activities. This would suggest that a certain concentration (threshold value) needs to be reached before any effects can be observed.

For the highest concentrations of the in-feed treatment tests, it was clear that ChE activity was rather high on day 3, decreased on day 5 to finally show no significant difference with the controls on day 14. It seems that when the fish are in contact with high concentrations of fluoroquinolones (above the threshold value), their ChE activity will be disturbed for few days. However, the fish seems to adapt after a short period (less than 5 days), such that ChE activity will return to normal. At the end of the test, the ChE activity returned to normal, the fish recovered totally from the test. This trend is also possible to see for the bath treatment test with enrofloxacin. However, in the bath treatment test with ciprofloxacin, the ChE activity seems to be affected at the highest concentrations but the recovery is not seen before the end of the experiment. Thus, when Nile tilapias are in contact with ciprofloxacin via the surrounding water, the tested biomarkers seem to be affected for a longer time.

II.3.b. Comparison of the CAT results with other studies

Only few studies have used CAT activity as a biomarker. However, this enzyme is an important defence system as it catalyzes H_2O_2 into inoffensive 0_2 and H_2O (Baudhuin *et al*, 1964; Wang *et al*, 2009). Wang *et al.* (2009) focused their interests on the adverse effects of enrofloxacin on Tra catfish comparing different fish densities. For this, all fish ate contaminated food (1g of enrofloxacin/kg of feed) during 7 days of exposure. They showed that CAT activity increased in the gills during the experimental period but return to the basal levels during the recovery period. They suggest that the recovery of CAT activity is fast because antibiotics like enrofloxacin have a moderate impact on oxidative stress. Gao *et al*. (2008) studied the effect of enrofloxacin (1 to 5g/kg of feed) on the earthworm *Eisenia fetida* during 14 days of exposure. They observed that CAT activity is inhibited when the concentration of enrofloxacin increases (up to 73% for 5g/kg of feed). They also observed a recovery of CAT activity for the lower concentrations from day 7 to day 14. Therefore, they suggest that there is a physiological adaptability of these earthworms to enrofloxacin stress.

Even if the studies presented above are different from the present experiments, they show similar results: in general, CAT activity increases with stress. However, it seems that above a certain concentration (threshold value), CAT activity decreases because it cannot cope anymore with the acute toxicity of enrofloxacin. The H_2O_2 is not catalyzed anymore, leading to cell damages.

II.3.c. Comparison of the ChE results with other studies

Up to date, only few scientists have studied the effects of fluoroquinolones on aquatic organisms using biomarkers. In fact, most studies have used ChE activity to assess the effects of pesticides on fish (Ansari *et al.*., 1987; Ozcan Oruc et Usta., 2007; Pan et Dutta., 1998). In the study of Tu *et al.* (2009), ChE activity was used as a biomarker of exposure in black tiger shrimps when exposed to 4g

of enrofloxacin/kg of feed. In muscles and gills, there was no significant effect between the treated and untreated test organisms. In the study of Wang *et al.* (2009) presented previously, they explained that when stress (i.e., fish density, in this study) increases, the ChE activity in the brain decreases. They suggest that this could be caused by lipid peroxidation leading to neuronal damages. In fact, ChE is a membrane bound enzyme such that any changes in its phospholipid environment will directly affect its activity (Sahoo *et al.*., 1999). When stress increases, ChE activity decreases such that acetylcholine is no more broken down and accumulates in the synapses leading to neurotoxicity symptoms.

The studies presented above show very different results from the current experiment performed with Nile tilapias. Moreover, it is the first time that the effects of fluoroquinolones added directly in the water or with different concentrations in the food are studied. Therefore, the arguments presented above cannot explain why ChE activity increases, when the concentration of antibiotics becomes more important. Moreover, the death of individuals has not been observed for the concentrations tested. This is the reason why other hypotheses need to be developed in order to elucidate the phenomenon seen for the present experiments.

II.3.d. Hypotheses on the increase of ChE activity

Before going into details on the possible hypotheses, it is important to describe the mechanisms of action of ChE. First, ChE is a family of enzymes that catalyze the hydrolysis of acetylcholine (ACh). Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE) are the only ChE present in the central nervous system (Savelev *et al*., 2004). In the following paragraph, the mechanisms of action of AChE will be described, but this explanation is valid for both ChE enzymes.
As shown in figure 28, ACh is synthesized in the pre-synaptic nerve by Choline Acetyl Transferase (ChAT), and will be further placed into synaptic vesicles. When an action potential reaches the pre-synaptic nerve terminal, the membrane will depolarize and the calcium channels will open. The calcium ions will enter the pre-synaptic neuron and further activate the set of proteins attached to the synaptic vesicles that contain ACh (Oda, 1999). Then, the vesicles will fuse with the pre-synaptic membrane and release the neurotransmitters into the synaptic cleft (exocytosis). A part of ACh will bind to muscarinic or nicotinic receptors and activate them. These receptors will often open ligand-gated ion channels such that ions will enter or exit the post-synaptic cells. As a result, local transmembrane potential will depolarize. If the depolarization exceeds a threshold value, an action potential will be created. The remaining ACh will attach to AChE or BChE and will be hydrolyzed into acetate and choline (Katzung, 2000; Sherwood, 2008).

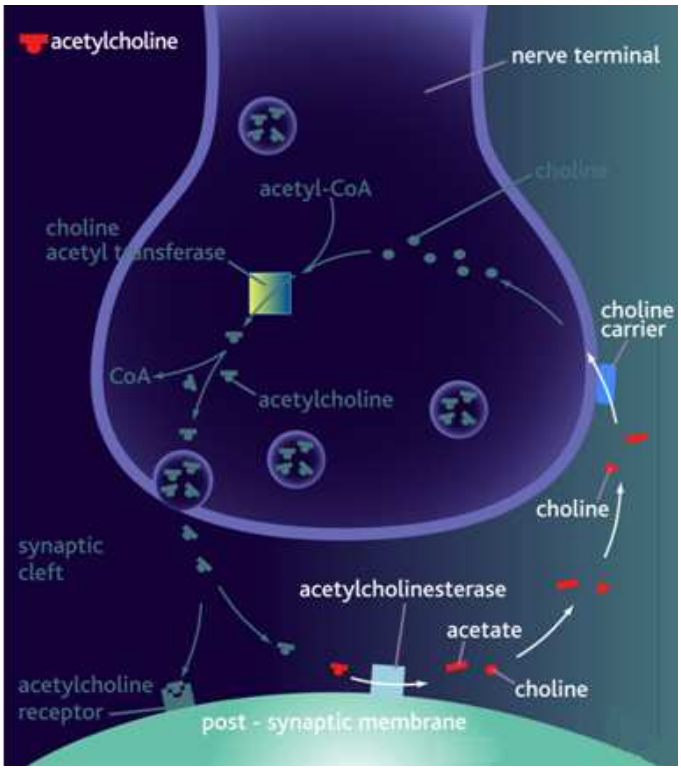


Figure 28: Mechanism of action of acetylcholinesterase (Katzung, 2000)

From this information, it is possible to draw two possible explanations for the increase of ChE activity with fluoroquinolone concentrations.

- ✓ Firstly, the quantity of ACh released in the synaptic cleft remains unchanged. However, it could be that the fluoroquinolones block or change the conformation of the muscarinic or nicotinic receptors. The antibiotics could bind in the orthosteric or allosteric binging sites of the receptors. Actually, all muscarinic ACh receptors have at least one or two extracellular allosteric sites that can recognize small molecules. Then, these ligands can turn into allosteric modulators and control the binding and function of the orthosteric sites (Gregory, 2007). Therefore, the fluoroquinolones could act as negative allosteric modulators and change the conformation of the orthosteric sites such that ACh cannot bind to the receptors anymore. Therefore, all ACh released in the synaptic cleft will only bind to ChE, such that its activity will increase in order to hydrolyze all ACh molecules.

- ✓ Secondly, ACh concentration is enhanced in the synaptic cleft when the concentration of enrofloxacin or ciprofloxacin increases. It could be that the fluoroquinolones are cholinergic agonists, such that they enhance the effects of ACh. In fact, Rawi *et al.* (2011) have shown that in vivo administration of cholinergic agonists increase the concentration of ACh. Another explanation could be that the antibiotics modify the gene coding for the ChAT, such that more ACh are synthesized. As more ACh is released in the synaptic cleft, more molecules will be hydrolyzed by ChE enzymes. Therefore, ChE activity will be more important than usual.

II.3.e. Hypotheses on the fish death

It is important to note that the fish only died when they were exposed to fluoroquinolones via the bath treatment tests.

The first hypothesis would be that the antibiotics in the water damaged the gills such that the oxygen concentration is decreased in the blood. The fish would die by hypoxia when they are exposed to the highest antibiotic concentrations. Moreover, the fish would swim slower when they are exposed to lower antibiotic concentrations. This hypothesis could explain the unexpected death and behavior seen in this experiment. However, the gill damages would probably recover slowly. In this experiment, the recovery was observed at the beginning of the recovery period. Therefore, another hypothesis need to be developed in order to explain the fish death for the highest tested concentration of antibiotics in the water.

