Population pharmacokinetics/pharmacodynamics modelling of enrofloxacin for the three major trout pathogens Aeromonas salmonicida, Flavobacterium psychrophilum and Yersinia ruckeri

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Abstract

Enrofloxacin is a fluoroquinolone antimicrobial agent used in freshwater rainbow trout against the main pathogenic bacteria *Aeromonas salmonicida*, *Yersinia ruckeri and Flavobacterium psychrophilum*. However, the current "standard" dose (10 mg/kg/day for 10 days) was based only on some old, rather limited experimental data, and needed to be re-assessed. Thus, a pharmacokinetic-pharmacodynamic (PKPD) approach was used by combining a population PK model with new epidemiological data (Minimum Inhibitory Concentrations (MIC)) of the three bacterial species to determine optimal enrofloxacin doses in rainbow trout.

Ninety-six rainbow trout (half diploid, half triploid) were randomly assigned to four different groups and received oral (gavage) and then intravenous administration of enrofloxacin at four

different doses (range 5-60 mg/kg). Individual blood samples were taken to develop a population PK model.

Enrofloxacin should be considered as a long-acting drug in trout due to the observed long plasma half-life (>100 h), which is therefore inadequate with the "standard" dosage based on daily oral administrations. Moreover, the fish ploidy had an impact on the PK of enrofloxacin with a longer persistence of enrofloxacin in triploid individuals, which raises the question of the withdrawal period to apply. The absolute bioavailability of oral enrofloxacin was estimated at ~88%.

For *F. psychrophilum*, the provisional epidemiological cut-off value (CO_{NRI}), calculated according to the NRI method, was equal to 0.03 µg/mL. For *A. salmonicida* and *Y. ruckeri*, however, no clear bimodal distribution of MIC could be observed, and therefore no relevant CO_{NRI} could be obtained.

According to our model, a single oral dose of ~5 mg/kg should provide sufficient exposure to treat the wild-type population of *F. psychrophilum* for 4 days, while complying with the PKPD breakpoints. Then, a maintenance dose of ~2.5 mg/kg could possibly be re-administered every 4 days. The absence of a CO_{NRI} did not allow to predict an optimal dose for the two other bacteria. As more than 70% of *A. salmonicida* isolates in our data set have an enrofloxacin MIC $\geq$ 0.25 µg/mL, it seems that enrofloxacin should not be recommended against this bacterium.

The PKPD approach allowed us to refine the dosing regimens in rainbow trout, for a more sustainable approach. These new dosing regimens have yet to be clinically confirmed.

Keywords: enrofloxacin, rainbow trout, *Aeromonas salmonicida*, *Yersinia ruckeri*, *Flavobacterium psychrophilum*, population pharmacokinetics

Abbreviations

AUC : Area under the plasma concentration versus time curve
cBIC : corrected Bayesian Information Criteria
Cl : Total body clearance
Cmax : Peak plasma drug concentration
CO_{NRI}: Provisional epidemiological cut-off calculated with the NRI method

ECOFF : Epidemiological cut-off
EMA : European Medicine agency
fu : unbound fraction
HPLC : High-performance liquid chromatography
IIV : inter-individual variability
IV : intra-venous
MCS : Monte Carlo simulation
MIC :Minimal inhibitory concentration
NLME : non-linear mixed effect
NRI : normalized resistance interpretation
pcVPC : Predicted-corrected Visual predictive checks
PK :Pharmacokinetic
PD :Pharmacodynamic
PTA : Probability of target achievement
RSE : relative standard error
SF : Scaling factor
$t1/2_{\beta}$: Plasma half-life
WT : wild-type

1. INTRODUCTION

In recent decades, international aquaculture has increased in terms of production volume, species diversity and economic value (FAO, 2020). Rainbow trout (*Oncorhynchus mykiss*) is an important species of salmonids farmed worldwide, with Europe as the main production area (FAO, 2020). As in any other intensive animal production system, salmonid production is confronted with the problem of bacterial infections. In Europe, yersiniosis (*Yersinia ruckeri*), furunculosis (*Aeromonas salmonicida* subps *salmonicida*) and cold water disease (*Flavobacterium psychrophilum*) are among the major bacterial infectious diseases of rainbow trout that causes economic losses (Furones *et al.*, 1993; Antaya, 2008; Austin and Austin, 2016). Antibiotics used to control these pathogens in aquaculture include enrofloxacin, a

fluoroquinolone widely used in veterinary practice around the world (Trouchon and Lefebvre, 2016). Of the 11 largest aquaculture producing countries, 6 use enrofloxacin (Lulijwa *et al.*, 2020) despite the great differences that exist between countries regarding the regulation of its use. In Europe, the European Medicine agency (EMA) recently classified enrofloxacin in the category B ("Restrict") regarding the risk of antimicrobial resistance, but highlighted the very few treatment alternatives against the three bacteria mentioned above (EMA, 2019). In order to obtain an optimal dosing regimen that provides a high probability of treatment success while minimizing the risk of resistance selection in the target species, the pharmacokinetic (PK) and pharmacodynamic (PD) properties of enrofloxacin should be properly characterized for rainbow trout.

Enrofloxacin PK has been studied in some farming fish species, such as salmonids (Bowser *et al.*, 1992; Stoffregen *et al.*, 1997; Lucchetti *et al.*, 2004; Koc *et al.*, 2009), some freshwater fish (Fang *et al.*, 2012; Xu *et al.*, 2013) and in marine species (Lewbart *et al.*, 1997; Intorre *et al.*, 2000; Della Rocca *et al.*, 2004). For salmonids, a wide range of terminal half-life has been reported (~19h to ~130h) which is overall longer compared to mammal species (Trouchon and Lefebvre, 2016). Moreover, some covariates as the water temperature have been shown to strongly influence the PK of enrofloxacin in fish (Bowser *et al.*, 1992; Liang *et al.*, 2012). The influence of the ploidy (i.e. diploid *vs* triploid), however, has never been investigated although the triploidy process, which consists in inducing sterility for fish, is increasingly used in rainbow trout due to numerous advantages for fish growth (Piferrer *et al.*, 2009).

Enrofloxacin displays a concentration-dependent bactericidal activity with a wide spectrum of action on aerobic bacteria, especially against Gram-negative bacteria (Brown, 1996). Its efficacy has been shown to be linked to the PKPD index defined by the ratio of the area under free plasma concentration–time curve (fAUC) over the MIC (fAUC/MIC) (Wright *et al.*, 2000). For rainbow trout, a wide range of enrofloxacin doses has been used (*e.g.* 1-50 mg/kg/day *per os*) (Reimschuessel *et al.*, 2013). The dose of 10 mg/kg/day, later be referred to as the "standard" dose, is the most commonly used dose. It is, however, based only on a few PK experiments, and on a few field trials carried out several decades ago involving only one or two different pathogenic strains (e.g. *Aeromonas*) (Bowser *et al.*, 1990; Bowser *et al.*, 1992; Hsu *et al.*, 1994; Hsu *et al.*, 1995). Curiously, the variability associated with PK parameters (clearance, bioavailability) and PD parameters (in terms of MIC) has not really been explored although it is a major influencing factor that must be taken into account to obtain optimal dosing (EMA, 2018). Moreover, a recent evaluation of the efficacy of enrofloxacin in rainbow trout

following an experimental challenge with *Y. ruckeri* showed that a treatment based on oral doses from 1 to 5 mg/kg in feed for 7 days was insufficient (Rostang *et al.*, 2021).

The aim of this study was to compare the "standard" dose of 10 mg/kg/day enrofloxacin in rainbow trout with the optimal dosing regimen calculated by a PKPD approach, including: (i) the development of a population PK model based on experiments with rainbow trout receiving oral and IV administration of enrofloxacin at different doses, and the identification of relevant physiological covariates (ploidy, weight); (ii) collection of MIC data of enrofloxacin from the three major pathogens *Y ruckeri, A salmonicida and F psychrophilum* (PD parameters); (iii) integration of the above-mentioned PK and PD parameters with serum protein binding data within Monte Carlo simulations to derive optimal dosing regimens in rainbow trout.

2. MATERIALS AND METHODS

2.1. Animals

Rainbow trout (48 diploid and 48 triploid individuals) were purchased from the experimental fish farm of INRA (Sizun, France). Fish were acclimatized for two weeks, before being allotted in batches of six fish in 200 L tanks with a homogenous weight repartition (weight means between 344 and 389 g with a CV ranging from 15% to 20%). Genetic profiles were separated. Water parameters such as temperature (11.0 ± 1°C), O_2 (90-100%) and flow rate (2m^3/h) were regulated and controlled daily. Outside the oral treatment period, fish received standard feed, pellet B Mega 19 (Le Gouessant, Lamballe, France), once a day at the rate of 0.5% of biomass. The pharmacokinetic experiments were performed in 2008, before the adoption of the European Directive 2010/63/EU while already respecting its general philosophy. This PK study was carried-out in a French veterinary school within a joint research unit of INRAE (National Research Institute for Agriculture, Food and Environment), within an certified experimental aquaculture facility with the approval number D44272. This experimental structure was managed by veterinarians and scientists with the required qualifications. Experimental design and animal welfare were assessed by local animal experts ensuring high ethical requirements. For this study, particular attention was paid to reduce the number of fish needed to conduct the experiment, as well as to limit stress for the animals during the study (environmental enrichment measures, noise limitation, anaesthesia during handling, blood samples taken by experienced veterinarians or technicians).

2.2. *In vivo* experiments

For each genetic profile, fish were randomly divided in four groups (12 individuals per group) corresponding to different dosages of enrofloxacin. In each group, fish received a single oral administration and then an intra-venous (IV) injection of enrofloxacin four days later. The different doses are specified in Table 1. The enrofloxacin concentration in the feed was verified (in triplicate) by the HPLC method described in the analytical method section.

For the oral experiment, in-house medicated feed was prepared by mixing standard feed, pellet B Mega 19, coating with oil fish (Le Gouessant) and a veterinary drug formulation of enrofloxacin, Baytril 10% (Bayer Sante, Loos, France). After preparation, enrofloxacin-supplemented pellets were stored at room temperature for 12 hours and then at 4°C. Fish were starved during 48 h before being anesthetized by bathing with 2-phenoxyethanol (0.2 mL/L) to receive the medicated feed by gavage. After gavage, each fish was transferred to an individual tank for few minutes to monitor regurgitation and none fish regurgitated more than two pellets. Fish were then transferred back to their initial tank.

Approximately 0.2 mL of blood were collected from the caudal vein of each fish at 1, 3, 6, 10, 26 and 96 h following gavage. For the group receiving the dose of 5 mg/kg, there was an additional sampling time at 72h after gavage. For each blood sampling, fish were first anesthetised by bathing with 2-phenoxyethanol (0.2 mL/L). The fish was then taken out of the water for a few minutes to perform the blood sampling. It was then placed back in a monitoring tank until it was fully awake. The anaesthesia, from its induction to the animal's awakening, was closely monitored by a dedicated veterinarian. For each animal, the whole handling did not exceed 15 minutes.

Immediately after the last sampling at 96h (no wash-out), fish received an IV administration of enrofloxacin from the same formulation (Baytril 10%) and blood samples were collected at 1, 4, 10, 14, 30, 100 and 120 h after injection. Fish were euthanized with an over-dose of 2-phenoxyethanol (0.6 mL/L) after the last sampling time (*i.e.* around 216 h after the first oral administration).

All blood samples were centrifuged at 2,000 *g* for 10 min at room temperature and plasma was stored at -20°C before assay.

2.3. *In vitro* experiments

2.3.1. Protein binding of enrofloxacin in plasma

Ultrafiltration method was used to determine the unbound fraction (fu) of enrofloxacin in plasma. Briefly, frozen plasma samples (pH between 7.2 and 7.4) from a pool of several untreated trout were thawed and supplemented with enrofloxacin (Fluka, Steinheim, Switzerland) to obtain final plasma enrofloxacin concentrations of 0.1, 1, 5 and 10 µg/mL. Samples were incubated under agitation at ambient temperature for 1 h. Then, 500 µL of each sample were transferred to a cartridge with a centrifuge filter (MICROCON YM-10, Millipore, USA) and centrifuged at 3,000 x *g* for 15 min at 22°C. Ultrafiltrates were then collected and stored at -20°C until assay (see analytical method). The same steps were performed in isotonic PBS instead of plasma in order to determine the non-specific binding (NSB). All experiments were performed in triplicate (technical replicates) and experiments in plasma were repeated two times (independent replicate). Plasma fu was expressed as in equation 1 for each tested concentrations:

$$fu = \frac{Ultrafiltrate\ concentration}{\left(100 - \frac{NSB}{100}\right) \times Initial\ plasma\ concentration} \quad (1)$$

2.3.2. Bacteria collection, MIC determination and MICs analysis

A collection of 280 *Y. ruckeri*, 151 *A. salmonicida* and 77 *F. psychrophilum* strains from our lab collection were used to get enrofloxacin MIC data by the microbroth-dilution method in accordance with CLSI recommendations for bacteria isolated from aquatic animals (CLSI, 2014) (Table 2). All our strains come from diseased fish samples (not from water samples). For the *A. salmonicida* isolates, bacteria that are difficult to identify, the identification was performed by Maldi-Tof and confirmed with the PCR method of (Byers *et al.*, 2002). Briefly, *Asalmonicida* ATCC 33658 and *Escherichia coli* ATCC 25922 were used as quality control strains. The range of enrofloxacin concentrations tested was 0.004-2 µg/mL. For *Y. ruckeri* and *A. salmonicida* isolates, the plates were incubated at 22°C for 24 h and for *F. psychrophilum* isolates plates were incubated at 17°C for 96 h. MICs were equal to the lowest concentration of enrofloxacin that inhibited visible bacterial growth.

