Preventive Veterinary Medicine 156 (2018) 16–21

Contents lists available at ScienceDirect

Preventive Veterinary Medicine

journal homepage: www.elsevier.com/locate/prevetmed

Assessing concentration of antibiotics in tissue during oral treatments against piscirickettsiosis

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ARTICLE INFO

Keywords:
Antibiotic concentration
MIC90
Oral treatment
In-feed treatment
Oxytetracycline
Florfenicol
Atlantic salmon
Rainbow trout

ABSTRACT

The use of antimicrobials in aquaculture is increasingly being scrutinized. In Chile, piscirickettsiosis accounts for approximately 90% of the total volume of antibiotics used in marine aquaculture. Treatment failures are frequently reported, but there is limited information on why this occurs. Fish producers have started assessing the level of antibiotics in fish tissues during and immediately after in-feed treatments to determine if they are adequately medicating their fish. In this study, we evaluated the probability of finding antibiotic concentrations in muscle tissue above the minimum inhibitory concentration for 90% of the *P. salmonis* isolates (MIC90) recently tested in Chile, for two antibiotics commonly used in aquaculture. We found that the proportion of fish with antibiotic concentrations above the MIC90 varied, depending on the product used, species, day of sample collection, and size category of fish within a cage. The proportion of fish above the MIC90 was lower in fish treated with florfenicol than in fish treated with oxytetracycline. Using a mixed-effects logistic model, we modeled the probability of antibiotic concentrations above MIC90 when fish were treated with florfenicol. Our model suggested lower probabilities of having concentrations above MIC90 in Atlantic salmon than in rainbow trout when samples were collected 14 days after the treatment started compared to 7 days, and in the smaller fish within a cage. We discuss these findings and hypothesize about potential issues with treating large populations of fish with in-feed antimicrobials.

1. Introduction

In Chile, the main reason for antibiotic use in salmon aquaculture is to treat piscirickettsiosis (Sernapesca, 2016a), also known as salmonid rickettsial septicemia (SRS), a disease caused by the intracellular bacterium *Piscirickettsia salmonis* (Fryer et al., 1992). In recent years, as the use of antibiotics has come under more scrutiny, it has become apparent that *P. salmonis* treatments are inconsistent and often unsuccessful (Price et al., 2016; Rozas and Enríquez, 2014). The reasons for treatment failure are not well understood, but antimicrobial resistance (AMR) has been suggested as a possible explanation; however, in a recent study which assessed the minimum inhibitory concentration (MIC) for florfenicol and oxytetracycline for 292 Chilean *P. salmonis* isolates collected between 2010 and 2014, the authors did not find a high level of resistance towards these two commonly used antimicrobial products (Henríquez et al., 2016; Sernapesca, 2016a). These findings suggest that there may be other causes of treatment failure for piscirickettsiosis. Furthermore, in our previous study, we detected differences in treatment efficacy between cages within a farm (Price et al., 2016), which cannot be explained by AMR.

Other possible reasons for treatment failure in Chile include: the disease is not properly diagnosed and there is a co-infection with a non-bacterial agent or with bacteria for which the chemotherapeutant is not effective; or the diagnostic is correct, but the chemotherapeutant concentration in the target tissue or the duration of the treatment is insufficient to eliminate the bacteria. It is unlikely that veterinarians in Chile misdiagnose *P. salmonis* often as it has typical lesions and the government requires a laboratory-confirmed diagnosis prior to initiating treatment (Sernapesca, 2012). Although infrequent, it is possible that some cases have co-infections with non-bacterial diseases, and even less frequent is the occurrence of co-infection with another species of bacteria; however, the prevalence of these events cannot explain the high level of treatment failures observed recently (Sernapesca, 2016b).

The other possibility for treatment failure is inadequate or subtherapeutic levels of antibiotic in the target tissues of treated fish.

