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

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19

20 **Abstract**

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

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

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

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

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

42 According to our model, a single oral dose of ~5 mg/kg should provide sufficient exposure to
43 treat the wild-type population of *F. psychrophilum* for 4 days, while complying with the PKPD
44 breakpoints. Then, a maintenance dose of ~2.5 mg/kg could possibly be re-administered every
45 4 days. The absence of a CO_{NRI} did not allow to predict an optimal dose for the two other
46 bacteria. As more than 70% of *A. salmonicida* isolates in our data set have an enrofloxacin MIC
47 ≥ 0.25 µg/mL, it seems that enrofloxacin should not be recommended against this bacterium.

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

50

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

53

54 **Abbreviations**

55 AUC : Area under the plasma concentration versus time curve

56 cBIC : corrected Bayesian Information Criteria

57 Cl : Total body clearance

58 C_{max} : Peak plasma drug concentration

59 CO_{NRI}: Provisional epidemiological cut-off calculated with the NRI method

60 ECOFF : Epidemiological cut-off
61 EMA : European Medicine agency
62 fu : unbound fraction
63 HPLC : High-performance liquid chromatography
64 IIV : inter-individual variability
65 IV : intra-venous
66 MCS : Monte Carlo simulation
67 MIC :Minimal inhibitory concentration
68 NLME : non-linear mixed effect
69 NRI : normalized resistance interpretation
70 pcVPC : Predicted-corrected Visual predictive checks
71 PK :Pharmacokinetic
72 PD :Pharmacodynamic
73 PTA : Probability of target achievement
74 RSE : relative standard error
75 SF : Scaling factor
76 $t_{1/2\beta}$: Plasma half-life
77 WT : wild-type

78

79

80

81 **1. INTRODUCTION**

82

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

92 fluoroquinolone widely used in veterinary practice around the world (Trouchon and Lefebvre,
93 2016). Of the 11 largest aquaculture producing countries, 6 use enrofloxacin (Lulijwa *et al.*,
94 2020) despite the great differences that exist between countries regarding the regulation of its
95 use. In Europe, the European Medicine agency (EMA) recently classified enrofloxacin in the
96 category B (“Restrict”) regarding the risk of antimicrobial resistance, but highlighted the very
97 few treatment alternatives against the three bacteria mentioned above (EMA, 2019). In order to
98 obtain an optimal dosing regimen that provides a high probability of treatment success while
99 minimizing the risk of resistance selection in the target species, the pharmacokinetic (PK) and
100 pharmacodynamic (PD) properties of enrofloxacin should be properly characterized for
101 rainbow trout.

102 Enrofloxacin PK has been studied in some farming fish species, such as salmonids (Bowser *et al.*
103 *et al.*, 1992; Stoffregen *et al.*, 1997; Lucchetti *et al.*, 2004; Koc *et al.*, 2009), some freshwater fish
104 (Fang *et al.*, 2012; Xu *et al.*, 2013) and in marine species (Lewbart *et al.*, 1997; Intorre *et al.*,
105 2000; Della Rocca *et al.*, 2004). For salmonids, a wide range of terminal half-life has been
106 reported (~19h to ~130h) which is overall longer compared to mammal species (Trouchon and
107 Lefebvre, 2016). Moreover, some covariates as the water temperature have been shown to
108 strongly influence the PK of enrofloxacin in fish (Bowser *et al.*, 1992; Liang *et al.*, 2012). The
109 influence of the ploidy (i.e. diploid *vs* triploid), however, has never been investigated although
110 the triploidy process, which consists in inducing sterility for fish, is increasingly used in
111 rainbow trout due to numerous advantages for fish growth (Piferrer *et al.*, 2009).

112 Enrofloxacin displays a concentration-dependent bactericidal activity with a wide
113 spectrum of action on aerobic bacteria, especially against Gram-negative bacteria (Brown,
114 1996). Its efficacy has been shown to be linked to the PKPD index defined by the ratio of the
115 area under free plasma concentration–time curve (fAUC) over the MIC (fAUC/MIC) (Wright
116 *et al.*, 2000). For rainbow trout, a wide range of enrofloxacin doses has been used (*e.g.* 1-50
117 mg/kg/day *per os*) (Reimschuessel *et al.*, 2013). The dose of 10 mg/kg/day, later be referred to
118 as the “standard” dose, is the most commonly used dose. It is, however, based only on a few
119 PK experiments, and on a few field trials carried out several decades ago involving only one or
120 two different pathogenic strains (*e.g.* *Aeromonas*) (Bowser *et al.*, 1990; Bowser *et al.*, 1992;
121 Hsu *et al.*, 1994; Hsu *et al.*, 1995). Curiously, the variability associated with PK parameters
122 (clearance, bioavailability) and PD parameters (in terms of MIC) has not really been explored
123 although it is a major influencing factor that must be taken into account to obtain optimal dosing
124 (EMA, 2018). Moreover, a recent evaluation of the efficacy of enrofloxacin in rainbow trout

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

127
128 The aim of this study was to compare the "standard" dose of 10 mg/kg/day enrofloxacin in
129 rainbow trout with the optimal dosing regimen calculated by a PKPD approach, including: (i)
130 the development of a population PK model based on experiments with rainbow trout receiving
131 oral and IV administration of enrofloxacin at different doses, and the identification of relevant
132 physiological covariates (ploidy, weight); (ii) collection of MIC data of enrofloxacin from the
133 three major pathogens *Y ruckeri*, *A salmonicida* and *F psychrophilum* (PD parameters); (iii)
134 integration of the above-mentioned PK and PD parameters with serum protein binding data
135 within Monte Carlo simulations to derive optimal dosing regimens in rainbow trout.

136

137 **2. MATERIALS AND METHODS**

138 **2.1. Animals**

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

157

158 **2.2. In vivo experiments**

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

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

172
173 Approximately 0.2 mL of blood were collected from the caudal vein of each fish at 1, 3, 6, 10,
174 26 and 96 h following gavage. For the group receiving the dose of 5 mg/kg, there was an
175 additional sampling time at 72h after gavage. For each blood sampling, fish were first
176 anesthetised by bathing with 2-phenoxyethanol (0.2 mL/L). The fish was then taken out of the
177 water for a few minutes to perform the blood sampling. It was then placed back in a monitoring
178 tank until it was fully awake. The anaesthesia, from its induction to the animal's awakening,
179 was closely monitored by a dedicated veterinarian. For each animal, the whole handling did not
180 exceed 15 minutes.