Prior developing possible explanations, the functioning of the cardiac rhythm (cardiac action potential) has to be reviewed. Figure 29 is presenting an action potential in the ventricular myocytes cells. This process can be divided into four phases (Katz, 1977; Luo and Rudy, 1994; Rhoades and Bell, 2009; Shepard 2007):

- ✓ *Phase 1: Rapid depolarization phase*
  The cell is electrically stimulated from an electric current from an adjacent cell. If the depolarization is large enough, the threshold is reached (≈ - 40mV) and the fast voltage-gated sodium channels open such that Na^+ ions enter rapidly into the cell (I_{Na}), following the electrical gradient.
- ✓ *Phase 2: Plateau phase*
  First, the fast voltage-gated sodium channels are inactivated. Then, the L-type calcium channels open such that an inward current of Ca^{2+} ions ($I_{Ca\text{-}L}$) is created. In the same time, an outward current of K^+ ions is produced (I_{KS}) when the slow delayed rectifier potassium channels open.

- ✓ *Phase 3: Rapid repolarization phase*
  First, the L-type calcium channels close while the slow delayed rectifier potassium channels remain open. The membrane potential becomes more negative such that the rapid delayed rectifier potassium channels (hERG channel) and the inward rectifying potassium current channels open and create two different K^+ currents I_{Kr} and $I_{K\text{-}ACh}$ respectively.
  The opening of the inward rectifying potassium channels is controlled by ACh. In fact, when the vagus nerve releases ACh in the synaptic cleft, the neurotransmitters bind to the muscarinic M_2 receptors. This interaction activates $I_{K\text{-}ACh}$ and potassium ions exit the cell which becomes hyperpolarized. The threshold will be more difficult to reach such that the neurons will not be able to fire actions potentials as fast. Therefore, the hyperpolarization of the membrane induces a reduction of the cardiac rhythm (Kunkel and Peralta, 1995; Mark and Herlitze, 2000; Yatani *et al.*, 1987).

- ✓ *Phase 4: Resting potential*

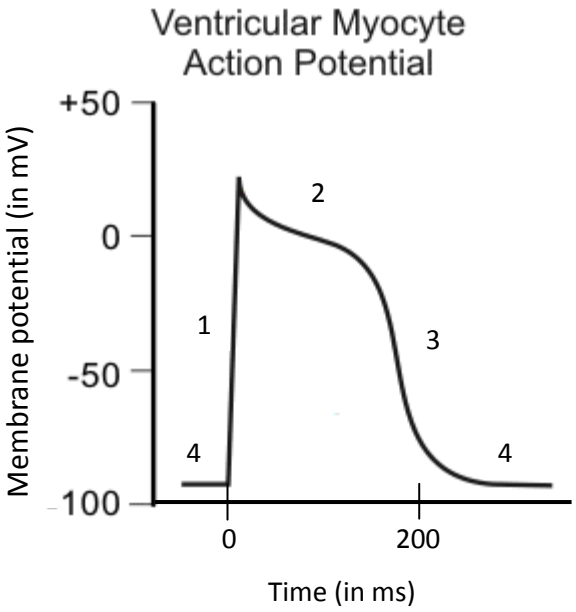


Figure 29: Action potential in ventricular myocytes cells (Rhoades and Bell, 2009)

In humans, fluoroquinolones are often used for antibacterial treatment. However, these antibiotics are known to block the hERG channels and thus the I_{Kr} currents during the action potential in the ventricular myocytes cells. This adverse effect leads to a prolongation of the QT interval on the electrocardiogram (arrhythmias). In the worst cases, it can result in a potentially fatal disorder called Torsade de Pointe because not enough blood is pumped to the brain and muscles (Khang *et al.*, 2001; Mitcheson 2003; Rubinstein and Camm, 2002; Zhang *et al.*, 2003). An overdose of the antibiotics given to the fish could have lead to a Torsade de Pointe disorder. In fact, Milan *et al.* (2006) have tested 4 drugs (astemizole,haloperidol, pimozide, terfenadine) on zebra fishes. All these pharmaceuticals, known to cause QT prolongation in humans, also caused QT prolongation in the tested fish.

Kang *et al.* (2001) have tested different fluoroquinolones on hERG potassium channel. They showed that all of these antibiotics inhibit the I_{Kr} currents but with different IC_{50} values (half-maximal inhibitory concentration). In fact, sparfloxacin has the smallest IC_{50} value (18 µM) which means that it is the compound that blocks most the hERG channels. However, ciprofloxacin can also inhibit I_{Kr} currents if its concentration is high enough (IC_{50} = 966 µM).

Also, Motcheson (2003) explains that fluoroquinolones binds on the aromatic residues in the inner cavity of the hERG channel (figure 24). Several structural characteristics can explain this nonspecific antibiotic-binging property. First, the hERG channel cavity is rather large compared with other potassium channels. Moreover, aromatic and polar residues in the cavity (black and grey respectively on figure 30) are relatively rare in other ion channels. They represent critical interaction sites for most compounds.

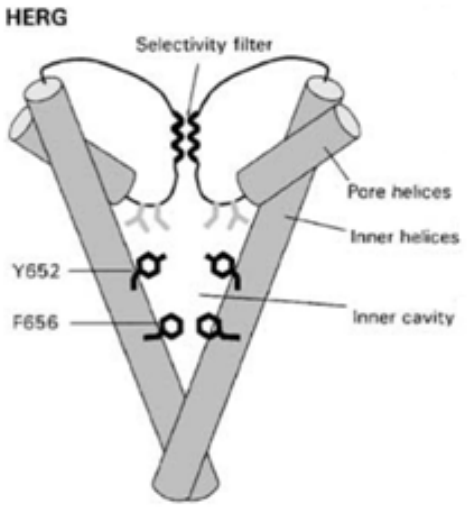


Figure 30: Scheme of an hERG channel (Mitcheson, 2003)

Looking at the molecules (figure 31), all fluoroquinolones have a fluor (F) on the carbon 6 and a carboxyl group (COOH) on the carbon 3. Rubinstein and Camm (2002) and Kang *et al.* (2001) showed that a radical on the carbon 5 is responsible for the prolongation of the QT interval. For example, a methyl group on the carbon 5 (sparfloxacin) is associated with a QT prolongation of 14ms. A methyl group at the same position (grepafloxacin) is related with a QT prolongation of 11ms. A proton (H) on the carbon 5 leads to a shorter QT prolongation of less than 2ms for ciprofloxacin (figure 31) (Rubinstein and Camm, 2002). Indeed, the administration method in the studies of Rubinstein and Camm (2002) and Kang *et al.* (2001) is different from this experiment. However, if the tested antibiotics reach the fish blood, the QT prolongation is highly possible.

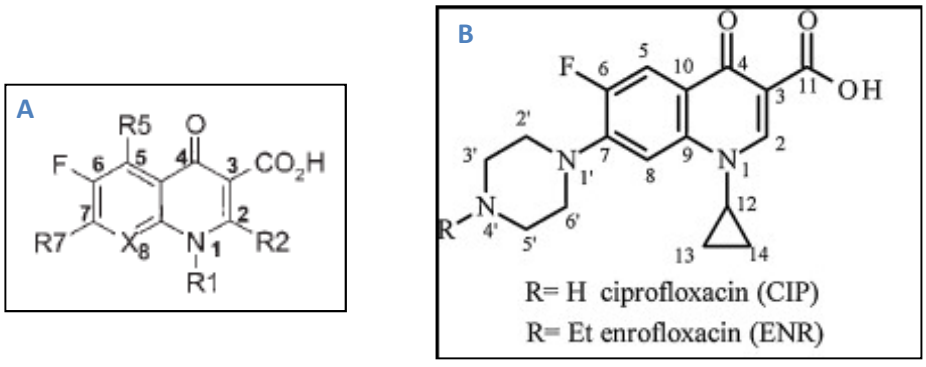


Figure 31: Molecular structure of A: fluoroquinolones and B: enrofloxacin and ciprofloxacin

It has to be mentioned that the specific structural features of fluoroquinolones contribute separately to their antimicrobial activity and to the hERG channel blockade (Khang *et al.*, 2001; Zhang *et al.*, 2003). Moreover, the potassium current inhibition is a reversible process; the compound has to be washed out of the channel (Mitcheson, 2003; Zhang *et al.*, 2003). This could explain why the fish exposed to enrofloxacin and ciprofloxacin in the water (bath treatment tests) recovered quickly after the exposure period ended.

More recently, it has been found that hERG channels are also situated in the central nervous system. Shepard (2007) and Zang *et al.* (2003) explain that pharmacological blockade of this specific potassium current can modify the neuronal excitability and thus the synaptic integration (threshold value that needs to be reached to obtain an action potential) but also induce abnormal neuronal discharges. Therefore, an increase of ACh release in the synapses could be possible leading to more important ChE activity in the brain.

Conclusions

In this project, the effects of enrofloxacin and ciprofloxacin were studied on non-target aquatic organisms which might be potentially exposed to these compounds in the surroundings of catfish farms. It can be concluded that *Chlorella sp.* was more sensitive to ciprofloxacin (EC_{50}-72h = 31.1 mg/L, which corresponds to 93.8 x 10^{-6} mol/L after 72h) than to enrofloxacin (EC_{50}-72h = 401 mg/L, which corresponds to 1117 x 10^{-6} mol/L after 72h). However, enrofloxacin was slightly more toxic to *Moina macrocopa* than ciprofloxacin (respectively EC_{50}-48h = 70 mg/L, which corresponds to 195 x 10^{-6} mol/L after 48h and EC_{50}-48h = 96.5 mg/L, which corresponds to 291 x 10^{-6} mol/L after 48h).