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https://doi.org/10.1016/j.prevetmed.2018.04.014
Received 18 May 2017; Received in revised form 10 April 2018; Accepted 20 April 2018

Antibiotics in aquaculture are typically incorporated in the feed and delivered to all fish in a cage. The product label directions for the daily dose and length of treatment are typically based on laboratory studies. These studies may not mimic the normal feeding behavior of fish populations in commercial operations nor do they capture the impact of feeding behavior of fish during outbreaks of SRS. Understanding whether fish are effectively dosed during in-feed antibiotic treatments would help determine whether this is one of the reasons for treatment failure in Chile.

Recently fish farmers in Chile have been measuring and recording the levels of antibiotics in muscle tissue of their fish during and immediately after treatments to determine whether they are treating their fish with adequate levels of antibiotics. The objectives of our study were to assess these data to determine whether antibiotic tissue concentrations in fish were above the inhibitory threshold for a representative sample of Chilean *P. salmonis* isolates and to identify factors associated with the probability of having concentrations above this threshold.

2. Material and methods

2.1. Study population

Salmon farming companies provided us with skin-on-muscle antibiotic tissue analyses from 87 treatments across 34 farms between June 2010 and February 2016. 5–10 Atlantic salmon (*Salmo salar*) or rainbow trout (*Oncorhynchus mykiss*) from one or two cages on treated farms were sampled midway through the treatment (approximately 97% obtained between day 7 and 8, and the remaining on day 9 of the treatment) and on the last day of their florfenicol or oxytetracycline treatment (98% of the samples were obtained on day 14). All treatments assessed in this study were for piscirickettsiosis and lasted between 10 and 14 days. Many farms were sampled more than once during the course of the study period; however, the time between treatments was always greater than the withdrawal period for the product used in the initial treatment (i.e., 300 degree-days for florfenicol treatments and 600 degree-days for Oxytetracycline).

2.2. Sample collection

Fish were collected using a dipnet at the start of the morning feeding. Skin-on muscle samples were submitted by the companies to commercial laboratories in Chile for high-performance liquid chromatography (HPLC) analysis for florfenicol (Hormazabal et al., 1993) or oxytetracycline (Reveurs and Díaz, 1994). This type of sample is routinely submitted by farmers in Chile for residue testing prior to harvesting their fish, and all laboratories used by the companies participating in this study were certified to run these analyses for regulatory purposes. The above methods, used to evaluate tissues in this study, were consistent with the government-sanctioned detection methods for the antibiotics used. For florfenicol, the method includes the detection of both florfenicol and the amine metabolite. Results of the individual fish HPLC analyses were provided to us in parts per billion (ppb).

Additional information provided by the companies included the farm and cage identification codes, treatment start date, date of sample collection, antimicrobial product administered (florfenicol or oxytetracycline), individual fish weight in kilograms, and the dose prescribed by the company veterinarian in mg/kg/day. The fish were prescribed either 100 mg/kg/day of oxytetracycline or 15, 20, 25, or 30 mg/kg/day of florfenicol. The average weight of the fish sampled from each cage was calculated. Fish that were 1SD above the average fish weight were labeled “large,” fish within 1 SD of the average weight were labeled “average,” and fish less than 1 SD of the average weight were labeled “small.”

2.3. Statistical analysis

Based on supplementary data provided in Henríquez et al. (2016), we determined the minimum concentration that inhibited 90% (MIC90) of the Chilean *P. salmonis* included in the above study. The MIC90 was set at 2 μg/ml (2000 ppb) for florfenicol and 1 μg/ml (1000 ppb) for oxytetracycline.

For our descriptive analysis, we computed the proportion of samples with tissue levels above the MIC90 for different antibiotics, species, relative weight groups (i.e., small, average and large), doses, and at different treatment stages (i.e., midway and at the end of the treatment). For florfenicol, we used a mixed effects logistic regression analysis with random effects for farm, treatment, and cage to determine what factors were associated with the probability of tissues being above the MIC90. We modeled only the probability of antibiotic concentrations being above MIC90 for florfenicol treatments because almost all fish treated with oxytetracycline were above MIC90 for this drug. Only 84 of 828 fish (10.15%) had levels of oxytetracycline below the MIC90.