Other enrofloxacin MIC data sets for the 3 bacteria species were found from literature search (using Scopus and Google Scholar) and only those following the CLSI recommendations for bacteria isolated from aquatic animals (CLSI, 2014) were kept (Table 2). Only data set issuing from a proper confirmation of the identification of *A. salmonicida* strains were considered.

The NRI method (Kronvall, 2010) was used with permission from the patent holder, Bioscand AB, TÄBY, Sweden (European patent No. 1,383,913, US Patent No. 7,465,559). Provisional epidemiological cut-off value (named CO_{NRI}) were calculated using the automatic Excel spreadsheet for MIC data accessed from http://www.bioscand.se/nri/. The acronym CO_{NRI} was chosen to avoid any confusion with the internationally recognized epidemiological cut-offs ECV and ECOFF used by CLSI and EUCAST, respectively. In data sets where a small percentage (<5%) of the wild-type (WT) observations were "below- scale," these observations were treated as having the MIC value immediately below the limit of the plate quantitation. When the percentage of the WT observations "below- scale" was >5%, the data set was considered as unsuitable for NRI analysis and excluded.

2.4. Analytic method

2.4.1. Chemicals and reagents

Enrofloxacin used for determination of analytical method was obtained as pharmaceutical-grade powders from Fluka (Steinheim, Switzerland). Stock standard solutions of enrofloxacin (1000 µg/mL) were prepared by dissolving 50 mg in 50 mL of sodium hydroxide 0.03 mol/L and stored at 4°C for 1 month. Acetonitrile was HPLC-solvent grade, trimethylamine, orthophosphoric acid were analytical-reagent grade (Merck, Lyon, France). Ultrapure water was obtained from a Milli-Q system from Millipore (Bedford, MA, USA). Zinc sulphate heptahydrate and sodium hydroxide 1 mol/L were obtained from Panreac QuimicaSA (Barcelona, Spain).

2.4.2. Enrofloxacin assay

Experiments were performed using an isocratic pump, an automatic injector with a 20 µL loop 307 pump, a 234 auto-injector (Gilson, Villiers Le Bel, France), and a cartridge oven CTO.10As VP (Shimadzu, Kyoto, Japan) coupled to a fluorescence detector (FP-1520, Jasco, Tokyo, Japan). Analytical separation was achieved on a Chromolith® performance RP-18 endcapped 100mm x4.6mm HPLC column, protected by a 5 x 4.6 mm guard column containing the same packing material. LC mobile phase was prepared by combining 840 mL of 0.02 mol/L orthophosphoric acid – 0.008 mol/L triethylamine (1:1, v/v) with 160 mL of acetonitrile and then filtering with a filtration unit SolVac using a GH-Polypro membrane of 0.45 µm porosity (Pall Corporation, Ann Arbor, MI, USA). The flow rate was 0.8 mL/min and the temperature

column oven 27°C, with detector set at an excitation wavelength of 280 nm and an emission wavelength of 470 nm.

For samples preparation, an amount of 150 µL of plasma sample was placed in a 1.5 mL microvial tube. 15 µL zinc sulphate solution 10%, 15 µL sodium hydroxide 0.1 mol/L and 300 µL acetonitrile were added. The mixture was homogenized for 10 min with the agitator and centrifuged for 10 min at 3000 x *g* at 4°C. The supernatant was transferred to a 10 mL glass tube and evaporated to dryness under a nitrogen stream at 40°C. The dry residue was dissolved in 150 µL mobile phase, sonicated for 0.5 min and filtered with Millex filter unit 0.45 µm. 20 µL were injected into the HPLC column. The calibration curves were drawn by plotting the peak heights of enrofloxacin against the known concentrations of enrofloxacin. LOD and LOQ were equal to 7 ng/mL and 20 ng/mL respectively.

2.5. Data analysis

2.5.1. Population PK model development

The assessment of dose-linearity after the oral treatment was carried out using a bioequivalence approach (Gough *et al.*, 1995). Briefly, a power model was fitted to the partial AUC_{0_96h} (just before the IV administration) and assessed by a linear regression. The slope (β_1) and its associated 90% confidence interval (CI) were compared to the reference interval, with a ratio of maximal to minimal dose r=8, and lower and upper acceptance limits equals to 0.8 and 1.25, respectively (Smith *et al.*, 2000).

Enrofloxacin plasma concentration time- courses from oral and IV dosing were analysed simultaneously thanks to a non-linear mixed effect (NLME) approach, allowing the estimation of population parameters, inter-individual variabilities (IIV) and residual errors (Bon *et al.*, 2018).

A structural model was chosen based on a good fit of the data and adequate diagnostic plots (Observed value vs Predicted value, residuals) as well as precision of the relative standard error (RSE) of the estimated parameters. Selection between different structural models was based on a decrease of the corrected Bayesian Information Criteria (cBIC). Different error models were tested (additive, proportional and combined).

A log-normal distribution of parameters was assumed for all parameters except those relative to the bioavailability (logit-normal distribution). IIV were estimated for all parameters and kept only if the eta-shrinkage was low (<35%) in addition to a decrease of cBIC and an acceptable

RSE value (< 30%). Potential correlation between random effects were evaluated thanks to visual inspection of the scatterplot of random effects sampled from the conditional distribution and tested if necessary with Pearson correlation tests (p-value < 0.05). Indeed, omitting the correlation could bias the simulation (Silber *et al.*, 2009).

Using classical equation for bi-compartmental model (Toutain and Bousquet-Melou, 2004), the terminal half-life ($t_{1/2}\beta$) was computed as a secondary parameter and expressed with harmonic mean.

The effect of covariates (BW and genetic) were evaluated using the automated Pearson's correlation test and the ANOVA method as implemented in Monolix (Monolix version 2019R1. Antony, France: Lixoft SAS, 2019). They were included with a significance of $p < 0.05$ and kept only if the IIV decreased > 5% associated to an acceptable RSE. The covariates were expressed as an exponential function. Hence for a discrete covariate (like ploidy status), the following equation was applied (Eq. 2):

$$log(\mathrm{X}i) = log(Xpop) + \beta_{\mathrm{X_{gen=T}}} + \eta i \quad (2)$$

where Xpop is the population value of the parameter X and $\beta_{\mathrm{X\_gen=T}}$ is the fixed effect of the categorical covariate (i.e., being triploid) on *X and* ηi the random effect for individual i. If $\beta_{\mathrm{X\_gen=T}}$ was significantly different from 0, the covariate was kept.

The continous covariate bodyweight (BW) was normalized by its median value (weight = 361g) and log-transformed to give the equation 3:

$$log(\mathrm{X}i) = log(Xpop) + \beta_{\mathrm{X_{WT}}} \times \log\left(\frac{WT}{361}\right) + \eta i \quad (3)$$

where Xpop is the population value of the parameter X and $\beta_{\mathrm{X\_WT}}$ is the fixed effect of the continous covariate WT on *X and* ηi the random effect for individual i.

The condition number was scrutinized during the whole model development to avoid any parameter correlation or over-parametrisation of the model (Mould and Upton, 2013).

2.5.2. PK model validation

Predicted-corrected visual predictive checks (pcVPC) were generated to validate the model. These kind of VPCs are particularly relevant when dealing with different covariates and a wide range of doses between groups (Bergstrand *et al.*, 2011). Briefly, n=500 simulations were

carried out from the initial dataset and the 10^{th} and 90^{th} percentiles were plotted with their respective confidence interval to verify if 80% of the (corrected) observed data were included within this interval.

The robustness of the model convergence was tested by a convergence assessment of Monolix where all parameters were estimated during eight successive runs with different, randomly generated, initial values of fixed effects as well as different seeds.

2.5.3. **PKPD integration and computation of dose by Monte Carlo simulation**

Monte Carlo simulation (MCS) generates a set of PK parameters values for each simulated individual by random sampling within the associated covariates and the estimated PK parameter distributions from the population PK model, taking into account the potential correlations between random-effects. Each MCS were carried-out with 5000 simulated individuals.

First, to explore the "standard" dosing regimen, the PTA (probability of target achievement) was calculated as the percentage of the 5,000 simulated fish who met the PKPD index target value for each MIC value. For fluoroquinolones like enrofloxacin, a PKPD index $fAUC_{24h}$/MIC greater than 100/125 h at steady-state is classically used (Wright *et al.*, 2000). For fish pathogenic bacteria, however, there is a lack of information about the relevance of these target PKPD index values (see discussion). Values of 50, 75, 100 and 125 h, therefore, were chosen, equivalent to a scaling factor (SF) ranging from ~2 to ~5 when divided by 24h (Toutain *et al.*, 2007). For each PKPD index value, the lowest MIC at which the PTA became ≥ 90% was considered as the PKPD cut-off ($PKPD_{CO}$) (Toutain *et al.*, 2017a)

Then, an overall weighted-PTA was calculated for the "standard" dose, taking into account the probability distribution derived from the MIC data of this study for each bacteria. Briefly, each PTA value previously calculated for a given MIC was multiplied by the percentage of the microbial population associated with that MIC value. The sum of these products gave the weighted-PTA (Drusano *et al.*, 2001).

Finally, for those bacterial species for which a CO_{NRI} could be calculated (see 2.3.2), optimisation of enrofloxacin doses was achieved through MCS with the following steps:

- (i) Determination of a maintenance dose

For the PKPD index fAUC/MIC, a maintenance dose (mg/kg) can be computed to insure sufficient exposure over any regular interval (named *tau*), i.e. 24, 48, 72 h, etc., using equation taken from Toutain *et al*. (4) (2017b):

$$\text{Maintenance Dose}_{tau} = \frac{\text{CL} \times tau \times \text{SF} \times \text{MIC}}{\text{fu} \times \text{F}} \quad (4)$$

where CL (mLh/kg) is the population distribution of the plasma clearance as obtained in our PK model; *tau*: the target dosing interval (h); SF is the scaling factor (unitless) related to the PKPD index and obtained by dividing the value of $fAUC_{24h}$/MIC by 24 h (Toutain *et al.*, 2007). The main advantage of the use of SF is to get rid of the time dimension, thus simplifying the expression of the PKPD index over any longer interval than the classical 24 h; MIC (µg/mL) is the provisional cut-off value (e.g CO_{NRI}); fu is the unbound fraction of drug computed as a uniform distribution between 0.47 and 0.64 (see 3.1.1) and F (%) is the population distribution of the bioavailability as obtained in our PK model.

- (ii) Determination of a single dose (equivalent to a loading dose)

Equation (4) is appropriate to compute daily dosage only when plasma steady state has been reached. For long terminal half-life drug as enrofloxacin, a loading dose should be required to reach the target steady-state plasma concentration more quickly. The loading dose can be derived using equation (5) taken from Toutain and Bousquet-Mélou (2004):

$$AUC_{\text{Loading Dose}_{tau}} = R_{tau} \times AUC_{\text{Maintenance Dose}_{tau}} \quad (5)$$

and assuming pharmacokinetic linearity (see results), we finally got equation (6) which is equivalent to an initial dose with a duration of effect equals to the target interval (*tau*):

$$\text{Loading Dose}_{tau} = R_{tau} \times \text{Maintenance Dose}_{tau} \quad (6)$$

Where R_{tau} is the accumulation ratio which depends on the target dosing interval *tau* (i.e. 24, 48, 72 h, etc). If the dosing interval is sufficiently large, *i.e*. doses are administered in the post-distributive phase (see 3.2.3), R_{tau} is equal to equation (7) (Toutain and Bousquet-Melou, 2004)

$$R_{tau} = \frac{1}{1 - e^{-\left(\frac{\ln 2}{t_{1/2}} \times tau\right)}} \quad (7)$$

With $t_{1/2}$ =terminal half-life (h); *tau* = dosing interval.

Achievement of a PTA=90% was considered as an appropriate threshold for these calculated doses (Toutain *et al.*, 2017a).