181
182 Immediately after the last sampling at 96h (no wash-out), fish received an IV administration of
183 enrofloxacin from the same formulation (Baytril 10%) and blood samples were collected at 1,
184 4, 10, 14, 30, 100 and 120 h after injection. Fish were euthanized with an over-dose of 2-
185 phenoxyethanol (0.6 mL/L) after the last sampling time (*i.e.* around 216 h after the first oral
186 administration).

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

189

190 **2.3. *In vitro* experiments**

191 **2.3.1. Protein binding of enrofloxacin in plasma**

192

193 Ultrafiltration method was used to determine the unbound fraction (f_u) of enrofloxacin in
194 plasma. Briefly, frozen plasma samples (pH between 7.2 and 7.4) from a pool of several
195 untreated trout were thawed and supplemented with enrofloxacin (Fluka, Steinheim,
196 Switzerland) to obtain final plasma enrofloxacin concentrations of 0.1, 1, 5 and 10 $\mu\text{g/mL}$.
197 Samples were incubated under agitation at ambient temperature for 1 h. Then, 500 μL of each
198 sample were transferred to a cartridge with a centrifuge filter (MICROCON YM-10, Millipore,
199 USA) and centrifuged at 3,000 $\times g$ for 15 min at 22°C. Ultrafiltrates were then collected and
200 stored at -20°C until assay (see analytical method). The same steps were performed in isotonic
201 PBS instead of plasma in order to determine the non-specific binding (NSB). All experiments
202 were performed in triplicate (technical replicates) and experiments in plasma were repeated two
203 times (independent replicate). Plasma f_u was expressed as in equation 1 for each tested
204 concentrations:
205

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

207
208

209 **2.3.2. Bacteria collection, MIC determination and MICs analysis**

210 A collection of 280 *Y. ruckeri*, 151 *A. salmonicida* and 77 *F. psychrophilum* strains from our
211 lab collection were used to get enrofloxacin MIC data by the microbroth-dilution method in
212 accordance with CLSI recommendations for bacteria isolated from aquatic animals (CLSI,
213 2014) (Table 2). All our strains come from diseased fish samples (not from water samples). For
214 the *A. salmonicida* isolates, bacteria that are difficult to identify, the identification was
215 performed by Maldi-Tof and confirmed with the PCR method of (Byers *et al.*, 2002). Briefly,
216 *Asalmonicida* ATCC 33658 and *Escherichia coli* ATCC 25922 were used as quality control
217 strains. The range of enrofloxacin concentrations tested was 0.004-2 $\mu\text{g/mL}$. For *Y. ruckeri* and
218 *A. salmonicida* isolates, the plates were incubated at 22°C for 24 h and for *F. psychrophilum*
219 isolates plates were incubated at 17°C for 96 h. MICs were equal to the lowest concentration of
220 enrofloxacin that inhibited visible bacterial growth.

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

225

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

236

237 **2.4. Analytic method**

238 **2.4.1. Chemicals and reagents**

239 Enrofloxacin used for determination of analytical method was obtained as pharmaceutical-
240 grade powders from Fluka (Steinheim, Switzerland). Stock standard solutions of enrofloxacin
241 (1000 µg/mL) were prepared by dissolving 50 mg in 50 mL of sodium hydroxide 0.03 mol/L
242 and stored at 4°C for 1 month. Acetonitrile was HPLC-solvent grade, trimethylamine,
243 orthophosphoric acid were analytical-reagent grade (Merck, Lyon, France). Ultrapure water
244 was obtained from a Milli-Q system from Millipore (Bedford, MA, USA). Zinc sulphate
245 heptahydrate and sodium hydroxide 1 mol/L were obtained from Panreac QuimicaSA
246 (Barcelona, Spain).

247

248 **2.4.2. Enrofloxacin assay**

249 Experiments were performed using an isocratic pump, an automatic injector with a 20 µL loop
250 307 pump, a 234 auto-injector (Gilson, Villiers Le Bel, France), and a cartridge oven CTO.10As
251 VP (Shimadzu, Kyoto, Japan) coupled to a fluorescence detector (FP-1520, Jasco, Tokyo,
252 Japan). Analytical separation was achieved on a Chromolith® performance RP-18 endcapped
253 100mm x4.6mm HPLC column, protected by a 5 x 4.6 mm guard column containing the same
254 packing material. LC mobile phase was prepared by combining 840 mL of 0.02 mol/L
255 orthophosphoric acid – 0.008 mol/L triethylamine (1:1, v/v) with 160 mL of acetonitrile and
256 then filtering with a filtration unit SolVac using a GH-Polypro membrane of 0.45 µm porosity
257 (Pall Corporation, Ann Arbor, MI, USA). The flow rate was 0.8 mL/min and the temperature

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

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

269

270 **2.5. Data analysis**

271 **2.5.1. Population PK model development**

272

273 The assessment of dose-linearity after the oral treatment was carried out using a bioequivalence
274 approach (Gough *et al.*, 1995). Briefly, a power model was fitted to the partial AUC_{0-96h} (just
275 before the IV administration) and assessed by a linear regression. The slope (β_1) and its
276 associated 90% confidence interval (CI) were compared to the reference interval, with a ratio
277 of maximal to minimal dose $r=8$, and lower and upper acceptance limits equals to 0.8 and 1.25,
278 respectively (Smith *et al.*, 2000).

279

280 Enrofloxacin plasma concentration time- courses from oral and IV dosing were analysed
281 simultaneously thanks to a non-linear mixed effect (NLME) approach, allowing the estimation
282 of population parameters, inter-individual variabilities (IIV) and residual errors (Bon *et al.*,
283 2018).