Regarding the biomarker results, it was found that fish exposed to high concentrations of the studied antibiotics (oral treatment: 5g/kg of feed for both fluoroquinolones, bath-treatment: 0.4mg of ciprofloxacin/L and 0.8mg of enrofloxacin/L) showed an increase in ChE activity three days after the beginning of the exposure period. However, the enzymatic activity returned to normal levels five days after the beginning of the experiment, which indicates that there is a physiological adaptability of the fish to antibiotic stress. For all the tests except the bath treatment test with ciprofloxacin, the ChE activity was not found to be significantly different to controls after a post-stress period of 9 days. After 5 days of exposure, the CAT activity decreased significantly for the bath-treatment experiment with ciprofloxacin (0.4 and 50mg/L) and the in-feed treatment with enrofloxacin (1 and 10mg/kg of feed).

In general, most biomarker changes have been observed for ChE activity. This is the reason why the assessment of ChE activity in the brain seems to be an appropriate biomarker for fluoroquinolones. However, the assessment of CAT activity in the liver or ChE activity in the gills would have been valuable for this experiment, especially for the bath-treatment tests, based on the information reported in previous studies and the hypothesis discussed in this study.

Moreover, changes in the enzymatic activities have been observed for concentrations in the range of 0.4-50 mg/L in this experiment. When the antibiotic concentration in the environment is in the range of µg/L (Tong *et al*., 2011; Zheng *et al*., 2011), ChE and CAT biomarker analysis is probably not the most suitable tool to monitor the effects of antibiotic residues to non-target fish species. However, ChE and CAT enzymatic activities are interesting endpoints to assess the potential mode of action of the tested antibiotics on the fish metabolism from a toxicological point of view.

Significant effects on the CAT and ChE activities in Nile tilapia were observed at levels of 10mg/L for enrofloxacin and 0.4 mg/L for ciprofloxacin. These concentrations are more than 10 times lower than the EC_{50} values assessed in the toxicity tests with *Chorella sp*. and *Moina macrocopa*. Therefore, this study suggests that the inclusion of sub-lethal effect assessments (i.e., biomarker effects in fish) should be taken into account in the risk assessment of antibiotics. In fact, effects on such biomarkers could lead to important behavioural and/or reproductive effects; however, little research has been conducted in this field to date. Therefore, studies are necessary to address this issue and mechanistically link physiological biomarker effects to organism's behaviour, reproduction and ultimately to observed effects in higher levels of biological organization (i.e., populations, communities).

From this study it can be concluded that the bath treatment application resulted in higher toxic effects than the in-feed administration method when considering the same applied dose. In fact, fish

died in the highest treatment level (100 mg of enrofloxacin/L and 50 mg of ciprofloxacin/L) of the bath-treatment tests. This fatal end is hypothesized to be caused by the blockage of the hERG channels, leading to a prolongation of the QT interval on the electrocardiogram, as observed in humans.

Finally, the concentrations of enrofloxacin and ciprofloxacin in the effluent discharge point of the catfish pond were found to be approximately 1000 times lower than the concentrations at which acute effects have been observed on the tested organisms. Therefore, the environmental pollution caused by the use of enrofloxacin and its main metabolite ciprofloxacin in aquaculture (at the particular dosages and environmental conditions of the monitored pond) is not likely to result in acute toxic effects on non-target aquatic organisms inhabiting ecosystems surrounding *Pangasius* catfish farms. Nevertheless, further investigations should be conducted in order to i) test the hypothesis suggested in this report regarding the mode of action of these antibiotics in fish, ii) study the potential chronic effects of these fluoroquinolones on larger assemblages of non-target aquatic organisms, and iii) study potential toxic effects of antibiotic mixtures used in Asian aquaculture farms, as these substances are very often applied together.

References

Ansari B.A., Aslam M., Kumar K. 1987. Diazinon toxicity: activities of acetylcholinesterase and phosphatases in the nervous tissue of zebra fish *Brachydanio rerio* (Cyprinidae). *Acta Hydrochimica et Hydrobiologica.* **15**, 301-306.

APHA, American Public Health Association. 1996. American Water Works Association and Federal Water Pollution Control Administration. *Standard method for the Examination of Water and Waste Water*. Washington, D.C. p 1193.

Ašperger D., Babić S., Mutavdžić Pavlović D., Dolar D., Košutić K., Horvat A., Kaštelan-Macan M. 2009. SPE-HPLC/DAD determination of trimethoprim, oxytetracycline and enrofloxacin in water samples. *International Journal of Environmental and Analytical Chemistry.* **89** (8-12), 809 – 819.

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, damino acid oxidase and catalase in rat-liver tissue. *Biochemical journal*. **92**, 179–184.

Bernard M., Latgea J.-P. 2001. *Aspergillus fumigatus* cell wall: composition and biosynthesis. *Medical Mycology* . 39 (Supplement 1), 9-17.

Cardoza L., Knapp C., Larive C., Belden J., Lydy M., Graham D. 2004. Factors Affecting the Fate of Ciprofloxacin in Aquatic Field Systems. *Water, Air, & Soil Pollution.* **161**(1-4), 383-398.

Crane M., Watts C., Boucard T. 2006. Review: Chronic aquatic environmental risks from exposure to human pharmaceuticals. *Science of the Total Environment*. **367**, 23-41.

Cuyers L., van Binh T. 2008. Aquaculture export development in Vietnam and the changing environment: the case of Pangasius in the Mekong Delta. *Centre for ASEAN Studies* and *Centre for International Management and Development Antwerp. Discussion Paper.* **59,** 27pp.

Danyi S., Widart J., Douny C., Dang P.K., Baiwir D., Wang N., Tu H.T., Tung V.T., Phuong N.-T., Kestemont P., Scippo M.-L. 2010. Determination and kinetics of enrofloxacin and ciprofloxacin in Tra catfish (*Pangasianodon hypophthalmus*) and giant freshwater prawn (*Macrobrachium rosenbergii*) using a liquid chromatography ⁄ mass spectrometry method. *Journal of Veterinary Pharmacology and Therapeutics*. **34**, 142–152.

Della Rocca G., Di Salvo A., Malvisi J., Sello M. 2004. The disposition of enrofloxacin in seabream (*Sparus aurata* L.) after single intravenous injection or from medicated feed administration. *Aquaculture.* **232** (1-4), 53-62.

Douny C., Widart J., De Pauw E., Scippo M.L., Silvestre F., Kestemont P., Maghuin-Rogister G. 2006. Determination of thirteen (fluoro)quinolones residues in shrimps by liquid chromatography-mass spectrometry. Fifth International Symposium on Hormone and Veterinary Drug Residue Analysis. Antwerpen, Belgium.

Ebert I., Bachmann J., Kühnen U., Küster A., Kussatz C., Maletzki D., Schlüter C. 2011. Toxicity of the fluoroquinolone antibiotics enrofloxacin and ciprofloxacin to photoautotrophic aquatic organisms. *Environmental toxicology and Chemistry.* **30** (12), 2786-2792.

Eckardt N.A. 2010. The Chlorella Genome: Big Surprises from a Small Package. *The Plant Cell*. **22** (2924).

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.

European External Action Service (EEAS). 2011. GREEN BOOK 2011 - EU Commercial Counsellor Report on Vietnam. Hanoi. 72p.

FAO Fisheries Department. 2010. The State of World Fisheries and Aquaculture (SOFIA). *FAO Fisheries and Aquaculture Department*. Rome. 218p.

FAO Fisheries Department. 2006. State of world aquaculture 2006. *FAO Fisheries Technical Paper*. Rome. **500**, 134p.

Ferreira C., Nunes B., Manuel J., de Melo Henriques-Almeida J.-M., Guilhermino L. 2006. Acute toxicity of oxytetracycline and florfenicol to the microalgae *Tetraselmis chuii* and to the crustacean *Artemia parthenogenetica. Ecotoxicology and Environmental Safety.* **67** (3), 452-458.

Gagliano G., Mc Namara F. 1996. Environmental Assessment for Enrofloxacin - BAYTRIL 3. 23% Concentrate Antimicrobial Solution. *Guideline 21 CFR Part 25*.

Gregory K.J., Sexton P.M., Christopoulos A. 2007. Allosteric modulation of muscarinic acetylcholine receptors. *Current Neuropharmacology*. **5**, 157-167.

Guidechem Chemical Network. <http://www.guidechem.com/>

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** (S1), 53-58.

Huang C.-H., Smeby K., Renew J. 2002. Occurrence and behavior of fluoroquinelone and sulfonamide antibacterial agents during municipal wastewater treatment. *Preprints of Extended Abstracts.* **42** (1).

Intorre L., Cecchini S., Bertini S., Cognetti Varriale A.M., Soldani G., Mengozzi G. 2000. Pharmacokinetics of enrofloxacin in the seabass (*Dicentrarchus labrax*). *Aquaculture.* **182** (1-2), 49-59.

Ji K., Kim Y., Oh S., Anh B., Jo H., Choi K. 2008. Toxicity of perfluorooctane sulfonic acid and perfluorooctanoic acid on freshwater macroinvertebrates (*Daphnia magna and Moina macrocopa*) and fish (*Oryzias latipes*). *Environmental Toxicology and Chemistry.* **27** (10), 2159-2168.

Kapaun E., Reisser W. 1995. A chitin-like glycan in the cell wall of a *Chlorella* sp. (Chlorococcales, Chlorophyceae). *Planta.* **197**, 577-582.