2.6. Software

Monolix was used to develop the population PK model (Monolix version 2019R1. Antony, France: Lixoft SAS, 2019). Simulx function from the Lixoft package "mlxR" (Lavielle, 2020) was used with R software version 3.5.2 (R Core Team, 2014) to perform the MCS and PKPD modelling.

3. RESULTS

3.1. *In vitro* experiment:

3.1.1. Protein binding

Unbound fraction of enrofloxacin in rainbow trout plasma was in the range of 0.47 to 0.63 (Table 3) for a concentration range of 0.1–10 µg/mL.

3.1.2. MICs analysis

A total of 346 isolates of *F. psychrophilum* (Smith *et al.*, 2016; Van Vliet *et al.*, 2017; Ngo *et al.*, 2018; Saticioglu *et al.*, 2019), 408 isolates of *Y. ruckeri* (Calvez *et al.*, 2014) and 151 isolates of *A. salmonicida* were pooled from this study and literature search (Table 2). Overall, these strains had mostly been isolated from rainbow trout. Their associated MIC_{90} were equals to 0.128 µg/mL for *Y. ruckeri*, 1 µg/mL for *F. psychrophilum*, and 1 µg/mL for *A. salmonicida*, (Figure 1)

Using the NRI method, a CO_{NRI} was obtained for *F. psychrophilum*, equals to 0.03 µg/mL (Supplementary Figure 1). Among the initial 346 collected isolates, only 31% (n = 108) would therefore be classified as wild-type (WT).

In contrast, for *A. salmonicida* and *Y. ruckeri* strains, no clear bimodal distribution could be observed that would allow the separation of WT isolates from those that are not wild-type (NWT) (Figure 1). Moreover, the NRI method failed to derive a relevant CO_{NRI} because the standard deviation of each MIC distribution was too large (*i.e.* $\geq$ 1.2 $\log_2$ µg/mL; Kronvall, 2010) .

3.2. *In vivo* study

3.2.1. Population PK model

The enrofloxacin concentrations were measured in the medicated feed and were equal to (mean ± SD) 4.75 ± 0.16, 8.51 ± 0.40, 19.80 ± 0.34 and 41.30 ± 2.54 mg/kg for the theoretical doses of 5, 10, 20 and 40 mg/kg respectively. Thus, corrected doses were used in the dataset used to develop the PK model.

A total of 1286 sampling times (none under LOQ) were simultaneously analysed with the PK model and the raw plasma data are presented in Figure 2.

After visual inspection of the data, different compartmental PK model were tested but the bi-compartmental model gave a better fit. The residual error was defined by a combined model of a constant term and a term proportional to the structural model. For the oral PK data, an atypical absorption profile was chosen following data observation at early time (Supplementary Figure 2). Indeed, the absorption process was modelled by two different first-order absorption constants (ka1 and ka2) separated by a lag-time (Tlag) (Figure 3). A fraction of the bioavailable dose (1-Frac_ka2) was absorbed early following ka1 and the remaining fraction (Frac_ka2) was absorbed more slowly following ka2 (ka1 > ka2). This atypical absorption model was supported by a huge decrease of the cBIC compared to a model with only one first-order absorption constant (delta = 340). Finally, inspection of the goodness-of-fit plots (Supplementary Figures 3-4) confirmed the adequacy of this structural model.

The linearity of the PK processes was assessed by fitting a power model to the partial $AUC_{0\text{-}96h}$ values (*i.e.*, between oral administration and IV injection) (Supplementary Figure 5). β_1 was equal to 1.076 (90% CI : 1.034-1.11), compared to the reference interval of [0.896-1.107]. Therefore, the 90% CI of β_1 was not completely included within the reference interval but we yet assumed pharmacokinetic linearity over this dose range.

All structural parameters were estimated with a very good confidence (RSE < 20%) and IIV could be estimated for all of them (except ka1) with a high level of confidence too (RSE < 20%)

(Table 4). The absolute bioavailability of enrofloxacin (Foral) was estimated at ~88%. The fraction of the absorbed dose during the early absorption phase (linked to ka1) was very low (< 5%). Additionally, four correlations between random parameters were also found to be significant (RSE < 40%).

For covariate analysis, (i) weight had a significant influence on clearance, central and peripheral volumes parameters; (ii) the genetic profile affected clearance (decreased for triploid) and oral absorption (slower absorption and longer lag-time for triploid) (Table 4). For instance, a typical individual of this study (BW = 361 g) being triploid will have a decrease ~30% of enrofloxacin clearance compared to the same individual being diploid (due to $\beta_{\mathrm{Cl\_gen=T}}$ = -0.34 in equation 2). The terminal half-life of enrofloxacin was 115h and 166h (with 32% IIV) for diploid and triploid fish, respectively, and differed significantly (Student t-test, $p < 0.001$).

Predicted-corrected VPC plots showed that the full model (including IIV, covariates and correlations) was able to describe adequately the observed data despite a slight underprediction during the absorption phase, around 24 h (Figure 4 and Supplementary Figure 6 for the pcVPC plots stratified by ploidy status). Moreover, the convergence assessment showed that the model was robust *i.e.*, not sensitive to the initial conditions (Supplementary Figure 7). Taking together, all these results validated the final PK model.

3.2.2. PKPD integration and exploration of the "standard" dose of enrofloxacin

For the PKPD integration, the effect of ploidy on clearance was considered negligible compared to the acceptable 2-fold uncertainty about MIC values when looking at Eq.4. We therefore only considered diploid individuals, as the "worst-case scenario" for enrofloxacin exposure (higher clearance). Using the "standard" oral maintenance dose of 10 mg/kg/day, PTA at steady-state was calculated for all MIC values covering the whole range of the 3 MIC distributions (from 0.004 to 4 µg/mL) (Figure 5).Whatever the chosen value of the PKPD index, this dosing regimen gave a PTA > 90% for all MIC ≤ 0.25 µg/ml . The $PKPD_{CO}$ associated to this dosing regimen were equal to 1, 0.5, 0.5 and 0.25 µg/mL for the SF ranging from 2 to 5 (or equivalently 50h to 125h), respectively (Figure 5).

When looking at the weighted-PTA at steady-state (Table 5), the standard dose appeared sufficient to cover the whole distribution of *Y. ruckeri.* For *A. salmonicida and F.*

psychrophilum, however, the weighted-PTA was ≥ 90% (or really close) only for a PKPD index SF of 3 or less.

These previous assessments were based on the values of fAUC/MIC between 2 doses over a 24 h-interval after steady-state was reached. However, as the t1/2 of enrofloxacin is long (between 4.5 and 6 days, see above), the time (or number of days of treatment) needed to reach the steady-state and therefore to attain the target value of fAUC/MIC (or equivalent SF value) is also long (Table 6 and Supplementary Figure 8). For instance, regarding the MIC of 0.25 µg/mL, the 90% PTA would be reached after at least 48 h and 144 h (*i.e.* 2 and 6 days of treatment) for the lowest and highest SF value of 2 and 5, respectively (Table 6). For MIC ≥ 1 µg/mL, the 90 % PTA would even never been achieved.

3.2.3. **Dose determination by MCS**

In view of the previous results, enrofloxacin acts as a long-acting drug in trout and therefore we considered the use of a single oral dose with a duration of effect of 96 or 120 h.

3.2.3.1. With the CO_{NRI} for *F. psychrophilum*

As we only got a provisional cut-off (CO_{NRI}) for *F. psychrophilum*, dose optimisation was carried-out solely for this bacteria species. Thanks to the equations 4-7, we could compute a single oral dose for the 2 durations of activity and the different values of fAUC/MIC (Table 7). For instance, a single dose of 4.9 mg/kg would give a PTA > 90% with SF = 4 over 96 h (i.e. $fAUC_{96h}$/CMI = 400 h). Then, a maintenance dose of 2.4 mg/kg could possibly be re-administered every 4 days (= 96 h) after this first loading dose to maintain a PTA > 90% in trout. All calculated doses for PTA values ranging from 10 to 90% are presented in Table S1 for the single doses and in Table S2 for the maintenance doses.

3.2.3.2. With the whole range of MIC for the three pathogens

The calculated oral single doses that insure a PTA ≥ 90% for all possible enrofloxacin MIC (from 0.004 to 4 µg/mL) of the 3 bacterial species are presented in Table 8. For the highest MIC value, very high and unrealistic doses of enrofloxacin would be needed (between 315 and 837 mg/kg, depending on the PKPD index value).

4. DISCUSSION

To our knowledge, this is the first time that a population PK model has been developed for enrofloxacin in rainbow trout based on longitudinal individual data. Moreover, thanks to some new MIC data and those from literature for the three major pathogens of rainbow trout, a PKPD integration was carried-out with MCS. This approach brings new insights on the pharmacological aspects of the use of enrofloxacin in rainbow trout.

4.1. MICs distributions of the three species

This study gave valuable and reliable new MIC data (following CLSI guidelines) about enrofloxacin for *A. salmonicida, Y. ruckeri* and *F. psychrophilum*. The first challenge was to find relevant cut-off values that could be used to compute doses by MCS (as discussed below). For trout (and overall fish) pathogenic bacteria, no epidemiological cut-off values from CLSI or EUCAST, nor clinical breakpoints are available concerning enrofloxacin. We attempted, therefore, to calculate a provisional cut-off value for enrofloxacin, the CO_{NRI}, for the three species.

The CO_{NRI} of 0.03 µg/mL that was calculated for *F. psychrophilum* in this study *(*Supplementary Figure 1) was in total agreement with the CO_{NRI} of 0.03 µg/mL previously published (Saticioglu *et al.*, 2019). Most part of the strains (~70%) from this pooled dataset should be therefore classified as NWT bacteria. Concerning *Y. ruckeri*, the MIC data did not allow us to propose a value of CO_{NRI}, despite a relatively large number of isolates (n = 408).

Regarding *A. salmonicida*, no clear bimodal distribution of the MIC could be observed (Figure 1). An enrofloxacin CO_{NRI} of 0.06 µg/mL (also obtained with the NRI method; Kronvall, 2010) was previously proposed for *A. salmonicida* (Baron *et al.*, 2017). This apparent huge discrepancy with our raw data could be explained by at least 3 factors : (i) The *A. salmonicida* isolated in the above-mentioned study were identified by Maldi Tof, whereas it was reported recently a poor performance of this technique for an accurate identification of *Aeromonas* at the species level (Pérez- Sancho *et al.*, 2018). On the contrary, all the *A. salmonicida* strains from our dataset were identified by PCR technique, which gives better confidence for the identification; (ii) Isolates from our dataset originated from clinically diseased animals, whereas isolates from the above-mentioned study were mainly from environmental water samples. It is still unclear whether *A. salmonicida* is a facultative or an obligate fish pathogen (Austin and Austin, 2016) ; (iii) There may be a selection bias in our data as the isolates came from samples

taken by field veterinarians, probably mostly when outbreaks had not been controlled by the first-line treatment. Thus, the proportion of NWT isolates may be over-represented.

Overall, genetic testing of isolates to screen for presence of resistance genes may be necessary, as a complementary approach to the usual MIC determination, to get robust epidemiological data and cut-offs for fish pathogens, useful to determine optimal enrofloxacin (and other antimicrobials) dosages.

4.2. Enrofloxacin PK parameters

The statistical approach using the power model showed that the assumption of dose-linearity could be reasonably accepted for the tested oral dose range. There are some limits of this analysis as we could only use partial AUCs over 96h (Supplementary Figure 5) instead of the AUC_{0_inf} because the extrapolated terminal part of AUC was higher than the usual threshold of 20%. We yet assumed pharmacokinetic linearity over this dose range.

The IV experiment of this study allowed us to estimate values of the true parameters (volumes, clearance) as opposed to studies using solely oral administrations, which only allow the calculation of apparent parameters. To our knowledge, there is only one other study presenting enrofloxacin PK data after an IV administration in rainbow trout (Bowser *et al.*, 1992) but without longitudinal individual data: they estimated a central volume of 93 – 141 mL (average values for a typical individual weighting 65 g). Results from our PK model were consistent: considering a typical individual of 65 g (diploid), the central volumes is equal to 137 mL.

A huge difference, however, is observed concerning the $t_{1/2,}$ which was ~ 4-5 times higher in our study compared to Bowser's study (still considering diploid). Several reasons could explain this discrepancy: (i) the sampling period was limited to 60 h post-IV injection in the study of Bowser *et al.* (1992) compared to 120 h here after IV injection, which probably concealed the terminal elimination phase and thus had an impact on the $t_{1/2}$ calculation (Toutain and Bousquet-Melou, 2004); (ii) their analytical method (microbiological assay) and their method of calculation of the $t_{1/2}$ (hybrid parameter) could also have a huge impact on its value (Toutain and Bousquet-Melou, 2004). With our sensitive analytical method and the population PK modelling approach, we are confident about the terminal $t_{1/2}$ calculation in this study.