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

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

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

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

299

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

306

$$307 \quad \log(X_i) = \log(X_{pop}) + \beta_{X_{gen=T}} + \eta_i \quad (2)$$

308

309 where X_{pop} is the population value of the parameter X and $\beta_{X_{gen=T}}$ is the fixed effect of the
310 categorical covariate (i.e., being triploid) on X and η_i the random effect for individual i . If
311 $\beta_{X_{gen=T}}$ was significantly different from 0, the covariate was kept.

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

$$314 \quad \log(X_i) = \log(X_{pop}) + \beta_{X_{WT}} \times \log\left(\frac{WT}{361}\right) + \eta_i \quad (3)$$

315

316 where X_{pop} is the population value of the parameter X and $\beta_{X_{WT}}$ is the fixed effect of the
317 continuous covariate WT on X and η_i the random effect for individual i .

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

320

321 **2.5.2. PK model validation**

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

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

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

331

332 2.5.3. PKPD integration and computation of dose by Monte Carlo simulation

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

337

338 First, to explore the “standard” dosing regimen, the PTA (probability of target achievement)
339 was calculated as the percentage of the 5,000 simulated fish who met the PKPD index target
340 value for each MIC value. For fluoroquinolones like enrofloxacin, a PKPD index $fAUC_{24h}/MIC$
341 greater than 100/125 h at steady-state is classically used (Wright *et al.*, 2000). For fish
342 pathogenic bacteria, however, there is a lack of information about the relevance of these target
343 PKPD index values (see discussion). Values of 50, 75, 100 and 125 h, therefore, were chosen,
344 equivalent to a scaling factor (SF) ranging from ~2 to ~5 when divided by 24h (Toutain *et al.*,
345 2007). For each PKPD index value, the lowest MIC at which the PTA became $\geq 90\%$ was
346 considered as the PKPD cut-off (PKPD_{CO}) (Toutain *et al.*, 2017a)

347

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

353

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

356 - (i) Determination of a maintenance dose

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

360

$$361 \quad \text{Maintenance Dose}_{\tau} = \frac{\text{CL} \times \tau \times \text{SF} \times \text{MIC}}{\text{fu} \times \text{F}} \quad (4)$$

362

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

371

372

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

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

378

$$379 \quad AUC_{\text{Loading Dose}_{\tau}} = R_{\tau} \times AUC_{\text{Maintenance Dose}_{\tau}} \quad (5)$$

380

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

383

$$384 \quad \text{Loading Dose}_{\tau} = R_{\tau} \times \text{Maintenance Dose}_{\tau} \quad (6)$$

385

386

387 Where R_{τ} is the accumulation ratio which depends on the target dosing interval τ (i.e. 24,
388 48, 72 h, etc). If the dosing interval is sufficiently large, i.e. doses are administered in the post-
389 distributive phase (see 3.2.3), R_{τ} is equal to equation (7) (Toutain and Bousquet-Melou, 2004)

390

391

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

392

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

394

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

397

398 **2.6. Software**

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

403

404 **3. RESULTS**

405 **3.1. *In vitro* experiment:**

406 **3.1.1. Protein binding**

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

409

410 **3.1.2. MICs analysis**

411 A total of 346 isolates of *F. psychrophilum* (Smith *et al.*, 2016; Van Vliet *et al.*, 2017; Ngo *et*
412 *al.*, 2018; Saticioglu *et al.*, 2019), 408 isolates of *Y. ruckeri* (Calvez *et al.*, 2014) and 151 isolates
413 of *A. salmonicida* were pooled from this study and literature search (Table 2). Overall, these
414 strains had mostly been isolated from rainbow trout. Their associated MIC₉₀ were equals to
415 0.128 µg/mL for *Y. ruckeri*, 1 µg/mL for *F. psychrophilum*, and 1 µg/mL for *A. salmonicida*,
416 (Figure 1)

417

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

421

422 In contrast, for *A. salmonicida* and *Y. ruckeri* strains, no clear bimodal distribution could be
423 observed that would allow the separation of WT isolates from those that are not wild-type
424 (NWT) (Figure 1). Moreover, the NRI method failed to derive a relevant CO_{NRI} because the
425 standard deviation of each MIC distribution was too large (*i.e.* $\geq 1.2 \log_2 \mu\text{g/mL}$; Kronvall,
426 2010).

427

428 **3.2. In vivo study**

429 **3.2.1. Population PK model**

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

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

436

437 After visual inspection of the data, different compartmental PK model were tested but the bi-
438 compartmental model gave a better fit. The residual error was defined by a combined model of
439 a constant term and a term proportional to the structural model. For the oral PK data, an atypical
440 absorption profile was chosen following data observation at early time (Supplementary Figure
441 2). Indeed, the absorption process was modelled by two different first-order absorption
442 constants (ka_1 and ka_2) separated by a lag-time (T_{lag}) (Figure 3). A fraction of the bioavailable
443 dose ($1 - \text{Frac}_{ka_2}$) was absorbed early following ka_1 and the remaining fraction (Frac_{ka_2}) was
444 absorbed more slowly following ka_2 ($ka_1 > ka_2$). This atypical absorption model was supported
445 by a huge decrease of the cBIC compared to a model with only one first-order absorption
446 constant ($\Delta = 340$). Finally, inspection of the goodness-of-fit plots (Supplementary Figures
447 3-4) confirmed the adequacy of this structural model.

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

453

454 All structural parameters were estimated with a very good confidence (RSE < 20%) and IIV
455 could be estimated for all of them (except ka_1) with a high level of confidence too (RSE < 20%)

456 (Table 4). The absolute bioavailability of enrofloxacin (Foral) was estimated at ~88%. The
457 fraction of the absorbed dose during the early absorption phase (linked to ka_1) was very low (<
458 5%). Additionally, four correlations between random parameters were also found to be
459 significant (RSE < 40%).