Katz A.M. 1977. Physiology of the heart. USA: Raven Press.

Katzung BG. 2000. Introduction to autonomic pharmacology. In: Basic and clinical pharmacology, 8th edition. USA: The McGraw Hill Companies. 75-91.

Khang J., Wang L., Chen X-L., Triggle D.J., Rampe D. 2001. Interactions of a Series of Fluoroquinolone Antibacterial Drugs with the Human Cardiac K1 Channel HERG. *Molecular Pharmacology.* **59**, 122–126.

Kim J., Park J., Kim P.-J,. 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.

Kim M.-S., Lim J.-H., Park B.-K., Hwang Y.-H., Yun H.-I. 2006. Pharmacokinetics of enrofloxacin in Korean catfish (Silurus asotus). *Journal of veterinary pharmacology and therapeutics*. **29**, 397–402.

Knapp C., Cardoza L., Hawes J., Wellington E., Larive C., Graham D. 2005. Fate and effects of enrofloxacin in aquatic systems under different light conditions. *Environmental Science & Technology*. **39**, 9140-9146.

Kunkel M.T., Peralta E.G. 1995. Identification of Domains Conferring G Protein Regulation on Inward Rectifier Potassium Channels. *Cell.* **83**, 443-449.

Liem P.T., Phong N.V., Phuong N.T. 2009. The use of drugs and chemicals in catfish farming in the Mekong River Delta, Vietnam: A review. *Scientific Journal of Can Tho University*, Viet Nam (in Vietnamese).

Lin J.-S., Pan H.-Y., Liu S.-M., Lai H.-T. 2010. Effects of light and microbial activity on the degradation of two fluoroquinolone antibiotics in pond water and sediment. *Journal of Environmental Science and Health*, Part B. **45** (5), 456-465.

Lowry O.H., Rosenbruogh N.J., Tarr A.I., Randall R.J. 1951. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*. **193**, 265–275.

Luo C.H. Rudy Y. 1994. A dynamic model of the cardiac ventricular action potential. I. Simulations of Ionic Currents and Concentration Changes.*Circulation research (American heart association).* **74**, 1071-1096.

Mark M.D., Herlitze S. 2000. G-protein mediated gating of inward-rectifier K^+ channels. *European Journal of Biochemistry*. **267**, 5830-5836.

Medeiros J. 2010. Enrofloxacin in soils: determination of sorption parameters by liquid chromatography. *Universitat de Barcelona* and *Universidade do Algarve*.

Mitcheson J.S. 2003. Drug binding to HERG channels: evidence for a 'non-aromatic' binding site for fluvoxamine. *British Journal of Pharmacology*. **139**, 883–884

National library of Medecine. (n.d.). Chemical information resources from the National Library of Medicine. <http://sis.nlm.nih.gov/chemical.html>

Nie X., Wang X., Chen J., Zitko V., An T. 2008. Response of the freshwater alga *Chlorella vulgaris* to trichloroisocyanuric acid and ciprofloxacin. *Environmental toxicology and chemistry.* **27** (1), 168-173.

Oda Y. 1999. Choline acetyltransferase: the structure, distribution and pathologic changes in the central nervous system. *Pathology International.* **49**, 921-937.

OECD: Organization for Economic Cooperation and Development, 2006. OECD Guidelines for the testing of chemicals. Freshwater alga and cyanobacteria, growth inhibition test. 25p.

OECD: Organization for Economic Cooperation and Development, 2004. OECD Guidelines for the testing of chemicals. *Daphnia sp.*, Acute immobilisation test. 12p.

Oh S., Choi K. 2011. Optimal conditions for three brood chronic toxicity test method using a freshwater macroinvertebrate Moina macrocopa. *Environmental Monitoring and Assessment*. 9p.

Ozcan Oruc E., Usta D. 2007. Evaluation of oxidative stress responses and neurotoxicity potential of diazinon in different tissues of *Cyprinus carpio*. *Environmental Toxicology and Pharmacology*. **23**, 48-55.

Pan G., Dutta H.M. 1998. The inhibition of brain acetylcholinesterase activity of juvenile largemouth bass *Micropterus salmoides* by sublethal concentrations of diazinon. *Environmental Research.* **79**, 133-137.

Pangasius exports in 2010. 2011. *Vietfish international*. **8** (01-39). <http://www.vietfish.org/ 201 10224115720348p49c65/pangasius-exports-in-2010.htm>

Park S., Choi K. 2008. Hazard assessment of commonly used agricultural antibiotics on aquatic ecosystems. *Ecotoxicology.* **17**, 526–538.

Pfizer Inc. 2002. Environmental Assessment - Danofloxacin 18% Injectable Solution for the Treatment of Respiratory Disease in Cattle. <http://www.fda.gov/downloads/AnimalVeterinary/DevelopmentApprovalProcess/EnvironmentalAssessments/UCM072389.pdf>

Phan L., Bui T., Nguyen T., Gooley G., Ingram B., Nguyen H., Nguyen P. De Silva S., 2009. Current status of farming practices of striped catfi sh, *Pangasianodon hypophthalmus* in the Mekong Delta, Vietnam. *Aquaculture*. **296**, 227-236.

Phuong N.T. 2010. Analytical and biological methods in support of sustainable aquaculture practices in Vietnam. A joint Vitenamese-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. 133–149.

Pico Y., Andreu V. 2007. Fluoroquinolones in soil - risks and challenges. *Analytical and Bioanalytical Chemistry*. **387** (4), 1287-1299.

Portunus Group Vietnam Delegation. 2011. Vietnam-Iceland business meeting in Reykjavik – Iceland, September 9th 2011. *Slideshare Present Yourself*. <http://www.slideshare.net/cacth uy/portunus-group-vietnam-delegation>

Qin H., Chen L., Lu N., Zhao Y., Yuan X. 2011. Toxic effects of enrofloxacin on *Scenedesmus obliquus*. *Frontiers of Environmental Science & Engineering in China.* **1/2007-5/2011**, 1-10.

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** (8). 993-1005.

Rhoades R.A. Bell D.R. 2009. Medical Physiology: Principles for Clinical Medicine. 3rd edition. USA: Lippincott Williams and Wilkins.

Rico A., Geng Y., Focks A., Van den Brink P. J. 2011. ERA-AQUA: a decision support system for the Environmental Risk Assessment of veterinary medicinal products used in Asian pond AQUAculture. Wageningen University.

Rico A., Satapornvanit K., Haque M.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.

Robinson A.A., Belden J.B., Lydy M.J. 2005. Toxicity of Fluoroquinolone antibiotics to aquatic organisms. *Environmental Toxicology and Chemistry*. **24**, 423-430.

Rubach M., Crum S., Van den Brink P. 2011. Variability in the dynamics of mortality and immobility responses of freshwater arthropods exposed to Chlorpyrifos. *Archives of environmental contamination and toxicology.* **60**, 708- 721.

Rubinstein E., Camm J. 2002. Cardiotoxicity of fluoroquinolones. *Journal of antimicrobial chemotherapy.* **49**, 593-596.

Sahoo, A., Samanta, L., Das, A., Patra, S.K., Chainy, G.B.N., 1999. Hexachlorocyclohexane-induced behavioural and neurochemical changes in rat. *Journal of applied toxicology*. **19**, 13-18.

Said H., Karickhoff S.W., Carreira L.A. 1995. A rigorous test for SPARC's chemical reactivity models: estimation of more than 4300 ionization pKa's. *Quantitative Structure*-activity *Relationships*. **14** (348).

Savelev S.U., Okello E.J., Perry E.K. 2004. Butyryl- and Acetyl-cholinesterase Inhibitory activities in essential oils of *Salvia* species and their constituents. *Phythotherapy research*. 18, 315-324.

SEAT Global. <http://seatglobal.eu/>

Shao Z. 2001. Aquaculture pharmaceuticals and biologicals: current perspectives and future possibilities. *Advanced Drug Delivery Reviews.* **50** (3), 229-243.

Shepard P.D., Canavier3 C.C, Levitan E.S. 2007. Ether-a-go-go–Related Gene Potassium Channels: What's All the Buzz About? *Schizophrenia Bulletin*. **33** (6), 1263–1269.

Sherwood L. 2008. Human Physiology: From Cells to Systems. 7th edition. USA: Brooks/Cole.

SPSS (Statistical Package for Social Sciences). 2010. SPSS Company. Chicago, USA. Version 19.0.

Sukul P., Spiteller M. 2007. Fluoroquinolone Antibiotics in the Environment. In: Ware G. 2007. Reviews of Environmental Contamination and Toxicology. *New York: Springer*. **191**, 131–162.

Tong C., Zhuo X., Guo Y. 2011. Occurrence and Risk Assessment of Four Typical Fluoroquinolone Antibiotics in Raw and Treated Sewage and in Receiving Waters in Hangzhou, China. *Journal of agriculture and food chemistry.* **59** (13), 7303–7309.

Toxrat (Toxicity Response Analysis and Testing). 2003. Toxrat Solutions GmbH. Aachen, Germany. Version 2.01.

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). Sci. J. Can Tho University. (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., Scippo M.-L., Thome J.-P., Phuong N.T., Kestemont P. 2009. Acetylcholinesterase activity as a biomarker of exposure to antibiotics and pesticides in the black tiger shrimp (*Penaeus monodon*). *Ecotoxicology and Environmental Safety.* **72** (Issue 5), 1463-1470.