Other studies with rainbow trout have also found a long $t_{1/2}$ after oral administration of enrofloxacin (*e.g.* 78 h; Kyuchukova *et al.* 2015), albeit at a higher water temperature. In a study with another salmonid (Atlantic salmon), carried out under experimental conditions

similar to ours (IV injection, similar fish weights and water temperature), the authors also found a long $t_{1/2}$ of 130 h (Stoffregen *et al.*, 1997). Overall, a meta-analysis of all published data using the NLME approach (Li *et al.*, 2015) would be necessary to better characterize the effect of important covariates as temperature.

The oral administration also provided interesting enrofloxacin PK information. First, the oral bioavailability of enrofloxacin was found to be high (~88%), in good agreement with other results issued from brown trout (~78%, at 10°C after gavage; Koc *et al.*, 2009) but higher than older results with rainbow trout (42-48% at 15°C after gavage, 24-35% at 10°C after gavage; Bowser *et al.*, 1992) and Atlantic salmon (~46-49% at 10 °C after gavage; Stoffregen *et al.*, 1997). An atypical absorption profile was observed in our data (Supplementary Figure S2) which was modelled by two successive absorption phases because this gave the best fit (Figure 3). However, the clinical relevance is probably weak as the early fast absorption phase (with ka1) only applied to a negligible fraction of the administered dose (< 5%). Anaesthesia used for gavage as well as for repeated blood sampling may have affected the gastro intestinal transit and thus may have affected the main absorption phase (Davies *et al.*, 2010). The existence of an enterohepatic recycling of enrofloxacin (Trouchon and Lefebvre, 2016) may also explain these absorption profiles although this has not been proven in fish. However, other atypical PK profiles with multiple plasma peaks have already been observed for orally administered enrofloxacin in rainbow trout (Kyuchukova *et al.*, 2015), Atlantic salmon (Stoffregen *et al.*, 1997) or red pacu (Lewbart *et al.*, 1997), without a clear explanation.

4.3. **Population PK modeling revealed the importance of ploidy**

The strength of the population PK using a NLME approach is the ability to analyse all data (i.e. all individuals and all dosing regimens) simultaneously and hence be able to discriminate the population parameters, the inter-individual variabilities and the residual errors (Bon *et al.*, 2018). In this study, all parameter values were estimated with very good confidence when looking at the rather low RSE (Table 4). Furthermore, the pcVPC showed a proper fitting of the data and thus validated the model (Figure 4).

The NMLE modelling approach helps to identify relevant covariates explaining some of the inter-individual variability (Bon *et al.*, 2018). To our knowledge, this is the first time that ploidy appears as a relevant covariate for several fundamental PK parameters such as clearance, and those related to oral absorption. Triploid individuals absorb and eliminate enrofloxacin more

slowly than their diploid congeners, and the molecule will therefore persist longer in plasma for triploids. This is underlined by the significant difference in terminal t1/2 between these 2 sub-populations (115 h vs. 166 h). While the influence of ploidy on clearance has an overall negligible impact on the calculation of enrofloxacin dose (see section 4.4), it may have consequence about the level of residues and the required withdrawal period after treatment that should be investigated. The reason for such difference between diploid and triploid is unknown and should deserve further investigations.

4.4. PKPD exploration and dose determination

Looking at the weighted PTA (Table 5), the "standard" dose of 10 mg/kg/day seems to provide an overall sufficient exposure for the 3 bacteria species, at least for the lowest PKPD indexes. There are 2 limitations for this finding: (i) the MIC distributions used for the calculation may not reflect the actual MIC distribution of each bacteria, especially for *Y. ruckeri* and A. *salmonicida* as all isolates originated from France; (ii) It does not take into account the delay to reach the adequate level of exposure (Table 6): for the CO_{NRI} for *F. psychrophilum,* only 24h would be needed. However, for a MIC of 0.25 µg/mL, this is rather long and varies from 48h to 144h, depending on the PKPD index value. These findings are confirmed by earlier studies using enrofloxacin at the "standard" dose of 10 mg/kg/day: enrofloxacin only had a significant impact on trout mortality after several days of treatment in the study of Hsu *et al.* (1995*)*, when the fish were naturally infected with *A. salmonicida*. Similar results were recently obtained for the treatment of trout infected with *F. psychrophilum,* where the mortality rate did not differ from that of the control group before 3 days of treatment (Boyacioğlu *et al.*, 2015).

While the "standard" dosage regimen will not necessary lead to a therapeutic failure, this is an issue concerning the risk of antimicrobial resistance. Indeed, changes in pathogen susceptibility can occur during the period of suboptimal drug exposure that lasts before a steady-state is reached (Martinez *et al.*, 2012). More, a long duration of treatment, *e.g*. over 10 days, as it is currently recommended (Bowser *et al.*, 1992; Giguère *et al.*, 2013) is also a factor of antimicrobial resistance selection (Martinez *et al.*, 2012) that could eventually increase the risk of treatment failure. In contrast, achieving sufficient antibiotic exposure of fish as soon as possible increases the chances of clinical recovery at the individual fish level while minimizing the risk of resistance selection.

In line with this previous statement, we illustrated the usefulness of the PKPD approach to optimize the enrofloxacin doses for *F. psychrophilum*, the only bacteria with a calculable CO_{NRI}. We considered enrofloxacin to be a long-acting drug in rainbow trout because of its long $t_{1/2}$ and chose to perform the analysis with a claimed duration of effect of 96 or 120 hours. Predicted single doses were well below the "standard" dose, even for a conservative SF of 5 (Table 7): for example, a single dose of 6.5 mg/kg would give a PTA ≥ 90% for diploid trout over 5 days, which could possibly be followed by a maintenance dose of 3.8 mg/kg if another 5-day exposure is required. Compared to the "standard" dose administered over 10 days of treatment (*e.g.* a total of 100 mg/kg), this represents a ~90% decrease in total antibiotic amounts while ensuring sufficient exposure to enrofloxacin over 10 days. However, this rationale applies only for the WT strains and our data showed that most of them were NWT (Fig 1). To decide whether or not to use enrofloxacin treatment therefore requires good situational knowledge or to measure the MIC of the infecting strain. The latter may be a limiting factor as *F. psychrophilum* needs about 72 h of culture growth for MIC testing.

Unfortunately, for *Y. ruckeri* and *A. salmonicida*, we could not find relevant thresholds (neither in this study nor in the literature) for calculating an optimal single dose of enrofloxacin. When an epidemiological cut-off will be available, the calculated doses from Table 8 could be used. For MIC ≥ 0.25 µg/mL, however, high oral doses would be needed with possible drawbacks: (i) the potential non-linearity of PK absorption processes, *e.g.* with a saturable absorption at these doses; (ii) the toxicity at this dose range is not well known for fish; (iii) the palatability of food is probably a limiting factor (Hsu *et al.*, 1994; Toften and Jobling, 1997), as already observed for sick animals (Rostang *et al.*, 2021). Therefore, based on the MIC distribution of *A. salmonicida* of our study with ~70% of isolates having a MIC ≥ 0.25 µg/mL (figure 1), enrofloxacin does not seem to be an antibiotic of choice for the treatment against this bacterium.

4.5. **Limits of the study**

The oral gavage represents an unrealistic feeding method for fish in rearing conditions. The natural feeding behaviour of trout is known to be "fast" (*i.e.* within few minutes), which means that a weakened or sick animal may not be able to eat its portion in the highly competitive environment of trout farms. Thus, additional dose-related inter-individual variability in intake is likely to be present under farming conditions due to the social rank and behaviour of the trout

(Ellis *et al.*, 2002), as already quantified in pigs (Soraci *et al.*, 2014). This may play a substantial role in the overall PK variability and therefore on the set up of the PKPD cut-offs.

Moreover, our study was conducted in healthy fish and results may be different for sick animals: (i) the pharmacokinetics of enrofloxacin may be different in diseased fish, as observed for crucian carp infected with *Aeromonas hydrophila*, with a lower systemic exposure (Fan *et al.*, 2017); (ii) fish showing clinical signs are often anorexic and are therefore less or not at all exposed to the antimicrobial when given through medicated feeds (Giguère *et al.*, 2013). However, in the context of metaphylaxis, which is the most frequent use of antimicrobial in fish production (Lulijwa *et al.*, 2020), the early initiation of an enrofloxacin treatment for the majority of fish would limit the influence of the infection on food intake and on the PK of enrofloxacin, while treating a rather low initial bacterial load. The use of other routes of administration, such as the intramuscular or the intraperitoneal route (as in vaccination campaigns) could be an alternative to the oral route, allowing better inter-individual reproducibility, and ensuring a sufficient dose to each diseased fish (Rostang *et al.*, 2021).

In a crossover experiment, it is common to let a wash-out of at least 4 times the $t_{1/2}$ between two administrations to ensure that (virtually) all drug has been eliminated (Gehring and Martinez, 2012). With the long $t_{1/2}$ of enrofloxacin, this wash-out would have lasted minimum 20 days, which is a limiting factor for such experiments. Thus we chose to use a particular design, known as the semi-simultaneous method, which remains relevant if the second administration is given during the post-distributive phase of the first administration (Karlsson and Bredberg, 1990). When combined to an NMLE approach and thanks to the numerous individual data, this experimental design has already proven its usefulness with other drugs (Karlsson and Bredberg, 1990; Lallemand *et al.*, 2007).

The PK experiments were only conducted with trout reared in a water at ~11°C. An effect of water temperature on the PK of enrofloxacin has been previously observed with increased bioavailability and absorption constants at 15°C compared to 10°C (Bowser *et al.*, 1992) but no significant effect on elimination processes was observed. As bioavailability and clearance are the main PK parameters influencing the dose calculation (see Eq. 4-6) and given the high oral bioavailability estimated in our study, it is likely that our calculated doses can be extrapolated to trout reared in warmer waters. These findings, however, have to be confirmed experimentally. In Turbot, an increase in water temperature lead to a decrease of the plasma $t_{1/2}$

of enrofloxacin (Liang *et al.*, 2012). Similar results have been observed with other antibiotic drugs and other fish species (Rairat *et al.*, 2019; Xu *et al.*, 2019).

Concerning the PKPD index, both Cmax/MIC and fAUC/MIC have been proposed as efficacy surrogates for fluoroquinolones (Wright *et al.*, 2000). We chose to perform the simulation with fAUC/MIC because it tends to be the most relevant index for antimicrobial with long terminal half-life (Nielsen and Friberg, 2013). One limitation concerns the use of frozen plasma to determine the unbound fraction of enrofloxacin as it was shown that freezing may impact serum protein binding (Banker & Clark, 2008). We chose a wide range of target value of fAUC/MIC, from 50 to 125 h (*i.e.* SF from 2 to 5), as an illustrating purpose of our PKPD analysis and to avoid being too conservative. Indeed, depending on the bacteria specie, great differences could be observed, especially as fish are poikilothermic animals. In addition, higher enrofloxacin MICs values were observed at 4°C compared to 15°C for *A. salmonicida* (Martinsen *et al.*, 1992). Further *in vitro* studies involving time-kill curves with these fish bacteria under specific condition are needed to better define the target values of the PKPD indexes for different level of efficacy (bacteriostase, bactericidal, virtual eradication) in trout,as already published for other bacteria and food-animal species (Dorey *et al.*, 2017; Paulin *et al.*, 2018; Pelligand *et al.*, 2019; Toutain *et al.*, 2019).

Another limit of our PKPD analysis concerns the optimal duration of the antimicrobial therapy which cannot be derived from this modelling approach (EMA, 2018). However, considering the use of a single oral dose covering 4 or 5 days of sufficient exposure, it is anticipated that a shorter duration would be necessary compared to the current 5 to 10 days used with the "standard" dose. If necessary (for a metaphylactic approach), maintenance doses can then be administered.

Finally, it should be stressed that alternatives to antibiotic therapy are possible. A vaccine against *Y. ruckeri* is available in some countries (Kumar *et al.*, 2015) as well as for *A. salmonicida* and the superiority of this practice over the use of successive antimicrobial treatments has been described (Du *et al.*, 2019). Overall, this preventive approach should be used preferably whenever possible, especially within the context of prudent antimicrobial use.