460

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

468

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

475

476 3.2.2. PKPD integration and exploration of the “standard” dose of enrofloxacin

477 For the PKPD integration, the effect of ploidy on clearance was considered negligible compared
478 to the acceptable 2-fold uncertainty about MIC values when looking at Eq.4. We therefore only
479 considered diploid individuals, as the “worst-case scenario” for enrofloxacin exposure (higher
480 clearance). Using the “standard” oral maintenance dose of 10 mg/kg/day, PTA at steady-state
481 was calculated for all MIC values covering the whole range of the 3 MIC distributions (from
482 0.004 to 4 $\mu\text{g/mL}$) (Figure 5). Whatever the chosen value of the PKPD index, this dosing
483 regimen gave a PTA > 90% for all MIC $\leq 0.25 \mu\text{g/ml}$. The PKPD_{CO} associated to this dosing
484 regimen were equal to 1, 0.5, 0.5 and 0.25 $\mu\text{g/mL}$ for the SF ranging from 2 to 5 (or equivalently
485 50h to 125h), respectively (Figure 5).

486

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

489 *psychrophilum*, however, the weighted-PTA was $\geq 90\%$ (or really close) only for a PKPD index
490 SF of 3 or less.

491 These previous assessments were based on the values of fAUC/MIC between 2 doses over a 24
492 h-interval after steady-state was reached. However, as the t_{1/2} of enrofloxacin is long (between
493 4.5 and 6 days, see above), the time (or number of days of treatment) needed to reach the steady-
494 state and therefore to attain the target value of fAUC/MIC (or equivalent SF value) is also long
495 (Table 6 and Supplementary Figure 8). For instance, regarding the MIC of 0.25 $\mu\text{g}/\text{mL}$, the
496 90% PTA would be reached after at least 48 h and 144 h (*i.e.* 2 and 6 days of treatment) for the
497 lowest and highest SF value of 2 and 5, respectively (Table 6). For MIC $\geq 1 \mu\text{g}/\text{mL}$, the 90 %
498 PTA would even never been achieved.

499

500 3.2.3. Dose determination by MCS

501

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

504

505 3.2.3.1. With the CO_{NRI} for *F. psychrophilum*

506

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

515

516

517 3.2.3.2. With the whole range of MIC for the three pathogens

518 The calculated oral single doses that insure a PTA $\geq 90\%$ for all possible enrofloxacin MIC
519 (from 0.004 to 4 $\mu\text{g}/\text{mL}$) of the 3 bacterial species are presented in Table 8. For the highest MIC
520 value, very high and unrealistic doses of enrofloxacin would be needed (between 315 and 837
521 mg/kg, depending on the PKPD index value).

522

523 4. DISCUSSION

524

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

530

531 **4.1. MICs distributions of the three species**

532

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

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

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

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

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

563

564 **4.2. Enrofloxacin PK parameters**

565

566 The statistical approach using the power model showed that the assumption of dose-linearity
567 could be reasonably accepted for the tested oral dose range. There are some limits of this
568 analysis as we could only use partial AUCs over 96h (Supplementary Figure 5) instead of the
569 AUC_{0_inf} because the extrapolated terminal part of AUC was higher than the usual threshold of
570 20%. We yet assumed pharmacokinetic linearity over this dose range.

571

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

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

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

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

595

596 The oral administration also provided interesting enrofloxacin PK information. First, the oral
597 bioavailability of enrofloxacin was found to be high (~88%), in good agreement with other
598 results issued from brown trout (~78%, at 10°C after gavage; Koc *et al.*, 2009) but higher than
599 older results with rainbow trout (42-48% at 15°C after gavage, 24-35% at 10°C after gavage;
600 Bowser *et al.*, 1992) and Atlantic salmon (~46-49% at 10 °C after gavage; Stoffregen *et al.*,
601 1997). An atypical absorption profile was observed in our data (Supplementary Figure S2)
602 which was modelled by two successive absorption phases because this gave the best fit (Figure
603 3). However, the clinical relevance is probably weak as the early fast absorption phase (with
604 k_{a1}) only applied to a negligible fraction of the administered dose (< 5%). Anaesthesia used for
605 gavage as well as for repeated blood sampling may have affected the gastro intestinal transit
606 and thus may have affected the main absorption phase (Davies *et al.*, 2010). The existence of
607 an enterohepatic recycling of enrofloxacin (Trouchon and Lefebvre, 2016) may also explain
608 these absorption profiles although this has not been proven in fish. However, other atypical PK
609 profiles with multiple plasma peaks have already been observed for orally administered
610 enrofloxacin in rainbow trout (Kyuchukova *et al.*, 2015), Atlantic salmon (Stoffregen *et al.*,
611 1997) or red pacu (Lewbart *et al.*, 1997), without a clear explanation.

612

613 **4.3. Population PK modeling revealed the importance of ploidy**

614

615 The strength of the population PK using a NLME approach is the ability to analyse all data (i.e.
616 all individuals and all dosing regimens) simultaneously and hence be able to discriminate the
617 population parameters, the inter-individual variabilities and the residual errors (Bon *et al.*,
618 2018). In this study, all parameter values were estimated with very good confidence when
619 looking at the rather low RSE (Table 4). Furthermore, the pcVPC showed a proper fitting of
620 the data and thus validated the model (Figure 4).

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

625 slowly than their diploid congeners, and the molecule will therefore persist longer in plasma for
626 triploids. This is underlined by the significant difference in terminal t_{1/2} between these 2 sub-
627 populations (115 h vs. 166 h). While the influence of ploidy on clearance has an overall
628 negligible impact on the calculation of enrofloxacin dose (see section 4.4), it may have
629 consequence about the level of residues and the required withdrawal period after treatment that
630 should be investigated. The reason for such difference between diploid and triploid is unknown
631 and should deserve further investigations.