Tuan, N.A., N.T. Phuong, P.T. Liem, and N.V. Thuong. 2003. Results of the study on Pangasius catfish and their future development. *Journal of Mekong Fisheries.* p. 129–134 (in Vietnamese). Agricultural publishing house. Ho Chi Minh city. Viet Nam.

Union Economic and commercial counsellors. 2011. 2011 Commercial counsellors Report on Vietnam. *The delegation of the European Union*. <www.vietnam.ahk.de/fileadmin/ahk_vietnam/Reports_and_position_papers/Greenbook_11.pdf>

Vaccaro E., Giorgi M., Longo V., Mengozzi G., Gervasi P.G. 2003. Inhibition of cytochrome P450 enzymes by enrofloxacin in the sea bass (*Dicentrarchus labrax)*. *Aquatic Toxicology.* **62** (Issue 1), 27-33.

Van der Oost R., Beyer J., Vermeulen N. 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental Toxicology and Pharmacology.* **13,** 57-149.

Veterinary substance database. 2011. < http://sitem.herts.ac.uk/aeru/vsdb/index. htm>

Walne PR. 1970. Studies on the food value of nineteen genera of algae to juvenile bivalves of the genera *Ostrea*, *Crassostrea*, *Mercenaria*, and *Mytilis*. *Fishery Investigation*. **26**, 162.

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*). C*hemosphere.* **77** (11), 1577-1584.

Washington S., Ababouch L. 2011. Private standards and certification in fisheries and aquaculture: current practice and emerging issues. *FAO Fisheries and Aquaculture Technical Paper.* No. 553. Rome, FAO. 181p.

Xu W., Zhu X., Wang X., Deng L., Zhang G. 2006. Residues of enrofloxacin, furazolidone and their metabolites in Nile tilapia (Oreochromis niloticus). *Aquaculture*. **254** (1–8).

Yang L.-H., Ying G.-G., Su H.C., Stauber J.L., Adams M.S., Binet M.T. 2008. Growth-inhibiting effects of 12 antibacterial agents and their mixtures on the freshwater microalga *Pseudokirchneriella subcapitata. Environmental toxicology and chemistry.* **27** (5), 1201-1208.

Yatani A., Codina J., Brown A.M., Birnbaumer L.1987. Direct activation of mammalian atrial muscarinic potassium channels by GTP regulatory protein Gk. Science, New Series. **235** (4785), 207-211.

Zhang L-R., Li M-H., Cheng N-N., Chen B-Y., Wang Y-M. 2003. Inhibition by fluoroquinolones of K+ currents in rat dissociated hippocampal neurons. *European Journal of Pharmacology*. **462**, 9– 13.

Zheng S., Qui X., Chen B., Yu X., Liu Z., Zhong G., Li H., Chen M., Sun G., Huang H., Yu W., Freestone D. 2011. Antibiotics pollution in Jiulong River estuary: Source, distribution and bacterial resistance. *Chemosphere*. **84** (issue 11), 1677-1685.

Annex

Annex 1: Composition of the Walne's medium (Walne, 1970)

Walne's Medium Composition

Reagents	Per Liter Seawater
Solution A	1.0 ml
Solution C	0.1 ml
Solution D (to add for diatoms)	2.0 ml
Solution A	
$FeCl_3 * 6H_2O$	1.3 g
$MnCl_2 * 4H_2O$	0.4 g
H_3BO_3	33.6 g
Na_2EDTA	45.0 g
$NaH_2PO_4 * 2H_2O$	20.0 g
$NaNO_3$	100.0 g
Solution B	1 ml
Solution B per 100 ml	
$ZnCl_2$	2.1 g
$CoCl_2 * 6H_2O$	2.0 g
$(NH_4)_6Mo_7O_{24} * 4H_2O$	0.9 g
$CuSO_4 * 5H_2O$	2.0 g
Concentrated HCl	10 ml
Solution C per 200 ml	
Thiamine HCl (Vitamin B_1)	0.2 g
Cyanocobalamin (Vitamin B_{12})	10 mg
Solution D (per liter)	
$Na_2SiO_3 * 5H_2O$	40.0 g

Annex 2: Amount of food to give daily to the fish

a) Test with enrofloxacin in the feed

Control (a)	mean fish weight ŵ = 8.75		
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food *W(d)*
1-2	19	0.03	2.49
3-4	16	0.03	2.10
5	13	0.03	1.71
6	13	0.05	2.84
7-9	10	0.05	2.19
10-13	7	0.05	1.53

Control (b)	mean fish weight ŵ = 9.13		
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food *W(d)*
1-2	19	0.03	2.60
3-4	16	0.03	2.19
5	13	0.03	1.78
6	13	0.05	2.97
7-9	10	0.05	2.28
10-13	7	0.05	1.60

Control (c)	mean fish weight ŵ = 10.76		
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food *W(d)*
1-2	19	0.03	3.07
3-4	16	0.03	2.58
5	13	0.03	2.10
6	13	0.05	3.50
7-9	10	0.05	2.69
10-13	7	0.05	1.88

1mg/kg of feed (a)	mean fish weight ŵ = 8.45		
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food *W(d)*
1-2	19	0.03	2.41
3-4	16	0.03	2.03
5	13	0.03	1.65
6	13	0.05	2.75
7-9	10	0.05	2.11
10-13	7	0.05	1.48

1mg/kg of feed (b)	mean fish weight ŵ = 10.78		
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food *W(d)*
1-2	19	0.03	3.07
3-4	16	0.03	2.59
5	13	0.03	2.10
6	13	0.05	3.50
7-9	10	0.05	2.70
10-13	7	0.05	1.89

1mg/kg of feed (c)	mean fish weight ŵ = 9.37		
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food *W(d)*
1-2	19	0.03	2.67
3-4	16	0.03	2.10
5	13	0.03	1.71
6	13	0.05	2.84
7-9	10	0.05	2.19
10-13	7	0.05	1.53

2.5 mg/kg of feed (a)	mean fish weight ŵ = 10.26		
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food *W(d)*
1-2	19	0.03	2.92
3-4	16	0.03	2.46
5	13	0.03	2.00
6	13	0.05	3.33
7-9	10	0.05	2.57
10-13	7	0.05	1.80

2.5 mg/kg of feed (b)	mean fish weight ŵ = 11.13		
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food *W(d)*
1-2	19	0.03	3.17
3-4	16	0.03	2.67
5	13	0.03	2.17
6	13	0.05	3.62
7-9	10	0.05	2.78
10-13	7	0.05	1.95

2.5 mg/kg of feed (c)	mean fish weight ŵ = 7.75		
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food *W(d)*
1-2	19	0.03	2.21
3-4	16	0.03	1.86
5	13	0.03	1.51
6	13	0.05	2.52
7-9	10	0.05	1.94
10-13	7	0.05	1.36

5 mg/kg of feed (a)	mean fish weight $\hat{w}$ = 9.65		
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food *W(d)*
1-2	19	0.03	2.75
3-4	16	0.03	2.32
5	13	0.03	1.88
6	13	0.05	3.14
7-9	10	0.05	2.41
10-13	7	0.05	1.69

5 mg/kg of feed (b)	mean fish weight $\hat{w}$ = 16.46		
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food *W(d)*
1-2	19	0.03	4.69
3-4	16	0.03	3.95
5	13	0.03	3.21
6	13	0.05	5.35
7-9	10	0.05	4.12
10-13	7	0.05	2.88

5 mg/kg of feed (c)	mean fish weight $\hat{w}$ = 8.32		
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food *W(d)*
1-2	19	0.03	2.37
3-4	16	0.03	2.00
5	13	0.03	1.62
6	13	0.05	2.70
7-9	10	0.05	2.08
10-13	7	0.05	1.46

10 mg/kg of feed (a)	mean fish weight $\hat{w}$ = 8.85		
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food *W(d)*
1-2	19	0.03	2.52
3-4	16	0.03	2.12
5	13	0.03	1.73
6	13	0.05	2.88
7-9	10	0.05	2.21
10-13	7	0.05	1.55

10 mg/kg of feed (b)	mean fish weight $\hat{w}$ = 9.28		
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food *W(d)*
1-2	19	0.03	2.64
3-4	16	0.03	2.23
5	13	0.03	1.81
6	13	0.05	3.02
7-9	10	0.05	2.32
10-13	7	0.05	1.62

10 mg/kg of feed (c)	mean fish weight $\hat{w}$ = 8.34		
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food *W(d)*
1-2	19	0.03	2.38
3-4	16	0.03	2.00
5	13	0.03	1.63
6	13	0.05	2.71
7-9	10	0.05	2.09
10-13	7	0.05	1.46

b) Test with ciprofloxacin in the feed

Control (a)	mean fish weight ŵ =	9.98	
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food *W(d)*
1-2	19	0.03	2.84
3-4	16	0.03	2.40
5	13	0.03	1.95
6	13	0.05	3.24
7-9	10	0.05	2.50
10-13	7	0.05	1.75

Control (b)	mean fish weight ŵ =	9.33	
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food *W(d)*
1-2	19	0.03	2.66
3-4	16	0.03	2.24
5	13	0.03	1.82
6	13	0.05	3.03
7-9	10	0.05	2.33
10-13	7	0.05	1.63

Control (c)	mean fish weight ŵ =	11.85	
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food *W(d)*
1-2	19	0.03	3.38
3-4	16	0.03	2.84
5	13	0.03	2.31
6	13	0.05	3.85
7-9	10	0.05	2.96
10-13	7	0.05	2.07