5. CONCLUSION

Based on our results on the pharmacokinetics of enrofloxacin and the epidemiological data on the MICs of the three main rainbow trout pathogenic bacteria, we were able to review the enrofloxacin dosing regimen using a PKPD approach. From our point of view, the current oral

enrofloxacin dosing regimens for rainbow trout are not optimal in terms of exposure but also concerning the risk of antibiotic resistance. We were able to calculate a dosing regimen for *F. psychrophilum* based on a provisional epidemiological cut-off, but not for *Y. ruckeri* and *A. salmonicida.* Regarding the MIC distribution of *A. salmonicida* in this study, enrofloxacin treatment should not be recommended. Our study highlights that a better understanding of the PKPD (target values of the PKPD index), as well as the epidemiology of each pathogenic bacteria (MIC distributions) is essential to establish better dosing regimens in rainbow trout and overall fish production. Finally, for the first time, the influence of the ploidy on the PK of an antibiotic was observed in trout. Further studies are now necessary to better characterize this effect and to see the potential implication for residue concerns.

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Figures legends:

Figure 1: Enrofloxacin MIC distribution of *Aeromonas salmonicida, Flavobacterium. psychrophilum and Yersinia ruckeri* obtained from literature search and from this work. The vertical dashed line represents the MIC90 of each specie.

Figure 2: Individual plasma concentration of enrofloxacin for the 96 trout over the whole experiment (oral administration at T=0h then IV injection at T=96h). Red: group 1; Blue: group 2; Green: group 3; Yellow: group 4.
The details of each group and dose are given in Table 1

Figure 3: Structural model used to describe the PK of enrofloxacin after oral and IV administration. Parameters that were estimated are in italics. See table 4 for description of each parameter. IV: intravenous

Figure 4: predicted corrected visual predictive checks (pcVPC) of the enrofloxacin plasma profiles. Observed data are the blue dots. Straight blue lines represent the empirical percentiles whereas the black dashed line represent the theoretical percentiles. Blue and red area represent the confidence intervals (with a level of 90%) around the 10,90th and the 50th percentiles, respectively. The use of pcVPC helps to diagnose model misspecification but makes the y-axis scale less intuitive as it transforms the original scale of observations and predictions.

Figure 5: PTA for the "standard" dose depending on the MIC value, the PKPD index target value and stratified by the ploidy status.
The horizontal dashed line represents the PTA 90%.

REFERENCES

Antaya, C.L. (2008). Current eco-economical impacts of *Flavobacterium psychrophilum*. *MMG 445 Basic Biotechnology eJournal* 4(1)**,** 16-21.

Austin, B., and Austin, D.A. (2016). "Aeromonadaceae representative (*Aeromonas salmonicida*)," in *Bacterial fish pathogens*. Springer), 215-321.

Baron, S., Granier, S.A., Larvor, E., Jouy, E., Cineux, M., Wilhelm, A., et al. (2017). Aeromonas diversity and antimicrobial susceptibility in freshwater—an attempt to set generic epidemiological cut-off values. *Frontiers in Microbiology* 8**,** 503.

Bergstrand, M., Hooker, A.C., Wallin, J.E., and Karlsson, M.O. (2011). Prediction-corrected visual predictive checks for diagnosing nonlinear mixed-effects models. *AAPS J* 13(2)**,** 143-151. doi: 10.1208/s12248-011-9255-z.

Bon, C., Toutain, P., Concordet, D., Gehring, R., Martin-Jimenez, T., Smith, J., et al. (2018). Mathematical modeling and simulation in animal health. Part III: Using nonlinear mixed-effects to characterize and quantify variability in drug pharmacokinetics. *Journal of Veterinary Pharmacology and Therapeutics* 41(2)**,** 171-183.

Bowser, P.R., Schachte Jr, J., Wooster, G., and Babish, J. (1990). Experimental treatment of Aeromonas salmonicida infections with enrofloxacin and oxolinic acid: field trails. *Journal of Aquatic Animal Health* 2(3)**,** 198-203.

Bowser, P.R., Wooster, G.A., St Leger, J., and Babish, J.G. (1992). Pharmacokinetics of enrofloxacin in fingerling rainbow trout (*Oncorhynchus mykiss*). *Journal of Veterinary Pharmacology and Therapeutics* 15(1)**,** 62-71. doi: 10.1111/j.1365-2885.1992.tb00987.x.

Boyacioğlu, M., Kum, C., KIRKAN, Ş., Sekkin, S., PARIN, U., KARADEMİR, Ü., et al. (2015). Comparison of in vitro and in vivo antibacterial efficacy for the control of *Flavobacterium psychrophilum* in rainbow trout (O*ncorhynchus mykiss*) fry: the first genotypical evidence in West Aegean region of Turkey. *Turkish Journal of Veterinary and Animal Sciences* 39(3)**,** 314-321.

Brown, S.A. (1996). Fluoroquinolones in animal health. *Journal of Veterinary Pharmacology and Therapeutics* 19(1)**,** 1-14. doi: 10.1111/j.1365-2885.1996.tb00001.x.

Byers, H.K., Gudkovs, N., and Crane, M.S.J. (2002). PCR-based assays for the fish pathogen *Aeromonas salmonicida.* I. Evaluation of three PCR primer sets for detection and identification. *Diseases of Aquatic Organisms* 49(2)**,** 129-138.

Calvez, S., Gantelet, H., Blanc, G., Douet, D.G., and Daniel, P. (2014). Yersinia ruckeri Biotypes 1 and 2 in France: presence and antibiotic susceptibility. *Diseases of Aquatic Organisms* 109(2)**,** 117-126. doi: 10.3354/dao02725.

CLSI (2014). "VET04-A2: Methods for Broth Dilution Susceptibility Testing of Bacteria Isolated from Aquatic Animals; Approved Guideline, 2nd Edn". Wayne,PA: Clinical and Laboratory Standards Institute.).

Davies, N.M., Takemoto, J.K., Brocks, D.R., and Yanez, J.A. (2010). Multiple peaking phenomena in pharmacokinetic disposition. *Clinical Pharmacokinetics* 49(6)**,** 351-377. doi: 10.2165/11319320-000000000-00000.

Della Rocca, G., Di Salvo, A., Malvisi, J., and 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.

Dorey, L., Pelligand, L., and Lees, P. (2017). Prediction of marbofloxacin dosage for the pig pneumonia pathogens *Actinobacillus pleuropneumonia*e and *Pasteurella multocida* by pharmacokinetic/pharmacodynamic modelling. *BMC Veterinary Research* 13(1)**,** 209. doi: 10.1186/s12917-017-1128-y.

Drusano, G., Preston, S., Hardalo, C., Hare, R., Banfield, C., Andes, D., et al. (2001). Use of preclinical data for selection of a phase II/III dose for evernimicin and identification of a preclinical MIC breakpoint. *Antimicrobial agents and chemotherapy* 45(1)**,** 13-22.

Du, X., Bayliss, S.C., Feil, E.J., Liu, Y., Wang, C., Zhang, G., et al. (2019). Real time monitoring of *Aeromonas salmonicida* evolution in response to successive antibiotic therapies in a

commercial fish farm. *Environmental Microbiology* 21(3), 1113-1123. doi: 10.1111/1462-2920.14531.
Ellis, T., North, B., Scott, A., Bromage, N., Porter, M., and Gadd, D. (2002). The relationships between stocking density and welfare in farmed rainbow trout. *Journal of Fish Biology* 61(3), 493-531.
EMA (2018). "Reflection paper on dose optimisation of established veterinary antibiotics in the context of SPC harmonisation", in: *EMA/CVMP/849775/2017.*).
EMA (2019). "Answer to the request from the European Commission for updating the scientific advice on the impact on public health and animal health of the use of antibiotics in animals - Categorisation of antimicrobials ", in: *EMA/CVMP/CHMP/682198/2017.*).
Fan, J., Shan, Q., Wang, J., Liu, S., Li, L., and Zheng, G. (2017). Comparative pharmacokinetics of enrofloxacin in healthy and *Aeromonas hydrophila*-infected crucian carp (*Carassius auratus gibelio)*. *Journal of Veterinary Pharmacology and Therapeutics* 40(5), 580-582. doi: 10.1111/jvp.12392.
Fang, X., Liu, X., Liu, W., and Lu, C. (2012). Pharmacokinetics of enrofloxacin in allogynogenetic silver crucian carp, Carassius auratus gibelio. *J Vet Pharmacol Ther* 35(4), 397-401. doi: 10.1111/j.1365-2885.2011.01337.x.
FAO (2020). *The State of World Fisheries and Aquaculture, 2010.* Food & Agriculture Organisation.
Furones, M., Rodgers, C., and Munn, C. (1993). *Yersinia ruckeri*, the causal agent of enteric redmouth disease (ERM) in fish. *Annual Review of Fish Diseases* 3, 105-125.
Gehring, R., and Martinez, M. (2012). Assessing product bioequivalence for extended-release formulations and drugs with long half-lives. *Journal of Veterinary Pharmacology and Therapeutics* 35 Suppl 1, 3-9. doi: 10.1111/j.1365-2885.2012.01372.x.
Giguère, S., Prescott, J.F., and Dowling, P.M. (2013). *Antimicrobial Therapy in Veterinary Medicine.* John Wiley & Sons.
Gough, K., Hutchison, M., Keene, O., Byrom, B., Ellis, S., Lacey, L., et al. (1995). Assessment of dose proportionality: report from the statisticians in the pharmaceutical industry/pharmacokinetics UK joint working party. *Drug Information Journal* 29(3), 1039-1048.
Hsu, H.-M., Wooster, G.A., and Bowser, P.R. (1994). Efficacy of enrofloxacin for the treatment of salmonids with bacterial kidney disease, caused by R*enibacterium salmoninarum*. *Journal of Aquatic Animal Health* 6(3), 220-223.
Hsu, H.M., Bowser, P., Schachte Jr, J., Scarlett, J., and Babish, J. (1995). Winter field trials of enrofloxacin for the control of *Aeromonas salmonicid*a infection in salmonids. *Journal of the World Aquaculture Society* 26(3), 307-314.
Intorre, L., Cecchini, S., Bertini, S., Varriale, A.C., Soldani, G., and Mengozzi, G. (2000). Pharmacokinetics of enrofloxacin in the seabass (*Dicentrarchus labrax*). *Aquaculture* 182(1-2), 49-59.
Karlsson, M.O., and Bredberg, U. (1990). Bioavailability estimation by semisimultaneous drug administration: a Monte Carlo simulation study. *Journal of Pharmacokinetics and Biopharmaceutics* 18(2), 103-120. doi: 10.1007/BF01063554.
Koc, F., Uney, K., Atamanalp, M., Tumer, I., and Kaban, G. (2009). Pharmacokinetic disposition of enrofloxacin in brown trout (*Salmo trutta fario*) after oral and intravenous administrations. *Aquaculture* 295(1-2), 142-144.
Kronvall, G. (2010). Normalized resistance interpretation as a tool for establishing epidemiological MIC susceptibility breakpoints. *Journal of Clinical Microbiology* 48(12), 4445-4452.
Kumar, G., Menanteau-Ledouble, S., Saleh, M., and El-Matbouli, M. (2015). Yersinia ruckeri, the causative agent of enteric redmouth disease in fish. *Veterinary Research* 46(1), 103. doi: 10.1186/s13567-015-0238-4.
Kyuchukova, R., Milanova, A., Pavlov, A., and Lashev, L. (2015). Comparison of plasma and tissue disposition of enrofloxacin in rainbow trout (*Oncorhynchus mykiss)* and common carp (*Cyprinus carpio*) after a single oral administration. *Food Additives & Contaminants: Part A Chem Anal Control Expo Risk Assess* 32(1), 35-39. doi: 10.1080/19440049.2014.983998.
Lallemand, E., Lespine, A., Alvinerie, M., BOUSQUET-MELOU, A., and TOUTAIN, P.L. (2007). Estimation of absolute oral bioavailability of moxidectin in dogs using a semi-simultaneous method:

influence of lipid co-administration. *Journal of veterinary pharmacology and therapeutics* 30(5), 375-380.
Lavielle, M. (2020). *mlxR: Simulation of Longitudinal Data. R package version 4.1 - https://CRAN.R-project.org/package=mlxR* [Online]. [Accessed].
Lewbart, G., Vaden, S., Deen, J., Manaugh, C., Whitt, D., Doi, A., et al. (1997). Pharmacokinetics of enrofloxacin in the red pacu (*Colossoma brachypomum*) after intramuscular, oral and bath administration. *Journal of Veterinary Pharmacology and Therapeutics* 20(2), 124-128. doi: 10.1046/j.1365-2885.1997.00814.x.
Li, M., Gehring, R., Lin, Z., and Riviere, J. (2015). A framework for meta-analysis of veterinary drug pharmacokinetic data using mixed effect modeling. *Journal of Pharmaceutical Sciences* 104(4), 1230-1239.
Liang, J., Li, J., Zhao, F., Liu, P., and Chang, Z. (2012). Pharmacokinetics and tissue behavior of enrofloxacin and its metabolite ciprofloxacin in turbot *Scophthalmus maximus* at two water temperatures. *Chinese Journal of Oceanology and Limnology* 30(4), 644-653.
Lucchetti, D., Fabrizi, L., Guandalini, E., Podesta, E., Marvasi, L., Zaghini, A., et al. (2004). Long depletion time of enrofloxacin in rainbow trout (*Oncorhynchus mykiss*). *Antimicrobial Agents and Chemotherapy* 48(10), 3912-3917. doi: 10.1128/AAC.48.10.3912-3917.2004.
Lulijwa, R., Rupia, E.J., and Alfaro, A.C. (2020). Antibiotic use in aquaculture, policies and regulation, health and environmental risks: a review of the top 15 major producers. *Reviews in Aquaculture* 12(2), 640-663.
Martinez, M.N., Papich, M.G., and Drusano, G.L. (2012). Dosing regimen matters: the importance of early intervention and rapid attainment of the pharmacokinetic/pharmacodynamic target. *Antimicrobial Agents and Chemotherapy* 56(6), 2795-2805. doi: 10.1128/AAC.05360-11.
Martinsen, B., Oppegaard, H., Wichstrom, R., and Myhr, E. (1992). Temperature-dependent in vitro antimicrobial activity of four 4-quinolones and oxytetracycline against bacteria pathogenic to fish. *Antimicrobial Agents and Chemotherapy* 36(8), 1738-1743. doi: 10.1128/aac.36.8.1738.
Mould, D., and Upton, R.N. (2013). Basic concepts in population modeling, simulation, and model-based drug development—part 2: introduction to pharmacokinetic modeling methods. *CPT: pharmacometrics & systems pharmacology* 2(4), 1-14.
Ngo, T.P.H., Smith, P., Bartie, K.L., Thompson, K.D., Verner-Jeffreys, D.W., Hoare, R., et al. (2018). Antimicrobial susceptibility of *Flavobacterium psychrophilum* isolates from the United Kingdom. *Journal of Fish Diseases* 41(2), 309-320. doi: 10.1111/jfd.12730.
Nielsen, E.I., and Friberg, L.E. (2013). Pharmacokinetic-pharmacodynamic modeling of antibacterial drugs. *Pharmacological Reviews* 65(3), 1053-1090. doi: 10.1124/pr.111.005769.
Paulin, A., Schneider, M., Dron, F., and Woehrle, F. (2018). Pharmacokinetic/pharmacodynamic evaluation of marbofloxacin as a single injection for Pasteurellaceae respiratory infections in cattle using population pharmacokinetics and Monte Carlo simulations. *Journal of Veterinary Pharmacology and Therapeutics* 41(1), 39-50.
Pelligand, L., Lees, P., Sidhu, P.K., and Toutain, P.-L. (2019). Semi-mechanistic modelling of florfenicol time-kill curves and in silico dose fractionation for calf respiratory pathogens. *Frontiers in microbiology* 10, 1237.
Pérez-Sancho, M., Cerdá, I., Fernández-Bravo, A., Domínguez, L., Figueras, M., Fernández-Garayzábal, J., et al. (2018). Limited performance of MALDI-TOF for identification of fish *Aeromonas* isolates at species level. *Journal of Fish Diseases* 41(10), 1485-1493.
Piferrer, F., Beaumont, A., Falguière, J.-C., Flajšhans, M., Haffray, P., and Colombo, L. (2009). Polyploid fish and shellfish: production, biology and applications to aquaculture for performance improvement and genetic containment. *Aquaculture* 293(3-4), 125-156.
R Core Team (2014). "R: A language and environment for statistical computing". (Vienna, Austria: R Foundation for Statistical Computing).
Rairat, T., Hsieh, C.-Y., Thongpiam, W., Sung, C.-H., and Chou, C.-C. (2019). Temperature-dependent pharmacokinetics of florfenicol in Nile tilapia (*Oreochromis niloticus*) following single oral and intravenous administration. *Aquaculture* 503, 483-488.

Reimschuessel, R., Miller, R., and Gieseker, C.M. (2013). "Antimicrobial drug use in aquaculture," in *Antimicrobial Therapy in Veterinary Medicine, 5th edition*. John Wiley & Sons), 645-661.

Rostang, A., Peroz, C., Fournel, C., Thorin, C., and Calvez, S. (2021). Evaluation of the efficacy of enrofloxacin in rainbow trout (*Oncorhynchus mykiss*) following experimental challenge with *Yersinia ruckeri*. *Veterinary Record*, e200.

Saticioglu, I.B., Duman, M., Smith, P., Wiklund, T., and Altun, S. (2019). Antimicrobial resistance and resistance genes in *Flavobacterium psychrophilum* isolates from Turkey. *Aquaculture* 512, 734293.

Silber, H.E., Kjellsson, M.C., and Karlsson, M.O. (2009). The impact of misspecification of residual error or correlation structure on the type I error rate for covariate inclusion. *Journal of Pharmacokinetics and Pharmacodynamics* 36(1), 81-99. doi: 10.1007/s10928-009-9112-1.

Smith, B.P., Vandenhende, F.R., DeSante, K.A., Farid, N.A., Welch, P.A., Callaghan, J.T., et al. (2000). Confidence interval criteria for assessment of dose proportionality. *Pharmaceutical Research* 17(10), 1278-1283.

Smith, P., Endris, R., Kronvall, G., Thomas, V., Verner-Jeffreys, D., Wilhelm, C., et al. (2016). Epidemiological cut-off values for *Flavobacterium psychrophilum* MIC data generated by a standard test protocol. *Journal of Fish Diseases* 39(2), 143-154.

Soraci, A.L., Amanto, F., Tapia, M.O., de la Torre, E., and Toutain, P.L. (2014). Exposure variability of fosfomycin administered to pigs in food or water: impact of social rank. *Research in Veterinary Science* 96(1), 153-159. doi: 10.1016/j.rvsc.2013.12.003.

Stoffregen, D.A., Wooster, G.A., Bustos, P.S., Bowser, P.R., and Babish, J.G. (1997). Multiple route and dose pharmacokinetics of enrofloxacin in juvenile Atlantic salmon. *Journal of Veterinary Pharmacology and Therapeutics* 20(2), 111-123. doi: 10.1046/j.1365-2885.1997.81531.x.

Toften, H., and Jobling, M. (1997). Feed intake and growth of Atlantic salmon, *Salmo salar L.*, fed diets supplemented with oxytetracycline and squid extract. *Aquaculture Nutrition* 3(3), 145-151.

Toutain, P.-L., Sidhu, P.K., Lees, P., Rassouli, A., and Pelligand, L. (2019). VetCAST method for determination of the pharmacokineticpharmacodynamic cut-off values of a long-acting formulation of florfenicol to support clinical breakpoints for florfenicol antimicrobial susceptibility testing in cattle. *Frontiers in Microbiology* 10, 1310.

Toutain, P.L., and Bousquet-Melou, A. (2004). Plasma terminal half-life. *Journal of Veterinary Pharmacology and Therapeutics* 27(6), 427-439. doi: 10.1111/j.1365-2885.2004.00600.x.

Toutain, P.L., Bousquet-Melou, A., Damborg, P., Ferran, A.A., Mevius, D., Pelligand, L., et al. (2017a). En route towards european clinical breakpoints for veterinary antimicrobial susceptibility testing: a position paper explaining the VetCAST approach. *Frontiers in Microbiology* 8, 2344. doi: 10.3389/fmicb.2017.02344.

Toutain, P.L., Bousquet-Melou, A., and Martinez, M. (2007). AUC/MIC: a PK/PD index for antibiotics with a time dimension or simply a dimensionless scoring factor? *Journal of Antimicrobial Chemotherapy* 60(6), 1185-1188. doi: 10.1093/jac/dkm360.

Toutain, P.L., Potter, T., Pelligand, L., Lacroix, M., Illambas, J., and Lees, P. (2017b). Standard PK/PD concepts can be applied to determine a dosage regimen for a macrolide: the case of tulathromycin in the calf. *Journal of Veterinary Pharmacology and Therapeutics* 40(1), 16-27.

Trouchon, T., and Lefebvre, S. (2016). A review of enrofloxacin for veterinary use. *Open Journal of Veterinary Medicine* 6(2), 40-58.

Van Vliet, D., Loch, T.P., Smith, P., and Faisal, M. (2017). Antimicrobial Susceptibilities of *Flavobacterium psychrophilum* Isolates from the Great Lakes Basin, Michigan. *Microbial Drug Resistance* 23(6), 791-798. doi: 10.1089/mdr.2016.0103.

Wright, D.H., Brown, G.H., Peterson, M.L., and Rotschafer, J.C. (2000). Application of fluoroquinolone pharmacodynamics. *Journal of Antimicrobial Chemotherapy* 46(5), 669-683. doi: 10.1093/jac/46.5.669.

Xu, L., Wang, H., Yang, X., and Lu, L. (2013). Integrated pharmacokinetics/pharmacodynamics parameters-based dosing guidelines of enrofloxacin in grass carp *Ctenopharyngodon idella* to

minimize selection of drug resistance. *BMC Veterinary Research* 9(1)**,** 126. doi: 10.1186/1746-6148-9-126.

Xu, N., Li, M., Fu, Y., Zhang, X., Dong, J., Liu, Y., et al. (2019). Effect of temperature on plasma and tissue kinetics of doxycycline in grass carp (*Ctenopharyngodon idella*) after oral administration. *Aquaculture* 511**,** 734204.

Figure 1

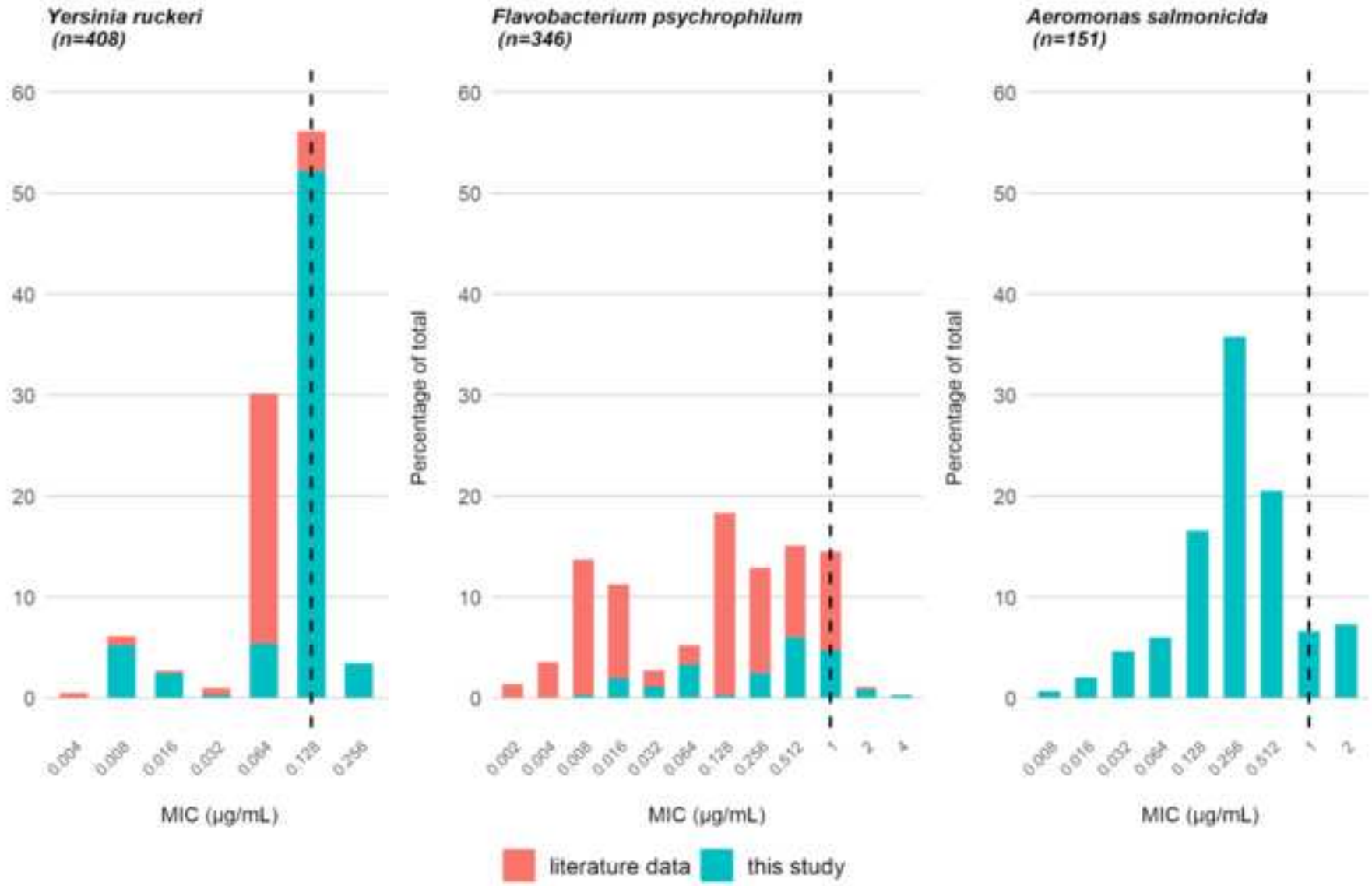

Yersinia ruckeri
(n=408)
Flavobacterium psychrophilum
(n=346)
Aeromonas salmonicida
(n=151)
Percentage of total
MIC (µg/mL)
literature data
this study