Our initial logistic regression model for florfenicol included the following fixed effects: dose, species, sample day (i.e., mid or end of treatment), and whether each fish was above, below, or within 1 SD of the average weight of the fish sampled from the same cage. We included the farm, treatment, and cage as random effects. We retained predictors with P value < 0.05 and predictors that were considered confounders. Confounding was assessed by examining whether the coefficients for factors in our model changed when a potential confounder was excluded from the analysis.

The cluster-specific odds ratios and their 95% confidence intervals were calculated by exponentiation of the logistic regression model coefficients. Because current regulations do not allow multiple species on a farm during a production cycle, the comparison between species was made at the population level. We used the farm-level random effect variance (σ_h^2) and the equation $\beta^{PA} \approx \beta^{SS}(1 + 0.346\,\sigma_h^2)^{\frac{1}{2}}$ to convert cluster-specific coefficients (β^{SS}) to population average coefficients (β^{PA}), as described in Dohoo et al. (2009, eq. 22.2, p.582) to estimate the effect of the species across farms. All statistical analyses were done in R 3.3.3 (R Core Team, 2017) with the package lme4 (Bates et al., 2015) to fit our mixed logistic models.

3. Results

In total, we analyzed data from 87 treatments against piscirickettsiosis. Florfenicol was used in 54 of these treatments and oxytetracycline was used in 33. Sixty-six of the 87 treatments were sampled twice, resulting in 1403 samples tested for florfenicol and 828 samples tested for oxytetracycline. A summary of the number of samples by species and antibiotic is presented in Table 1. We found that 58.1% and 89.8% of all the fish sampled had levels of florfenicol and oxytetracycline above their respective MIC90s.

For florfenicol, the proportion of individuals with antibiotic tissue levels above the MIC90 varied based on the species (Fig. 1 and Table 2), the relative size of the fish (Fig. 2 and Table 2), and the day the sample was collected during the treatment period (Fig. 3 and Table 2). Although the raw data suggested a correlation between the proportion of animals above the MIC90 and the dose of florfenicol (Fig. 4), this

Table 1
Number of farms, cages, treatments, sample events, and samples by data source, species, and antibiotic product.

Species	Antibiotic product	Farms	Cages	Treatments	Fish
Atlantic salmon	Florfenicol	22	64	44	1179
	Oxytetracycline	19	50	30	767
Rainbow trout	Florfenicol	7	18	10	224
	Oxytetracycline	3	6	3	61
Total		34	119	87	2231