0.5 mg/kg of feed (a)	mean fish weight ŵ =	9.57	
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food *W(d)*
1-2	19	0.03	2.73
3-4	16	0.03	2.30
5	13	0.03	1.87
6	13	0.05	3.11
7-9	10	0.05	2.39
10-13	7	0.05	1.67

0.5 mg/kg of feed (b)	mean fish weight ŵ =	11.00	
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food *W(d)*
1-2	19	0.03	3.14
3-4	16	0.03	2.64
5	13	0.03	2.15
6	13	0.05	3.58
7-9	10	0.05	2.75
10-13	7	0.05	1.93

0.5 mg/kg of feed (c)	mean fish weight ŵ =	9.95	
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food *W(d)*
1-2	19	0.03	2.84
3-4	16	0.03	2.40
5	13	0.03	1.95
6	13	0.05	3.24
7-9	10	0.05	2.50
10-13	7	0.05	1.75

1.12 mg/kg of feed (a)	mean fish weight ŵ =	9.70	
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food *W(d)*
1-2	19	0.03	2.76
3-4	16	0.03	2.33
5	13	0.03	1.89
6	13	0.05	3.15
7-9	10	0.05	2.43
10-13	7	0.05	1.70

1.12 mg/kg of feed (b)	mean fish weight ŵ =	10.00	
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food *W(d)*
1-2	19	0.03	2.85
3-4	16	0.03	2.40
5	13	0.03	1.95
6	13	0.05	3.25
7-9	10	0.05	2.50
10-13	7	0.05	1.75

1.12mg/kg of feed (c)	mean fish weight ŵ =	9.46	
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food *W(d)*
1-2	19	0.03	2.70
3-4	16	0.03	2.27
5	13	0.03	1.84
6	13	0.05	3.07
7-9	10	0.05	2.37
10-13	7	0.05	1.66

2.5 mg/kg of feed (a)	mean fish weight $\hat{w}$ = 10.45		
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food *W(d)*
1-2	19	0.03	2.98
3-4	16	0.03	2.51
5	13	0.03	2.04
6	13	0.05	3.40
7-9	10	0.05	2.61
10-13	7	0.05	1.83

2.5 mg/kg of feed (b)	mean fish weight $\hat{w}$ = 8.33		
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food *W(d)*
1-2	19	0.03	2.37
3-4	16	0.03	2.00
5	13	0.03	1.62
6	13	0.05	2.71
7-9	10	0.05	2.08
10-13	7	0.05	1.46

2.5 mg/kg of feed (c)	mean fish weight $\hat{w}$ = 10.02		
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food *W(d)*
1-2	19	0.03	2.86
3-4	16	0.03	2.40
5	13	0.03	1.95
6	13	0.05	3.26
7-9	10	0.05	2.51
10-13	7	0.05	1.75

5 mg/kg of feed (a)	mean fish weight $\hat{w}$ = 9.08		
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food *W(d)*
1-2	19	0.03	2.59
3-4	16	0.03	2.18
5	13	0.03	1.77
6	13	0.05	2.95
7-9	10	0.05	2.27
10-13	7	0.05	1.59

5 mg/kg of feed (b)	mean fish weight $\hat{w}$ = 9.38		
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food *W(d)*
1-2	19	0.03	2.67
3-4	16	0.03	2.25
5	13	0.03	1.83
6	13	0.05	3.05
7-9	10	0.05	2.35
10-13	7	0.05	1.64

5 mg/kg of feed (c)	mean fish weight $\hat{w}$ = 11.35		
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food *W(d)*
1-2	19	0.03	3.23
3-4	16	0.03	2.72
5	13	0.03	2.21
6	13	0.05	3.69
7-9	10	0.05	2.84
10-13	7	0.05	1.99

c) Test with enrofloxacin in the water

Control (a)	mean fish weight ŵ =	10.35	
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food W(d)
1-2	19	0.03	2.95
3-4	16	0.03	2.48
5	13	0.03	2.02
6	13	0.05	3.36
7-9	10	0.05	2.59
10-13	7	0.05	1.81

Control (b)	mean fish weight ŵ =	11.97	
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food W(d)
1-2	19	0.03	3.41
3-4	16	0.03	2.87
5	13	0.03	2.33
6	13	0.05	3.89
7-9	10	0.05	2.99
10-13	7	0.05	2.09

Control (c)	mean fish weight ŵ =	11.26	
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food W(d)
1-2	19	0.03	3.21
3-4	16	0.03	2.70
5	13	0.03	2.20
6	13	0.05	3.66
7-9	10	0.05	2.82
10-13	7	0.05	1.97

0.1 mg/L (a)	mean fish weight ŵ =	10.58	
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food W(d)
1-2	19	0.03	3.02
3-4	16	0.03	2.54
5	13	0.03	2.06
6	13	0.05	3.44
7-9	10	0.05	2.65
10-13	7	0.05	1.85

0.1 mg/L (b)	mean fish weight ŵ =	9.15	
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food W(d)
1-2	19	0.03	2.61
3-4	16	0.03	2.20
5	13	0.03	1.78
6	13	0.05	2.97
7-9	10	0.05	2.29
10-13	7	0.05	1.60

0.1 mg/L (c)	mean fish weight ŵ =	11.70	
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food W(d)
1-2	19	0.03	3.33
3-4	16	0.03	2.48
5	13	0.03	2.02
6	13	0.05	3.36
7-9	10	0.05	2.59
10-13	7	0.05	1.81

0.8 mg/L (a)	mean fish weight ŵ =	10.42	
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food W(d)
1-2	19	0.03	2.97
3-4	16	0.03	2.50
5	13	0.03	2.03
6	13	0.05	3.39
7-9	10	0.05	2.61
10-13	7	0.05	1.82

0.8 mg/L (b)	mean fish weight ŵ =	10.50	
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food W(d)
1-2	19	0.03	2.99
3-4	16	0.03	2.52
5	13	0.03	2.05
6	13	0.05	3.41
7-9	10	0.05	2.63
10-13	7	0.05	1.84

0.8 mg/L (c)	mean fish weight ŵ =	8.93	
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food W(d)
1-2	19	0.03	2.55
3-4	16	0.03	2.14
5	13	0.03	1.74
6	13	0.05	2.90
7-9	10	0.05	2.23
10-13	7	0.05	1.56

10 mg/L (a)	mean fish weight ŵ =	8.63	
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food W(d)
1-2	19	0.03	2.46
3-4	16	0.03	2.07
5	13	0.03	1.68
6	13	0.05	2.80
7-9	10	0.05	2.16
10-13	7	0.05	1.51

10 mg/L (b)	mean fish weight ŵ =	11.60	
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food W(d)
1-2	19	0.03	3.31
3-4	16	0.03	2.78
5	13	0.03	2.26
6	13	0.05	3.77
7-9	10	0.05	2.90
10-13	7	0.05	2.03

10 mg/L (c)	mean fish weight ŵ =	9.93	
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food W(d)
1-2	19	0.03	2.83
3-4	16	0.03	2.38
5	13	0.03	1.94
6	13	0.05	3.23
7-9	10	0.05	2.48
10-13	7	0.05	1.74

100 mg/L (a)	mean fish weight ŵ =	11.15	
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food W(d)
1-2	19	0.03	3.18
3-4	16	0.03	2.68
5	13	0.03	2.17
6	13	0.05	3.62
7-9	10	0.05	2.79
10-13	7	0.05	1.95

100 mg/L (b)	mean fish weight ŵ =	11.40	
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food W(d)
1-2	19	0.03	3.25
3-4	16	0.03	2.74
5	13	0.03	2.22
6	13	0.05	3.71
7-9	10	0.05	2.85
10-13	7	0.05	2.00

100 mg/L (c)	mean fish weight ŵ =	8.25	
Day	number fishes left *F(d)*	body mass *BM(d)*	weight of food W(d)
1-2	19	0.03	2.35
3-4	16	0.03	1.98
5	13	0.03	1.61
6	13	0.05	2.68
7-9	10	0.05	2.06
10-13	7	0.05	1.44

d) Test with ciprofloxacin in the water

Control (a)	mean fish weight ŵ =	15.10	
Day	number fishes left *F(d)*	% body mass *BM(d)*	weight of food W(d)
1-2	19	0.03	4.30
3-4	16	0.03	3.62
5	13	0.03	2.94
6	13	0.05	4.91
7-9	10	0.05	3.78
10-13	7	0.05	2.64

Control (b)	mean fish weight ŵ =	17.68	
Day	number fishes left *F(d)*	% body mass *BM(d)*	weight of food W(d)
1-2	19	0.03	5.04
3-4	16	0.03	4.24
5	13	0.03	3.45
6	13	0.05	5.75
7-9	10	0.05	4.42
10-13	7	0.05	3.09

Control (c)	mean fish weight ŵ =	9.60	
Day	number fishes left *F(d)*	% body mass *BM(d)*	weight of food W(d)
1-2	19	0.03	2.74
3-4	16	0.03	2.30
5	13	0.03	1.87
6	13	0.05	3.12
7-9	10	0.05	2.40
10-13	7	0.05	1.68