632

633 **4.4. PKPD exploration and dose determination**

634

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

648

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

658

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

673

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

684

685

686 4.5. Limits of the study

687

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

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

695 Moreover, our study was conducted in healthy fish and results may be different for sick
696 animals: (i) the pharmacokinetics of enrofloxacin may be different in diseased fish, as observed
697 for crucian carp infected with *Aeromonas hydrophila*, with a lower systemic exposure (Fan *et al.*,
698 2017); (ii) fish showing clinical signs are often anorexic and are therefore less or not at all
699 exposed to the antimicrobial when given through medicated feeds (Giguère *et al.*, 2013).
700 However, in the context of metaphylaxis, which is the most frequent use of antimicrobial in fish
701 production (Lulijwa *et al.*, 2020), the early initiation of an enrofloxacin treatment for the
702 majority of fish would limit the influence of the infection on food intake and on the PK of
703 enrofloxacin, while treating a rather low initial bacterial load. The use of other routes of
704 administration, such as the intramuscular or the intraperitoneal route (as in vaccination
705 campaigns) could be an alternative to the oral route, allowing better inter-individual
706 reproducibility, and ensuring a sufficient dose to each diseased fish (Rostang *et al.*, 2021).

707

708 In a crossover experiment, it is common to let a wash-out of at least 4 times the $t_{1/2}$ between two
709 administrations to ensure that (virtually) all drug has been eliminated (Gehring and Martinez,
710 2012). With the long $t_{1/2}$ of enrofloxacin, this wash-out would have lasted minimum 20 days,
711 which is a limiting factor for such experiments. Thus we chose to use a particular design, known
712 as the semi-simultaneous method, which remains relevant if the second administration is given
713 during the post-distributive phase of the first administration (Karlsson and Bredberg, 1990).
714 When combined to an NMLE approach and thanks to the numerous individual data, this
715 experimental design has already proven its usefulness with other drugs (Karlsson and Bredberg,
716 1990; Lallemand *et al.*, 2007).

717

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

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

728

729 Concerning the PKPD index, both C_{max}/MIC and fAUC/MIC have been proposed as efficacy
730 surrogates for fluoroquinolones (Wright *et al.*, 2000). We chose to perform the simulation with
731 fAUC/MIC because it tends to be the most relevant index for antimicrobial with long terminal
732 half-life (Nielsen and Friberg, 2013). One limitation concerns the use of frozen plasma to
733 determine the unbound fraction of enrofloxacin as it was shown that freezing may impact serum
734 protein binding (Banker & Clark, 2008). We chose a wide range of target value of fAUC/MIC,
735 from 50 to 125 h (*i.e.* SF from 2 to 5), as an illustrating purpose of our PKPD analysis and to
736 avoid being too conservative. Indeed, depending on the bacteria specie, great differences could
737 be observed, especially as fish are poikilothermic animals. In addition, higher enrofloxacin
738 MICs values were observed at 4°C compared to 15°C for *A. salmonicida* (Martinsen *et al.*,
739 1992). Further *in vitro* studies involving time-kill curves with these fish bacteria under specific
740 condition are needed to better define the target values of the PKPD indexes for different level
741 of efficacy (bacteriostase, bactericidal, virtual eradication) in trout, as already published for
742 other bacteria and food-animal species (Dorey *et al.*, 2017; Paulin *et al.*, 2018; Pelligand *et al.*,
743 2019; Toutain *et al.*, 2019).

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

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

755

756 5. CONCLUSION

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

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

770

771

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776 analysis.

777

778 **Figures legends:**

779

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

783

784 Figure 2: Individual plasma concentration of enrofloxacin for the 96 trout over the whole
785 experiment (oral administration at T=0h then IV injection at T=96h). Red: group 1; Blue:
786 group 2; Green: group 3; Yellow: group 4.

787 The details of each group and dose are given in Table 1

788

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

792

793

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

800

801 Figure 5: PTA for the “standard” dose depending on the MIC value, the PKPD index target
802 value and stratified by the ploidy status.