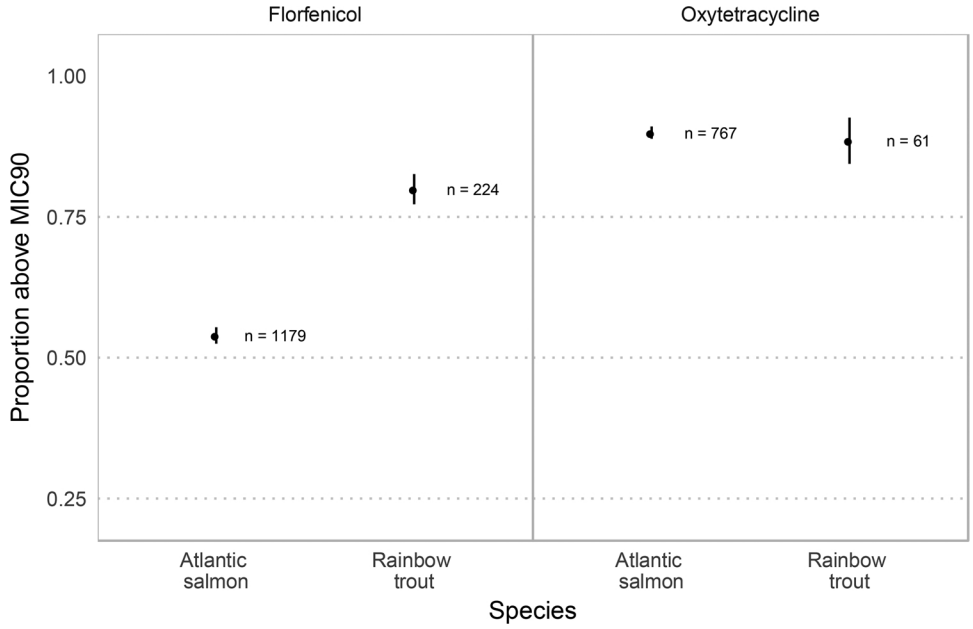

Fig. 1. Proportion of individuals with concentration of antibiotic above MIC90 by species and antibiotic product. Bars indicate the standard error for the proportions based on the normal approximation calculation method, and n is the number of observations in the category.

Table 2
Mixed-effects logistic model for the probability of florfenicol concentration above MIC90 in samples (N = 1249).

Mixed-effects logistic model: Fixed effects					
Term	Coefficient	SE	P value	OR	(95% Conf. Interval)
(Intercept)	−0.46	1.06	0.66	0.63	(0.05–6.68)
Sample Day			< 0.01*		
Day 14	(reference)				
Day 7	0.71	0.14		2.04	(1.53–2.82)
Relative size			< 0.01*		
Small	(reference)				
Average	0.31	0.18	0.08	1.36	(0.92–1.91)
Large	0.72	0.24	< 0.01	2.06	(1.29–3.36)
Dose (mg/kg/day)			0.55*		
15	(reference)				
20	−0.18	1.07	0.87	0.84	(0.08–9.98)
25	0.82	1.28	0.52	2.26	(0.11–87.76)
30	−0.77	1.44	0.60	0.46	(0.02–12.16)
Species					
Atlantic salmon	(reference)				
Rainbow trout	1.72	0.40	< 0.01	4.76	(2.49–13.31)

Mixed-effects logistic model: Random effects		
Term	N	Variance
Farm	26	3.1×10^{-7}
Treatment	52	0.64
Cage	79	0.45

(*)Overall P-value obtained with a Likelihood ratio test.

relationship was not statistically significant in our mixed model after correcting for other factors (Table 2). For oxytetracycline, the proportion of animals above the MIC90 did not vary greatly by species, the day of sampling or relative size of the fish (Figs. 1–3).

Our logistic regression model suggested the probability of having a concentration of florfenicol in tissue above the MIC90 was significantly higher for rainbow trout than for Atlantic salmon ($P < 0.01$). The odds of rainbow trout having florfenicol tissue concentrations above MIC90 was, on average, 4.76 times greater than Atlantic salmon (Table 2). The odds of being above the MIC90 was almost twice as high for fish sampled mid-treatment ($P < 0.01$) compared to fish sampled at the end of their treatment (Table 2). The odds of being above the MIC90 was approximately 1.4 times greater for average weight fish ($P = 0.08$) and approximately 2.1 times greater for large fish ($P < 0.01$) when compared to small fish in the sample (Table 2). Weight information was not recorded for all individuals resulting in the exclusion of 154 samples from the analysis. We retained dose of florfenicol in our final model despite the fact that it was not statistically associated with the probability of being above the MIC90 ($P = 0.55$) to correct for the slight confounding effect this predictor had on species of fish.