0.05 mg/L (a)	mean fish weight ŵ =	13.91	
Day	number fishes left *F(d)*	% body mass *BM(d)*	weight of food W(d)
1-2	19	0.03	3.96
3-4	16	0.03	3.34
5	13	0.03	2.71
6	13	0.05	4.52
7-9	10	0.05	3.48
10-13	7	0.05	2.43

0.05 mg/L (b)	mean fish weight ŵ =	13.99	
Day	number fishes left *F(d)*	% body mass *BM(d)*	weight of food W(d)
1-2	19	0.03	3.99
3-4	16	0.03	3.36
5	13	0.03	2.73
6	13	0.05	4.55
7-9	10	0.05	3.50
10-13	7	0.05	2.45

0.05 mg/L (c)	mean fish weight ŵ =	12.88	
Day	number fishes left *F(d)*	% body mass *BM(d)*	weight of food W(d)
1-2	19	0.03	3.67
3-4	16	0.03	3.62
5	13	0.03	2.94
6	13	0.05	4.91
7-9	10	0.05	3.78
10-13	7	0.05	2.64

0.4 mg/L (a)	mean fish weight ŵ =	11.12	
Day	number fishes left *F(d)*	% body mass *BM(d)*	weight of food W(d)
1-2	19	0.03	3.17
3-4	16	0.03	2.67
5	13	0.03	2.17
6	13	0.05	3.61
7-9	10	0.05	2.78
10-13	7	0.05	1.95

0.4 mg/L (b)	mean fish weight ŵ =	9.67	
Day	number fishes left *F(d)*	% body mass *BM(d)*	weight of food W(d)
1-2	19	0.03	2.76
3-4	16	0.03	2.32
5	13	0.03	1.89
6	13	0.05	3.14
7-9	10	0.05	2.42
10-13	7	0.05	1.69

0.4 mg/L (c)	mean fish weight ŵ =	9.18	
Day	number fishes left *F(d)*	% body mass *BM(d)*	weight of food W(d)
1-2	19	0.03	2.62
3-4	16	0.03	2.20
5	13	0.03	1.79
6	13	0.05	2.98
7-9	10	0.05	2.30
10-13	7	0.05	1.61

5 mg/L (a)	mean fish weight ŵ =	9.31	
Day	number fishes left *F(d)*	% body mass *BM(d)*	weight of food W(d)
1-2	19	0.03	2.65
3-4	16	0.03	2.23
5	13	0.03	1.82
6	13	0.05	3.03
7-9	10	0.05	2.33
10-13	7	0.05	1.63

5 mg/L (b)	mean fish weight ŵ =	12.73	
Day	number fishes left *F(d)*	% body mass *BM(d)*	weight of food W(d)
1-2	19	0.03	3.63
3-4	16	0.03	3.06
5	13	0.03	2.48
6	13	0.05	4.14
7-9	10	0.05	3.18
10-13	7	0.05	2.23

5 mg/L (c)	mean fish weight ŵ =	9.30	
Day	number fishes left *F(d)*	% body mass *BM(d)*	weight of food W(d)
1-2	19	0.03	2.65
3-4	16	0.03	2.23
5	13	0.03	1.81
6	13	0.05	3.02
7-9	10	0.05	2.33
10-13	7	0.05	1.63

50 mg/L (a)	mean fish weight ŵ =	10.72	
Day	number fishes left *F(d)*	% body mass *BM(d)*	weight of food W(d)
1-2	19	0.03	3.06
3-4	16	0.03	2.57
5	13	0.03	2.09
6	13	0.05	3.48
7-9	10	0.05	2.68
10-13	7	0.05	1.88

50 mg/L (b)	mean fish weight ŵ =	8.50	
Day	number fishes left *F(d)*	% body mass *BM(d)*	weight of food W(d)
1-2	19	0.03	2.42
3-4	16	0.03	2.04
5	13	0.03	1.66
6	13	0.05	2.76
7-9	10	0.05	2.13
10-13	7	0.05	1.49

50 mg/L (c)	mean fish weight ŵ =	8.98	
Day	number fishes left *F(d)*	% body mass *BM(d)*	weight of food W(d)
1-2	19	0.03	2.56
3-4	16	0.03	2.16
5	13	0.03	1.75
6	13	0.05	2.92
7-9	10	0.05	2.25
10-13	7	0.05	1.57

Annex 3: Cell number of *Chlorella* sp.

a) Test on enrofloxacin

T= 0h

[enrofloxacin] in mg/L	0	21.28	40.16	97.17	208.65	384.29	728.18
replicate a	0.75	0.77	0.80	0.81	0.76	0.82	0.76
replicate b	0.73	0.81	0.78	0.81	0.72	0.83	0.74
replicate c	0.79	0.83	0.84	0.75	0.73	0.74	0.67
Mean	0.76	0.80	0.81	0.79	0.73	0.80	0.73
Std.Dev.	0.03	0.03	0.03	0.03	0.02	0.05	0.05

T= 36h

[enrofloxacin] in mg/L	0	21.28	40.16	97.17	208.65	384.29	728.18
replicate a	6.31	4.24	4.61	3.76	3.19	3.17	1.34
replicate b	7.01	4.42	4.68	3.64	3.38	3.62	1.36
replicate c	6.19	4.24	4.59	3.81	3.39	3.15	1.29
Mean	6.50	4.30	4.63	3.74	3.32	3.32	1.33
Std.Dev.	0.44	0.10	0.05	0.08	0.11	0.26	0.04

T= 72h

[enrofloxacin] in mg/L	0	21.28	40.16	97.17	208.65	384.29	728.18
replicate a	8.72	5.95	5.44	3.94	3.67	4.00	1.40
replicate b	7.73	6.20	5.41	4.29	3.53	3.72	1.29
replicate c	8.30	5.90	5.37	4.21	3.30	4.11	1.30
Mean	8.25	6.02	5.41	4.15	3.50	3.94	1.33
Std.Dev.	0.49	0.16	0.04	0.18	0.18	0.20	0.06

b) Test on ciprofloxacin

T= 0h

[ciprofloxacin] in mg/L	0	3.81	6.69	17.22	26.79	57.68	103.49
a	0.85	0.83	0.92	0.71	0.88	0.77	0.76
b	0.78	0.79	0.90	0.77	0.88	0.75	0.73
c	0.80	0.76	0.85	0.69	0.87	0.78	0.75
Mean	0.81	0.79	0.89	0.73	0.88	0.76	0.75
Std.Dev.	0.04	0.04	0.03	0.04	0.00	0.01	0.02

T= 36h

[ciprofloxacin] in mg/L	0	3.81	6.69	17.22	26.79	57.68	103.49
a	5.53	4.23	5.92	3.53	3.95	1.10	0.80
b	5.66	4.37	5.60	4.14	3.63	1.00	0.63
c	4.95	4.21	5.25	4.13	3.81	1.10	0.80
Mean	5.38	4.27	5.59	3.93	3.80	1.07	0.74
Std.Dev.	0.38	0.09	0.33	0.35	0.16	0.06	0.10

T= 72h

[ciprofloxacin] in mg/L	0	3.81	6.69	17.22	26.79	57.68	103.49
a	6.18	5.44	5.39	4.10	3.73	0.68	0.76
b	6.86	5.69	5.69	4.23	3.72	0.79	0.75
c	7.20	5.67	5.52	4.61	3.95	0.85	0.72
Mean	6.75	5.60	5.53	4.31	3.80	0.77	0.74
Std.Dev.	0.52	0.14	0.15	0.26	0.13	0.09	0.02

Annex 4: Immobilization of *Moina macrocopa*

a) Test on enrofloxacin

T= 0h

[enrofloxacin] in mg/L	0	35.58	63.67	126.22	289.5	599.22	1115.56
replicate a	0	0	0	2	2	1	3
replicate b	0	0	0	0	3	3	3
replicate c	0	0	0	1	2	3	3
replicate d	0	0	0	1	1	2	3
replicate e	0	0	0	0	1	3	3
Mean	0	0	0	0.8	1.8	2.4	3
Std.Dev.	0	0	0	0.84	0.84	0.89	0

T= 24h

[enrofloxacin] in mg/L	0	35.58	63.67	126.22	289.5	599.22	1115.56
replicate a	0	0	0	2	3	3	3
replicate b	0	0	0	3	3	3	3
replicate c	1	0	1	3	3	3	3
replicate d	0	1	0	3	3	3	3
replicate e	0	0	1	3	3	3	3
Mean	0.2	0.2	0.4	2.8	3	3	3
Std.Dev.	0.45	0.45	0.55	0.45	0	0	0

T= 48h

[enrofloxacin] in mg/L	0	35.58	63.67	126.22	289.5	599.22	1115.56
replicate a	0	0	0	3	3	3	3
replicate b	0	0	1	3	3	3	3
replicate c	1	0	1	3	3	3	3
replicate d	0	2	0	3	3	3	3
replicate e	0	0	1	3	3	3	3
Mean	0.2	0.4	0.6	3	3	3	3
Std.Dev.	0.45	0.89	0.55	0	0	0	0

b) Test on ciprofloxacin

T= 0h

[ciprofloxacin] in mg/L	0	35.58	63.67	126.22	289.5	599.22	1115.56
replicate a	0	0	0	0	0	0	0
replicate b	0	0	0	0	0	0	0
replicate c	0	0	0	0	0	0	0
replicate d	0	0	0	0	0	0	0
replicate e	0	0	0	0	0	0	0
Mean	0	0	0	0	0	0	0
Std.Dev.	0	0	0	0	0	0	0