Figure 2

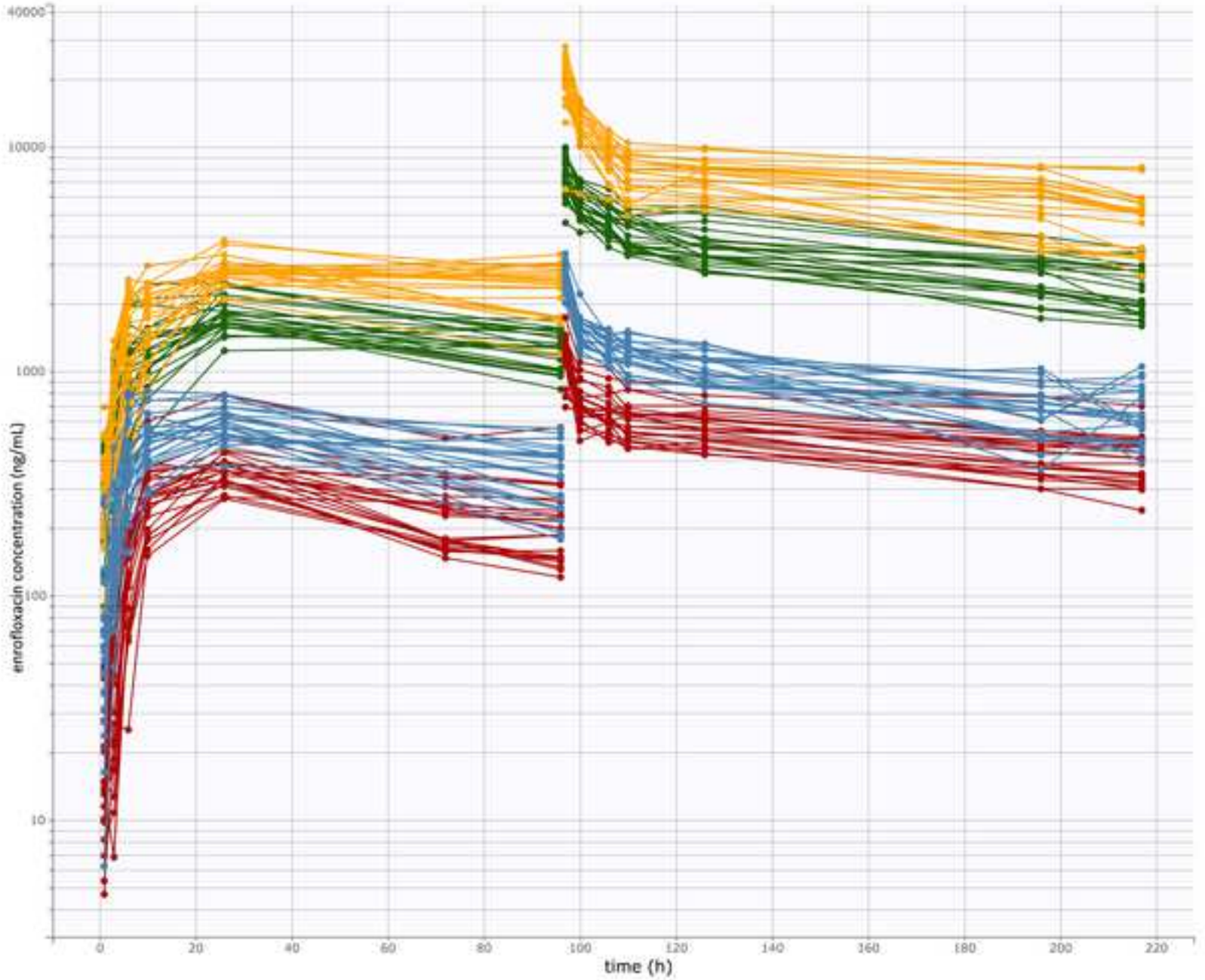

40000
10000
1000
100
10
enrofloxacin concentration (ng/mL)
0
20
40
60
80
100
120
140
160
180
200
220
time (h)

Figure 3

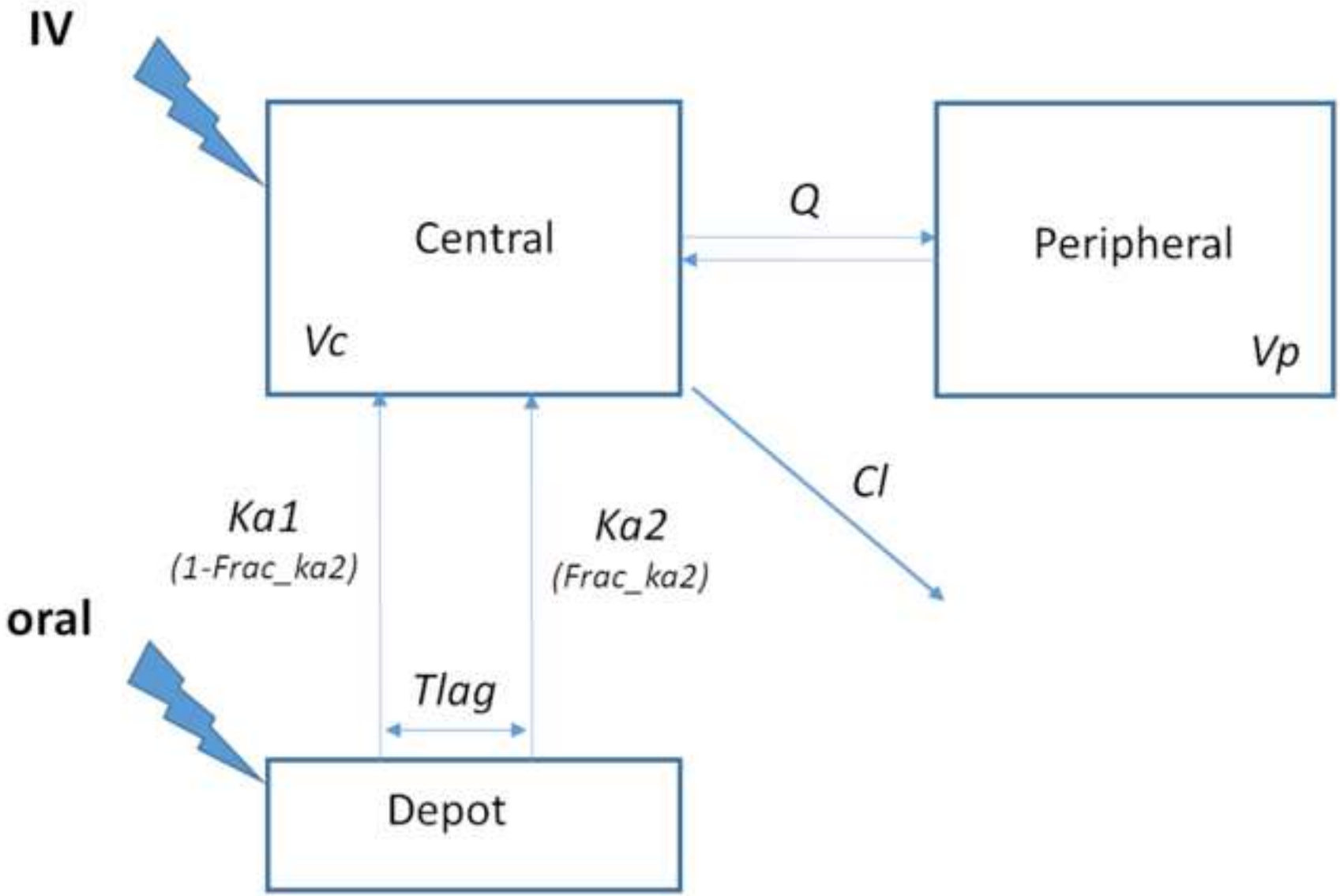

IV
Central
Vc
Q
Peripheral
Vp
Cl
Ka1
(1-Frac_ka2)
Ka2
(Frac_ka2)
oral
Tlag
Depot

Figure 4

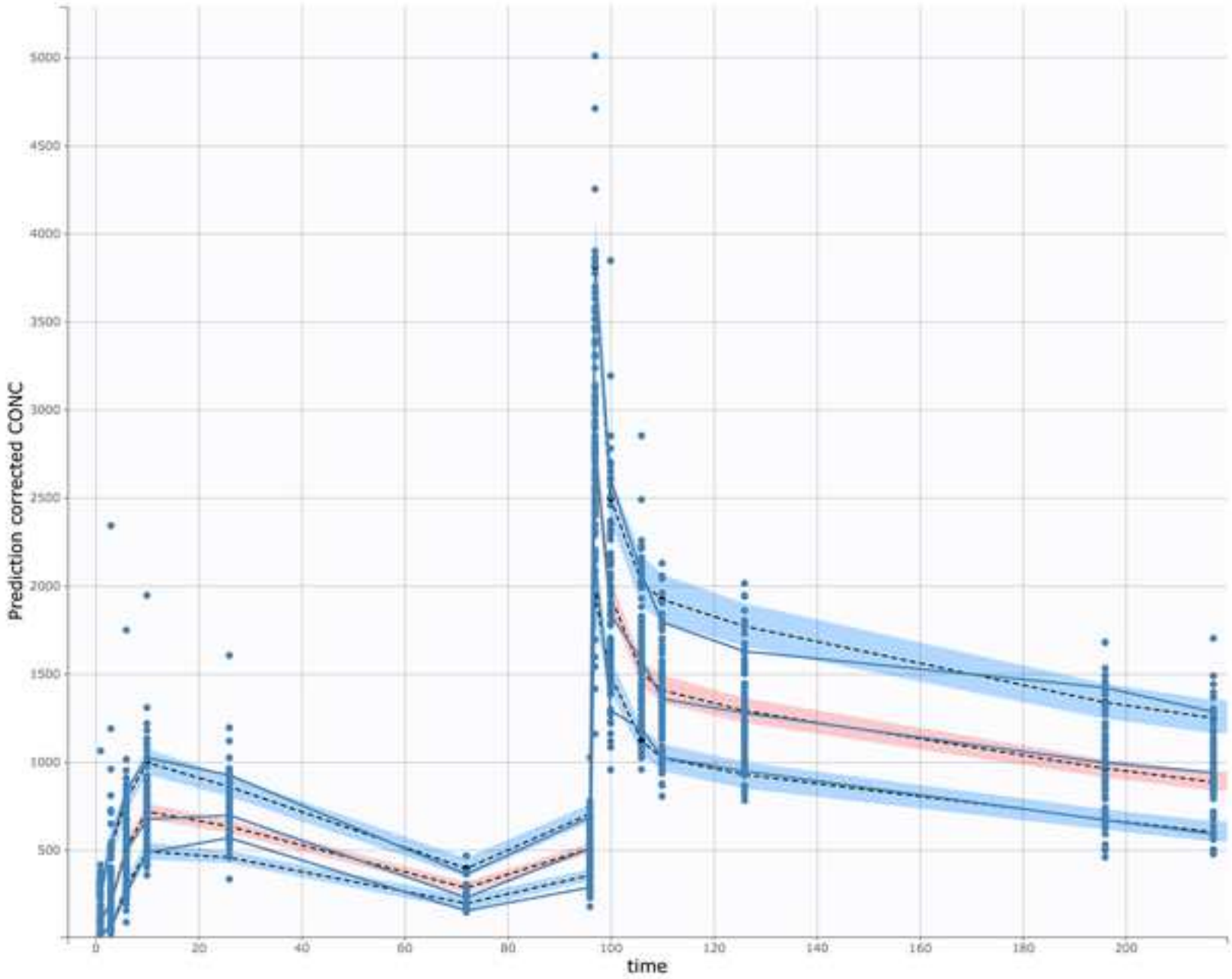
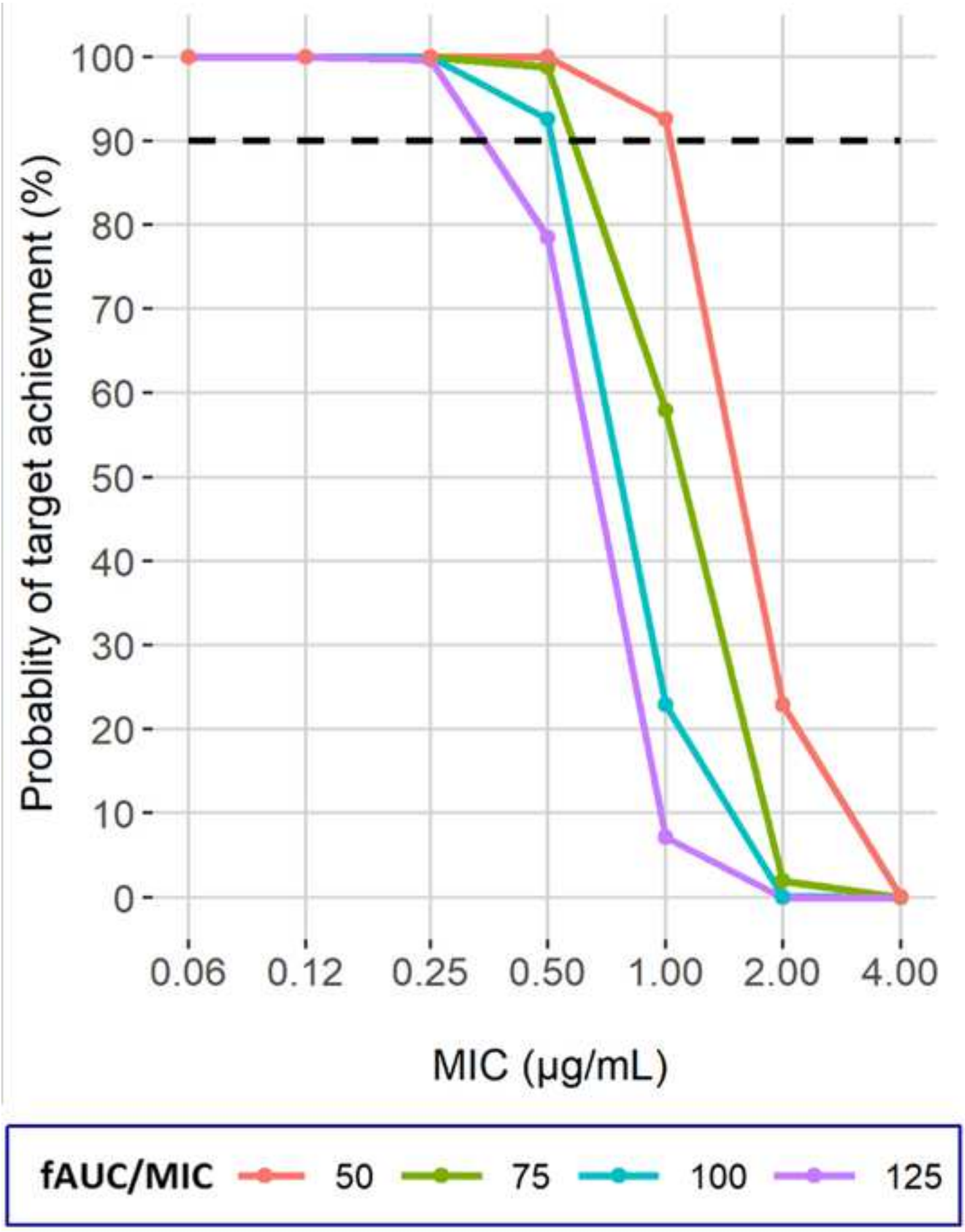