4. Discussion

We found that almost 90% of the fish treated with oxytetracycline were at or above the MIC90 threshold for this product, but less than 60% of the fish treated with florfenicol had levels at or above the MIC90 for florfenicol. In other words, approximately 40% of the fish sampled during or immediately after their florfenicol treatments did not have sufficient florfenicol concentrations in their muscle tissue to inhibit 90% of the Chilean isolates evaluated by Henríquez et al. (2016). Because we did not know the therapeutic tissue concentration required to treat *P. salmonis* for either florfenicol or oxytetracycline, and this dose may vary depending on the isolate, we felt the MIC90, which in this study was based on the 292 isolates tested by the Henríquez et al. (2016), represented a conservative minimum tissue concentration that fish should attain during treatment. The low percentage of fish achieving this threshold is likely even more significant, given that florfenicol levels provided in this study included the inactive amine metabolite. Further, the sampling strategy of using dipnets at the top of the water column to collect fish during the first morning feed may have biased towards sampling dominant and well-fed fish, as the vertical position of fish in the cage has been shown to depend on hunger level (Juell et al., 1994). The well-fed fish would have had the highest levels of antibiotics. Sick fish, which typically go off feed, would have been less likely to be sampled in this study. Given the bias towards finding antibiotics in our fish samples, the actual proportion of fish that had muscle tissue concentrations at or above the MIC90 for florfenicol in our populations was likely lower than our estimates.

The fact that a large proportion of fish treated with florfenicol had levels below the MIC90 threshold suggests that at least for a period of

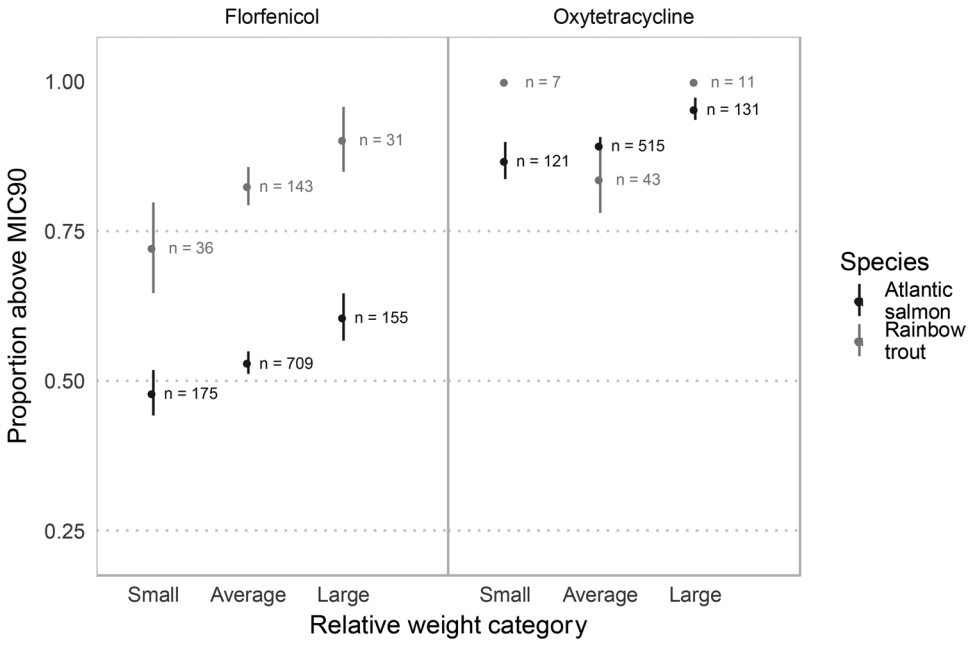

Fig. 2. Proportion of individuals with concentration of antibiotic above MIC90 by weight category (i.e., Average = mean ± 1 SD, Small = 1 SD below mean, Large = 1 SD above mean) within the sample event. Bars indicate the standard error for the proportions based on the normal approximation calculation method, and n is the number of observations in the category.

time, antibiotic levels in these fish were below the therapeutic threshold. Our study did not assess the period of time during the treatment when tissues were above the MIC90, but the fact that we found levels to be low on our single time-point estimates suggests that tissue concentrations, in at least some fish, dropped below this inhibitory threshold. Maintaining concentrations above this threshold for an extended period of time is important for antibiotics with time-dependent effects such as florfenicol and oxytetracycline (Martinez et al., 2013). Fish with tissue concentrations that are not adequate to eliminate *P. salmonis* could explain why treatments are not effective at a population level. The results of this study also indicate that the industry may be treating a large proportion of fish with sub-therapeutic levels of florfenicol, which could increase the risk of developing antimicrobial resistance (Andersson and Hughes, 2014).