T= 24h

[ciprofloxacin] in mg/L	0	35.58	63.67	126.22	289.5	599.22	1115.56
replicate a	0	0	0	0	3	3	3
replicate b	0	0	0	0	1	3	3
replicate c	0	0	0	0	2	3	3
replicate d	0	0	0	1	1	2	3
replicate e	0	0	0	0	1	3	3
Mean	0	0	0	0.2	1.6	2.8	3
Std.Dev.	0	0	0	0.45	0.89	0.45	0

T= 48h

[ciprofloxacin] in mg/L	0	35.58	63.67	126.22	289.5	599.22	1115.56
replicate a	0	0	0	0	3	3	3
replicate b	0	0	0	1	3	3	3
replicate c	0	0	0	1	3	3	3
replicate d	0	0	0	1	2	3	3
replicate e	0	0	0	3	3	3	3
Mean	0	0	0	1.2	2.8	3	3
Std.Dev.	0	0	0	1.1	0.45	0	0

Annex 5: Tables presenting the fish death during the different experiments

Table a: Fish death during the in-feed experiment with enrofloxacin

Concentration of enrofloxacin in the food (in g/kg of feed)	Number of fish that died during the experiment (Day of the death)
0	1 (D5)
1	1 (D2) 1 (D3) 1 (D7)
2.5	0
5	0
10	0
Total	4

Table b: Fish death during the in-feed experiment with ciprofloxacin

Concentration of ciprofloxacin in the food (in g/kg of feed)	Number of fish that died during the experiment (Day of the death)
0	1 (D9)
0.5	1 (D5) 1 (D7) 1 (D8) 1 (D13)
1	1(D10) 1(D12)
2.5	0
5	1 (D2) 1 (D9)
Total	9

Table c: Fish death during the bath treatment test with enrofloxacin

Concentration of enrofloxacin in the water (in mg/L)	Number of fish that died during the experiment (Day of the death)
0	1 (D2) 1 (D9)
0.1	2 (D2) 1 (D3) 1 (D11)
0.8	1 (D2) 2 (D3) 1 (D10)
10	1 (D3) 1 (D7) 1 (D9)
100	63 (D2)
Total	76

Table 3: Fish death during the bath treatment test with ciprofloxacin

Concentration of ciprofloxacin in the water (in mg/L)	Number of fish that died during the experiment (Day of the death)
0	0
0.05	1 (D9) 1 (D10)
0.4	0
5	1 (D12)
50	18 (D5) 15 (D6)
Total	36

Annex 6: Concentrations of enrofloxacin and ciprofloxacin measured with HPLC (bath treatment)

Table a: Concentrations of enrofloxacin measured with HPLC (bath treatment)

nominal concentration of enrofloxacin (in mg/L)	measured concentration of enrofloxacin (in mg/L)				
	day 1 (after application)	day 2	day 5	day 7	day 14
0	0	/	0	/	/
0.1	0.13	0.05	0.11	0.02	0
0.8	0.50	1.09	1.20	0.10	0.01
10	6.06	7.03	13.52	8.52	0.11
100	95.79	96.06	/	/	/

Table b: Concentrations of ciprofloxacin measured with HPLC (bath treatment)

nominal concentration of ciprofloxacin (in mg/L)	measured concentration of ciprofloxacin (in mg/L)				
	day 1 (after application)	day 2	day 5	day 7	day 14
0	0	/	0	/	/
0.05	0.02	0.05	0.04	0.24	0
0.4	0.27	0.35	0.58	0.21	0.02
5	4.93	9.10	11.50	2.15	0.03
50	70.58	127.75	136.34	6.19	1.89

Annex 7: Extra toxicity test with pure enrofloxacin on *Moina macrocopa*

As explained previously, the formulated compounds were used in the toxicity tests because the dilution of the pure antibiotics with water alone was impossible for the concentrations wanted. However, the EC_{50} value from the experiment on *Moina macrocopa* with enrofloxacin is lower than the one found by Park and Choi (2008). Therefore, an extra test has been performed with pure enrofloxacin to examine if the solvent in the formulated compound adds an extra toxicity to the antibiotic.

For this experiment, the methodology was the same as the one used for the toxicity tests on *Moina macrocopa.* The following concentrations of enrofloxacin were decided to be tested: 0, 20, 40, 80, 160, 320, 640 mg/L. For this, a stock solution of 620 mg of antibiotic/L was prepared. In order to increase the solubility of the compound in the water, 0.6 mg/L of ammonia (1 M) was added to the solution. Then, the Erlenmeyer was placed into a sonicator (branson 2510) for 1h and further in a hot water bath (45°C during 30 minutes) to remove the excess of ammonia. This solution was finally diluted to prepare the different concentrations of enrofloxacin.

After 0h (following the application), 24h and 48h, the number of immobilized daphnids per cuvette was recorded (table a). In general, the immobility of *Moina macrocopa* is also enhanced when the antibiotic concentration increases.

The dose-response curve for the toxicity experiment performed with "pure" enrofloxacin with *Moina macrocopa* is shown in figure a. The slope is 2.429 and the calculated EC_{50}-48h value is 153.9 mg/L (108.2 – 218.7).

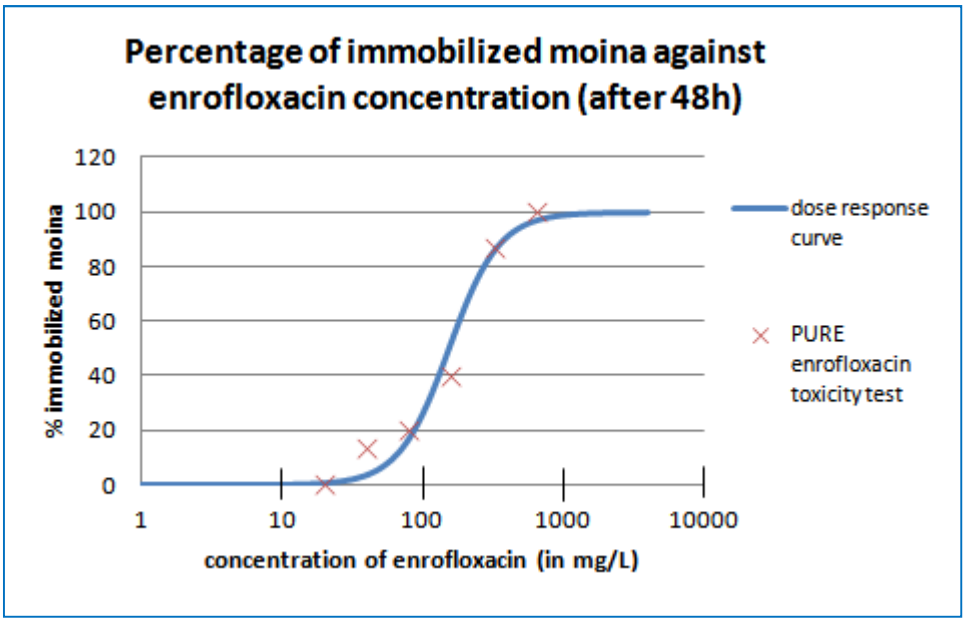


Figure a: Percentage of immobilized moina against enrofloxacin concentration (after 48h) – extra test with "pure" enrofloxacin

Table a: Number of immobilized *Moina macrocopa* when exposed to "pure" enrofloxacin

T= 0h

[enrofloxacin] in mg/L	0	20	40	80	160	320	640
[ammonia] in mg/L	0	0.02	0.04	0.08	0.15	0.30	0.60
replicate a	0	0	0	0	0	0	3
replicate b	0	0	0	0	1	0	3
replicate c	0	0	0	0	0	1	3
replicate d	0	0	0	0	0	0	3
replicate e	0	0	0	0	0	2	3
Mean	0	0	0	0	0	0	3
Std.Dev.	0	0	0	0	0	0	0

T= 24h

[enrofloxacin] in mg/L	0	20	40	80	160	320	640
[ammonia] in mg/L	0	0.02	0.04	0.08	0.15	0.30	0.60
replicate a	0	0	0	0	0	0	3
replicate b	0	0	0	0	1	3	3
replicate c	0	0	0	0	0	2	3
replicate d	0	0	1	1	1	2	3
replicate e	0	0	0	1	0	2	3
Mean	0	0	0.2	0.4	0.4	1.8	3.0
Std.Dev.	0	0	0.4	0.5	0.5	1.1	0

T= 48h

[enrofloxacin] in mg/L	0	20	40	80	160	320	640
[ammonia] in mg/L	0	0.02	0.04	0.08	0.15	0.30	0.60
replicate a	0	0	0	0	1	2	3
replicate b	0	0	0	0	1	3	3
replicate c	0	0	0	1	2	3	3
replicate d	0	0	1	1	2	2	3
replicate e	0	0	1	1	0	3	3
Mean	0	0	0.4	0.6	1.2	2.6	3.0
Std.Dev.	0	0	0.5	0.5	0.8	0.5	0

The EC_{50}-48h value given above is nearly two times higher than the one calculated for the test with formulated enrofloxacin. Therefore, the solvent present in Vimenro 200® clearly adds an extra toxicity on the *Moina macrocopa* apart from the antibiotic. However, this value is still smaller than the one given in the study of Park and Choi (2008). In fact, a fraction of the ammonia used to increase the solubility of enrofloxacin in the water, could remain in the stock solution. This compound could add an extra toxicity, apart from the antibiotic.