Prediction corrected CONC
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
20
40
60
80
100
120
140
160
180
200
time

Figure 5


Probablity of target achievment (%)
100
90
80
70
60
50
40
30
20
10
0
0.06
0.12
0.25
0.50
1.00
2.00
4.00
MIC (µg/mL)
fAUC/MIC
50
75
100
125

TABLES :

Table 1: Experimental design of the four groups of trout.

* the theoretical concentrations in feed were checked with HPLC analysis (see 3.4)

Groups	dosing regimens	Number of individuals and genetic profile
Group 1	Oral : 5* mg/kg IV : 5mg/kg	12 diploid and 12 triploid fish per group
Group 2	Oral :10* mg/kg IV : 10mg/kg	
Group 3	Oral : 20* mg/kg IV : 30mg/kg	
Group 4	Oral : 40* mg/kg IV : 60 mg/kg	

Table 2: Characteristics and source of the isolates used to get enrofloxacin MIC data, either from literature or from this study.

* the proportions are not known. RT : rainbow trout

Bacteria	Number of isolates	Range of tested enrofloxacin concentrations (µg/ml)	Fish species (proportion of isolates related to)	Reference
A. salmonicida	151	0.004 - 2	RT (> 90%) + others	This study
Y. ruckeri	280	0.004 - 2	RT (> 90%) + others	This study
	128		RT (> 95%) + others	(Calvez *et al.*, 2014)
F. psychrophilum	77	0.004 - 4	RT (> 90%)	This study
	61	0.002–1	RT (> 80%) + others	(Smith *et al.*, 2016)
	50		RT (36%) + other salmonids	(Van Vlie*, et al.*, 2017)
	133		RT (88%) + other salmonids	(Ngo *et al.*, 2018)
	25	0.008 - 256	RT (92%) + other salmonids	(Saticioglu *et al.*, 2019)

Table 3: Protein binding of enrofloxacin in plasma

NSB : non specific binding; fu : unbound fraction of enrofloxacin (between 0 and 1).

Initial enrofloxacin concentration (µg/ml)	**NSB% (mean± SD)**	fu **(mean± SD)**
0.1	6.7 ±0.22	0.47 ± 0.025
1	8.8 ±0.55	0.63 ±0.049
5	7.6 ±1.01	0.64 ±0.078
10	7.0 ±1.85	0.62 ±0.064

. Table 4: All parameters of the PK model. Parameters of the structural model have a log-normal distribution (bold font), except for the bioavailability (F_{oral}) and delayed fraction absorbed (Frac_ka2) which have a logit-normal distribution.

_ : Not concerned ; NA : not identifiable

*IIV value represents the standard deviation associated to the logit-normal distribution (the CV is not analytically calculable). For these parameters, the range [10th percentile – 90th percentile] is given.

Parameter	Symbol	Unit	Population estimate *(RSE %)*	IIV as CV % *(RSE %)*
Clearance	**Cl**	ml/h	21.5 *(4.5)*	28 *(10)*
Coefficient related to the effect of being triploid on Clearance	beta_Cl_Genetique_T	/	-0.34 *(19.8)*	–
Coefficient related to the effect of weight on Clearance	beta_Cl_tWT	/	0.70 *(28.4)*	–
Central Volume	**V1**	**ml**	**1400 *(3.7)***	**31 *(9)***
Coefficient related to the effect of weight on central volume	beta_V1_tWT	/	1.4 *(13)*	–
Peripheral volume	**V2**	**ml**	**2140 (4.3)**	**30 (12)**
Coefficient related to the effect of weight on peripheral volume	beta_V2_tWT	/	0.76 *(26.7)*	–
Inter-compartmental clearance	**Q**	**ml/h**	**259 (0.1)**	**–**
Bioavailibilty for the oral route	**F_{ORAL}***	**%**	**88.4 (2.3)**	**0.7* (12) [80-92]**
Absorption constant (early absorption)	**ka1**	**1/h**	**1.6 (0.02)**	**NA**
Fraction absorbed following ka2	**Frac_ka2***	**%**	**96 (0.4)**	**0.99* (10.5) [90-98]**
2d absorption constant (delayed absorption)	**ka2**	**1/h**	**0.102 (6.3)**	**37 (10)**
Coefficient related to the effect of being triploid on the 2d absorption constant	beta_ka2_Genetique_T	/	-0.55 *(14)*	–
Lag_time between the 2 absorption phases	**Tlag**	**h**	**1.9 (7.3)**	**42 (10)**
Coefficient related to the effect of being triploid on the 2d absorption constant	beta_Tlag_Genetique_T	/	0.42 *(23.3)*	–
Correlation between random effects				
Correlation bewteen V1 and Cl	corr1_V1_Cl	%	33.3 *(37.6)*	–
Correlation bewteen ka2 and Cl	corr1_ka2_Cl	%	33.4 *(37)*	–
Correlation bewteen ka2 and V1	corr1_ka2_V1	%	57.6 *(16.8)*	–
Correlation bewteen V2 and F	corr1_V2_F	%	66.2 *(13.4)*	–
Error Model Parameters				
Additive parameter for the error model	a	ng/ml	15.7 *(16.2)*	–
Proportional parameter for the error model	b	%	0.115 *(4.14)*	–

Table 5: weighted PTA with the "standard" dosing regimen (10 mg/kg/day for 10 days) and the distribution of MIC for each bacterial species. SF is equivalent to the PKPD index fAUC24h/CMI/24h (see 2.5.3).

Bacteria	Weighted PTA (%)			
	Value of SF (PKPD index)			
	2	**3**	**4**	**5**
F. psychrophilum	97.8	92.4	86.3	81.7
Y. ruckeri	100	100	100	99.9
A. salmonicida	93.9	89.8	86.1	82.0

Table 6: time to reach the target SF value for at least 90% of animals (i.e. achieving a PTA ≥ 90%) for all the possible MIC values with the "standard" dosing regimen (10 mg/kg/day for 10 days). SF is equivalent to the PKPD index fAUC24h/CMI/24h (see 2.5.3). Only diploid individuals were considered as "worst case scenario".

NA: not attainable

		Value of SF (PKPD index)			
		2	**3**	**4**	**5**
MIC (µg/ml)	≤0.03	24h	24h	24h	24h
	0.06	24h	24h	48h	48h
	0.12	48h	48h	48h	72h
	0.25	48h	72h	120h	144h
	0.5	120h	168h	>192h	*NA*
	1	>192h	*NA*	*NA*	*NA*
	2	>192h	*NA*	*NA*	*NA*
	≥4	*NA*	*NA*	*NA*	*NA*

Table 7: Predicted single dose and oral dose (mg/kg) to achieve a PTA > 90% for *Flavobacterium psychrophilum* according to the target value of the PKPD index (SF, scaling factor equals to fAUC/MIC/24h) and the duration of action (96 or 120h).

The calculations were carried-out with the CO_{NRI} derived for *F. psychrophilum*.

	Duration of activity							
	96h				**120h**			
	Value of SF (PKPD index)				**Value of SF (PKPD index)**			
	2	**3**	**4**	**5**	**2**	**3**	**4**	**5**
Single dose	2.5	3.7	4.9	6.2	2.6	3.9	5.2	6.5
Maintenance dose	1.2	1.8	2.4	3.0	1.5	2.3	3.0	3.8

Table 8 : Calculated single dose (mg/kg) to achieve a PTA ≥ 90% according to the target value of the PKPD index (SF, scaling factor equals to fAUC/MIC/24h), the duration of action (96 or 120h) and for all possible MIC values of any target bacteria.

Bold values corresponds to the CO_{NRI} of *Flavobacterium psychrophilum* (0.03 µg/ml)

MIC (µg/ml)	Duration of activity							
	96h				**120h**			
	Value of SF (PKPD index)				**Value of SF (PKPD index)**			
	2	**3**	**4**	**5**	**2**	**3**	**4**	**5**
0.004	0.3	0.5	0.6	0.8	0.3	0.5	0.7	0.8
0.008	0.6	0.9	1.2	1.5	0.7	1.0	1.3	1.6
0.015	1.2	1.8	2.5	3.1	1.3	2.0	2.6	3.3
0.03	**2.5**	**3.7**	**4.9**	**6.2**	**2.6**	**3.9**	**5.2**	**6.5**
0.06	4.9	7.4	9.8	12.3	5.2	7.8	10.5	13.1
0.12	9.8	14.8	19.7	24.6	10.5	15.7	20.9	26.2
0.25	19.7	29.5	39.4	49.2	20.9	31.4	41.9	52.3
0.5	39.4	59.1	78.7	98.4	41.9	62.8	83.7	104.6
1	78.7	118.1	157.5	196.9	83.7	125.6	167.4	209.3
2	157.5	236.2	315.0	393.7	167.4	251.1	334.8	418.5
4	315.0	472.5	630.0	787.5	334.8	502.2	669.7	837.1

Credit Author Statement

Alexis Viel: Data curation; Formal analysis, Software, Writing – original draft; Writing – review & editing; **Antoine Rostang**: Formal analysis ,Writing – original draft; Writing – review & editing; **Marie-Line Morvan**: Investigation; Resources; **Catherine Fourne**l: Investigation; Resources; **Patrick Daniel** : Investigation; Resources; **Chantal Thorin** **Sandrine Baron** : Investigation; Resources, Writing – review & editing ;**Pascal Sanders**: Formal analysis, Software, Writing – review & editing ; **Ségolène Calvez**: Conceptualization, Supervision ,Writing – review & editing

Supplementary Material (8 figures and 2 tables)