The fact that so many fish did not achieve this tissue concentration may reflect a problem with the delivery of florfenicol using standard industry in-feed treatments. Interestingly, the same issue was not apparent with oxytetracycline. The difference in the proportion of fish that met the MIC90 for florfenicol and oxytetracycline could be related to the differences in the half-lives of the products. Florfenicol has the shorter half-life of the two, with the concentration of this drug in tissue reduced by half in approximately 12 h at 11 °C (Martinsen et al., 1993). Based on the pharmacokinetics of this drug, fish would need to consume feed regularly to maintain levels in their tissues constantly elevated and fish should have had relatively stable steady-state concentrations in their tissues by day 4. For oxytetracycline, the fluctuation in the serum and tissue levels would be even less than for florfenicol given this antimicrobial has a longer half-life of 50-h half-life at 8 °C (Elema et al., 1996). This permits fish to maintain levels of this drug even when feeding is irregular. Our findings suggest that understanding

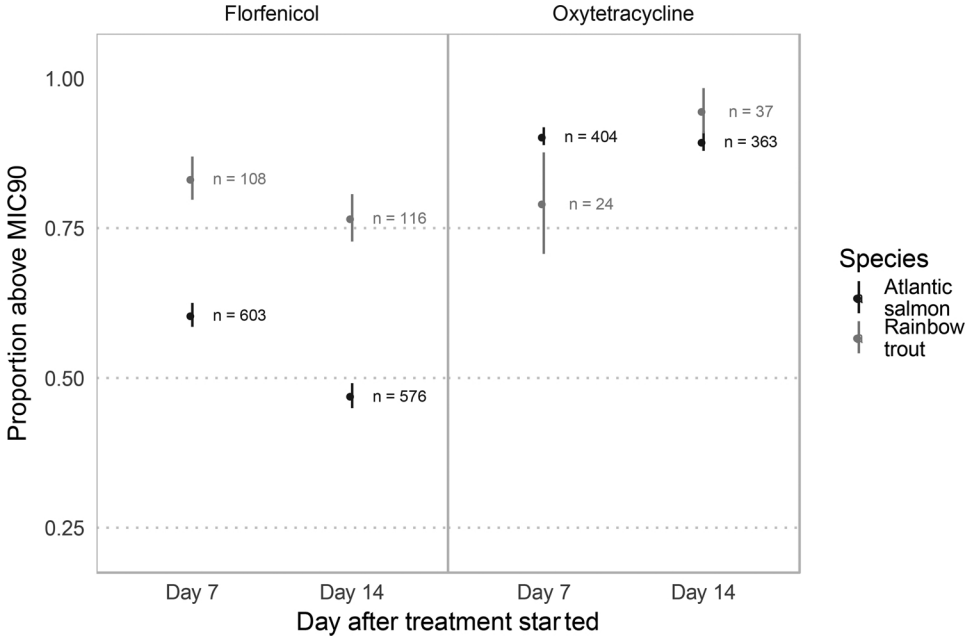

Fig. 3. Proportion of individuals with concentration of antibiotic above MIC90 by species, antibiotic product, and sample day. Bars indicate the standard error for the proportions based on the normal approximation calculation method, and n is the number of observations in the category.