803 The horizontal dashed line represents the PTA 90%.

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Figure 1

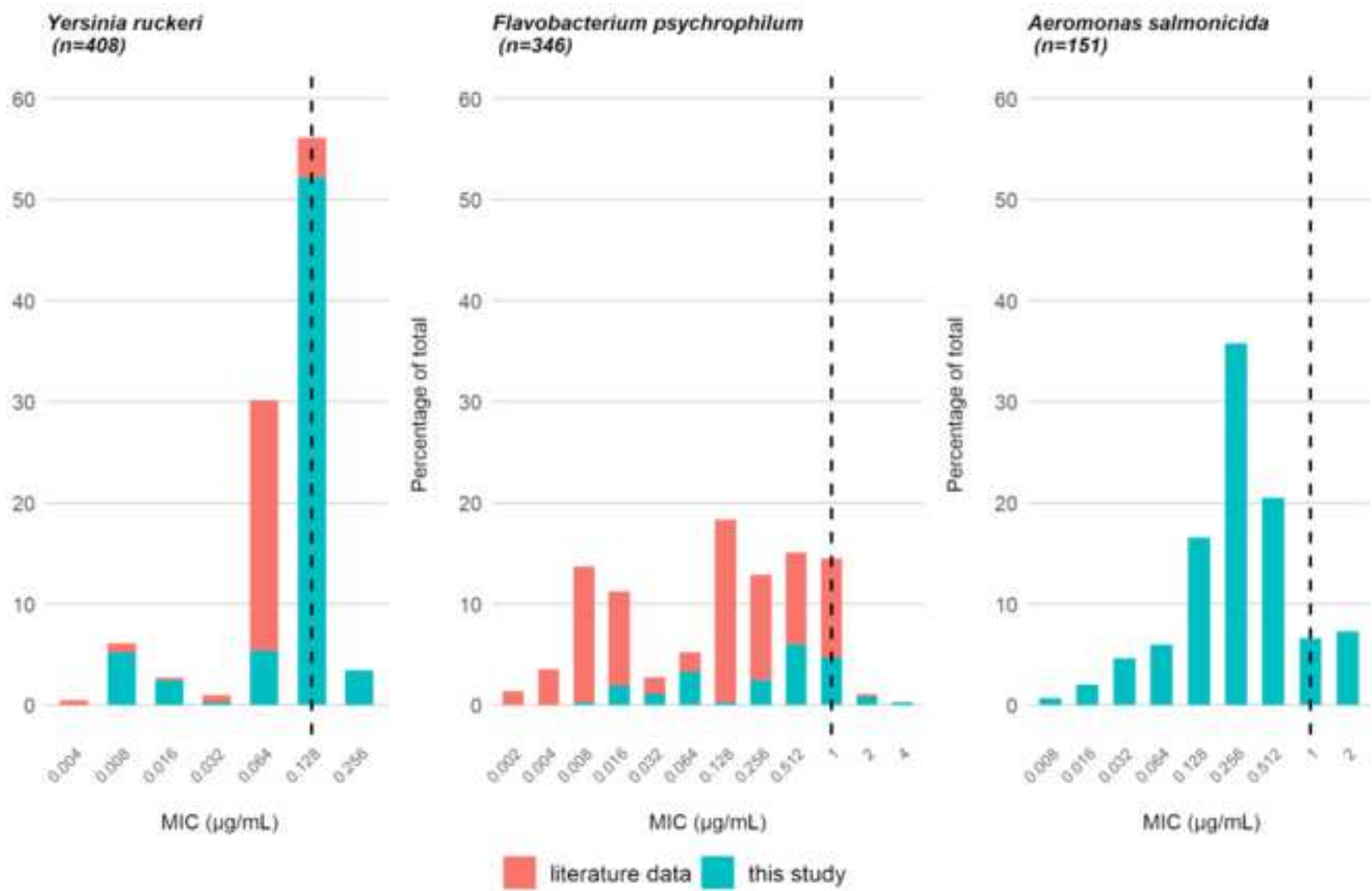


Figure 2

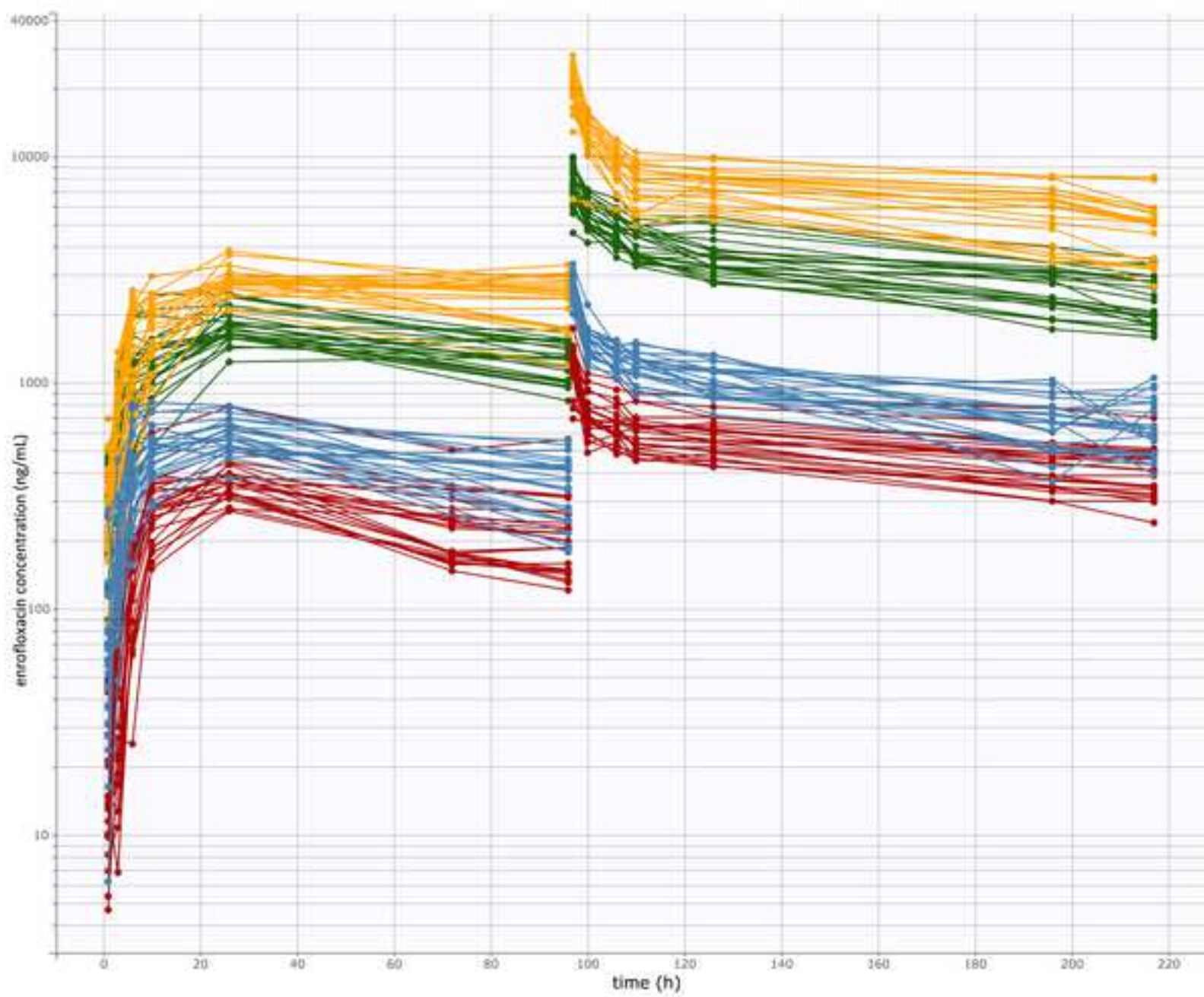


Figure 3

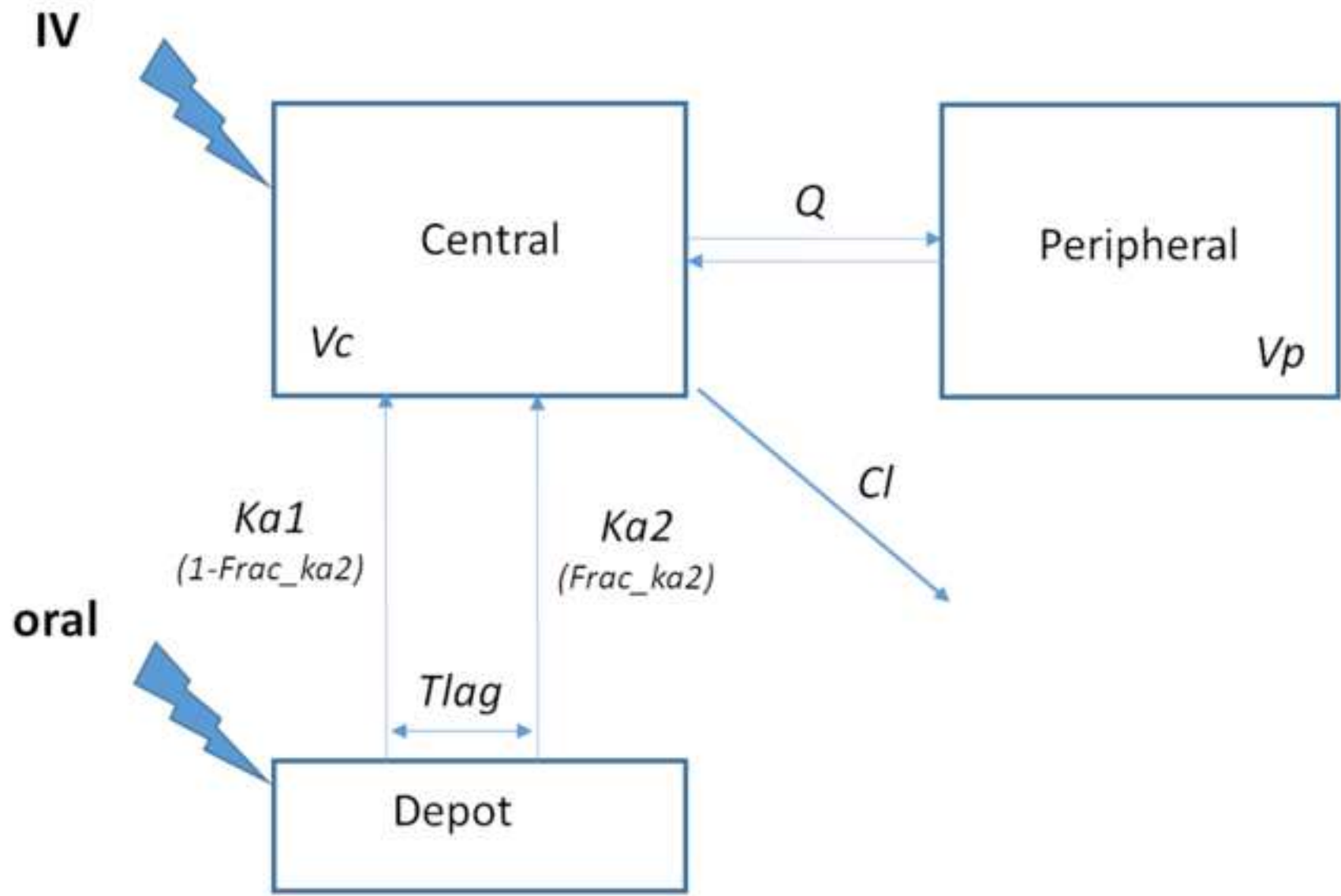


Figure 4

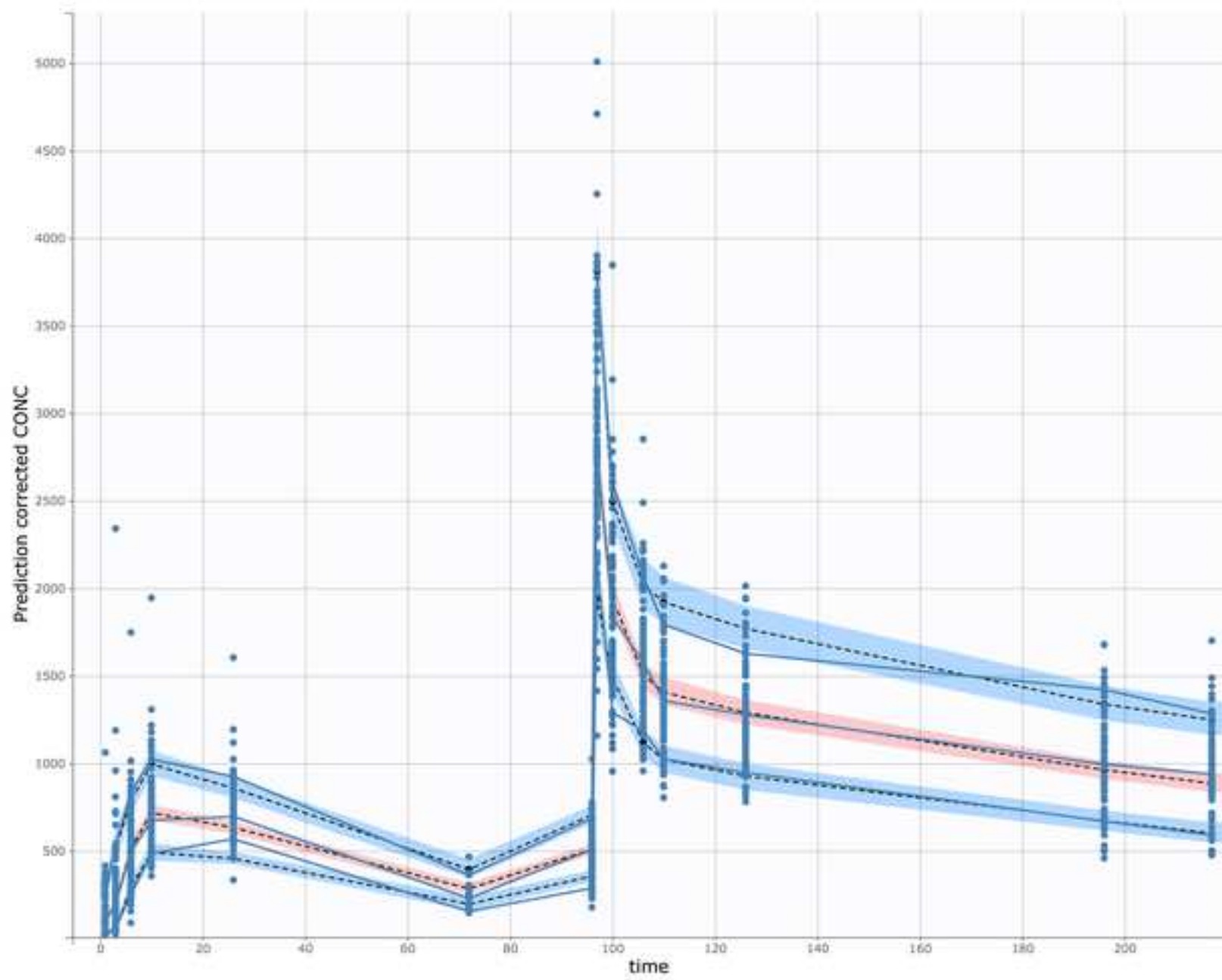
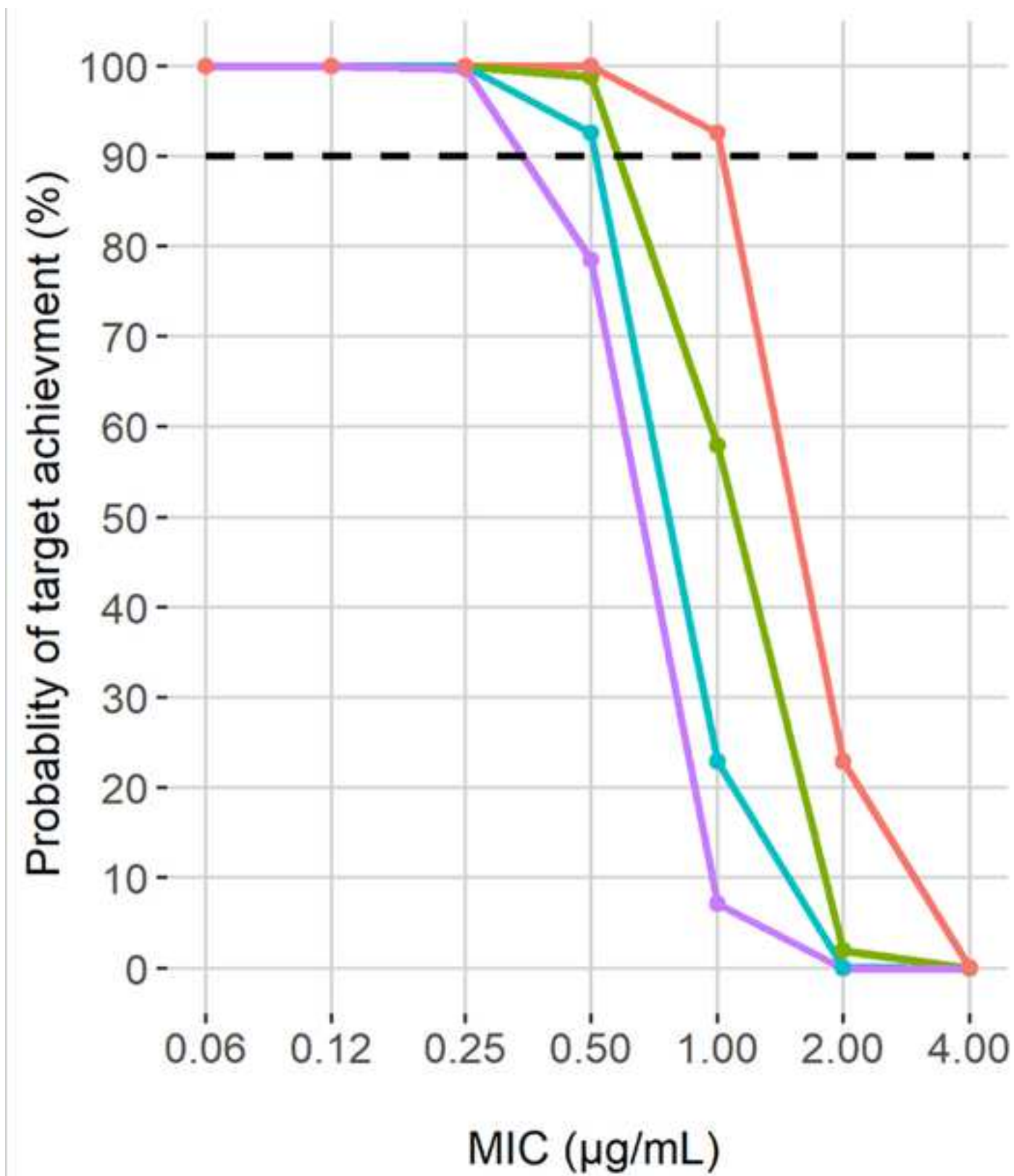


Figure 5



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 ($\mu\text{g/ml}$)	Fish species (proportion of isolates related to)	Reference
<i>A. salmonicida</i>	151	0.004 - 2	RT (> 90%) + others	This study
<i>Y. ruckeri</i>	280	0.004 - 2	RT (> 90%) + others	This study
	128		RT (> 95%) + others	(Calvez <i>et al.</i> , 2014)
<i>F. psychrophilum</i>	77	0.004 - 4	RT (> 90%)	This study
	61	0.002–1	RT (> 80%) + others	(Smith <i>et al.</i> , 2016)
	50		RT (36%) + other salmonids	(Van Vlie, <i>et al.</i> , 2017)
	133		RT (88%) + other salmonids	(Ngo <i>et al.</i> , 2018)
	25		0.008 - 256	RT (92%) + other salmonids

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)	–
Bioavailability 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 between V1 and Cl	corr1_V1_Cl	%	33.3 (37.6)	–
Correlation between ka2 and Cl	corr1_ka2_Cl	%	33.4 (37)	–
Correlation between ka2 and V1	corr1_ka2_V1	%	57.6 (16.8)	–
Correlation between 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
<i>F. psychrophilum</i>	97.8	92.4	86.3	81.7
<i>Y. ruckeri</i>	100	100	100	99.9
<i>A. salmonicida</i>	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 \geq 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 ($\mu\text{g/ml}$)	≤ 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

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