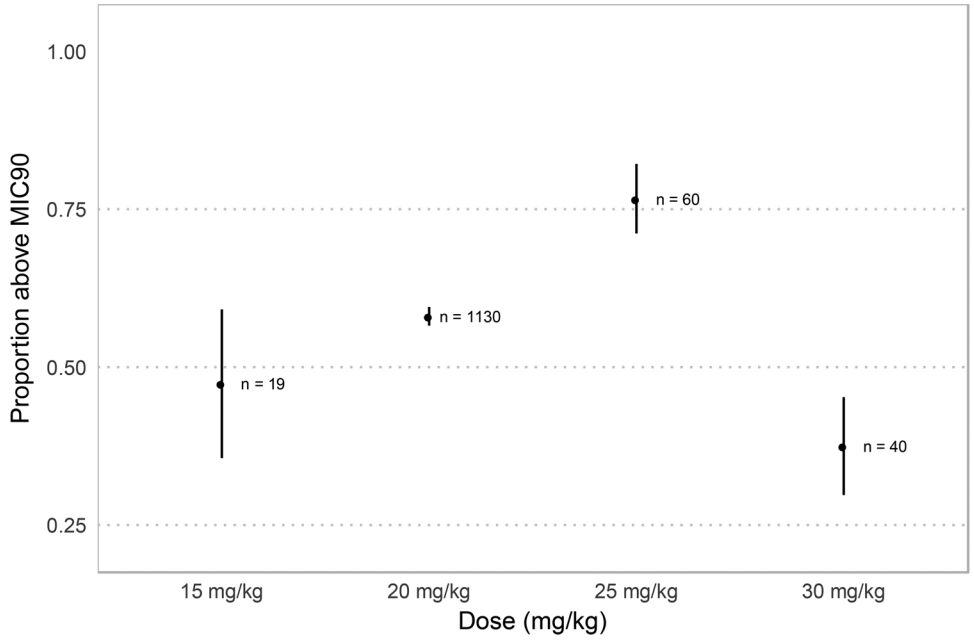

Fig. 4. Proportion of individuals with florfenicol concentration above MIC90 by prescribed dose (mg/kg/day). Bars indicate the standard error for the proportions based on the normal approximation calculation method, and n is the number of observations in the category.

the interactions between pharmacokinetics and feeding practices may be important to achieve sufficient concentrations of antibiotics to effectively treat pathogens in fish.

The fact that almost 90% of the fish treated with oxytetracycline in this study had concentrations at or above the MIC90, and the fact that there are reports of high treatment failure rates with this drug (Price et al., 2016), suggests that either the MIC90 is a gross underestimate of the therapeutic dose required to treat *P. salmonis* in vivo, or there are other causes of treatment failure besides inadequate tissue concentration of antibiotics. Other issues could include inadequate duration of treatment (i.e. the period of time above the MIC was not sufficient for an effective treatment at the population level), resistance with particular isolates of *P. salmonis*, and inadequate drug availability at the target tissue site(s). The tissue sampled in this study was skin-on-muscle which correlates relatively well to internal organ tissue concentrations, but is likely to be an overestimation of the drug concentration in the brain (Armstrong et al., 2005; Horsberg et al., 1994), where *P. salmonis* can be detected in chronic stages of infection (Skarmeta et al., 2000).

Because the issue with attaining consistent and sufficient levels of antibiotics in fish appeared to be with the florfenicol, we focused our investigation on these treatments. Our statistical analyses suggested that the proportion of individuals above MIC90 was lower for Atlantic salmon than rainbow trout. Our model suggests rainbow trout have, on average, 4.76 times greater odds of having concentrations of florfenicol above the MIC90 than Atlantic salmon, after controlling for other factors. This difference may be explained by differences in feeding behaviors between species. Trout feed at the surface and eat more rapidly than Atlantic salmon (Talbot et al., 1999), and Atlantic salmon may seek food less aggressively. For example, Atlantic salmon fed with self-feeders have lower growth rates than fish receiving their feed from preprogrammed feeders (Thomassen and Lekang, 1993), while in rainbow trout it is necessary to restrict access to feed to obtain good feed conversion ratios when using self-feeders (Alanärä, 1992). The sampling bias, mentioned above, associated with dip netting hungry fish during the first morning feed may have been greater for rainbow trout than for Atlantic salmon, given their more aggressive feeding behaviors.

Our data on size relative to the average size within the sample also suggested a feeding issue with the delivery of medicated feed. Our model revealed that the larger fish in the sample were more likely to have tissue concentrations of antibiotics above the MIC90. Further, we also observed a trend suggesting a size effect response. However, our model suggested that this observation was only significant for individuals with weights greater than 1 SD above the average (i.e., large fish). These individuals had 2.06 times greater odds of having antibiotic concentrations above MIC90 than the small fish in the sample (i.e., 1 SD smaller than the average). This finding may reflect the hierarchical behavior patterns often described in salmonids (Symons, 1968), which would indirectly relate to feed consumption (Ruzzante, 1994). Hierarchical behaviors and their role in access to food for subordinate fish should be further investigated, especially given the regulations in Chile, which prevent the grading of fish in salt water cages (Subpesca, 2001) and may, therefore, increase these types of behaviors.

An unexpected finding in our study was that the proportion of individuals with florfenicol levels above the MIC90 was significantly higher mid-way (~day 7) through the treatment than on the last day of the treatment (~day 14). This suggests that feed consumption in the population either declined as the treatment progressed or the biomass gain due to growth during the treatment was underestimated. Fish may go off-feed or decrease feed consumption during treatment if it is not working or if the medication is causing a palatability problem. The latter is not known to occur with florfenicol at the labeled dose, but all florfenicol treatments in this study were above the labeled dose (MSD, "Aquafen 50%: Detalle de producto"). It is also possible that fish grow over the course of the treatments and the dose is not adjusted to account for the increase in biomass, resulting in under-dosing towards the end of treatment. During the course of a treatment, fish growth can be significant (2 kg fish at 10 °C can gain up to 0.8% body weight daily (Austreng et al., 1987); however, most producers are well aware of this and adjust their prescriptions to account for this phenomenon. Interestingly, there was no trend in the raw data to indicate that this occurred for oxytetracycline treatments, but this antibiotic has a much longer half-life (Elema et al., 1996) so would be less susceptible to changes in dose or consumption over time.

We observed a positive trend in the raw data between the treatment dose of florfenicol and the proportion of individuals with antibiotic concentrations above MIC90 levels for all doses except the highest dose of florfenicol; however, this trend was not statistically significant in our

mixed model. The lack of statistical significance may have been due to the fact that we only analyzed 52 florfenicol treatments, and most of these treatments were at a 20 mg/kg/day dose (n = 47). A larger sample size with greater variability in dosage would help clarify the association between dose and tissue concentrations. With a larger sample size, we also could have controlled for the source of antibiotics, as there may be differences in the quality of the products. Given the low tissue concentrations in the group of fish treated at a dose of 30 mg/kg/day, producers should be cautious about increasing the dose of florfenicol, as a management strategy to address the low proportion of fish above the MIC90 level, until this has been further investigated.

One of the limitations of this study is that we did not know the outcomes of the treatments (i.e., failure or success), so we could not establish whether the proportion of fish above the MIC90 was directly related to treatment success. A better understanding of this relationship would help farmers interpret the clinical significance of data pertaining to antibiotic tissue concentrations. Another limitation of our study, which limits its external validity, is the fact that we only had data from a few companies and, although these data came from 34 farms located in 11 different management areas and it represented 10–15% of the total number of farms that operated during our study period, it did not represent many of the feeding strategies used by the industry. In fact, it did not include samples from fish fed using micro-rations, which is now commonly used by the industry (Skretting, 2016). This is a feeding method in which the delivery of the daily ration is divided into multiple meals to minimize the competition for food and improve feed distribution. Research should be conducted to assess the potential benefit of this feed delivery method for achieving adequate antibiotic concentrations in fish tissues under commercial conditions.

5. Conclusions

Although our study had constraints that limit the interpretations of our findings, it still identifies an issue with antibiotic delivery in aquaculture and may help explain the poor florfenicol treatment responses reported for SRS in Chile. Based on our analysis, the low proportion of fish with tissue concentrations above the MIC90 during a florfenicol treatment is likely associated with the short half-life of this product and the inconsistent consumption of feed within cages of fish. This preliminary study warrants further detailed investigation to determine how to obtain better distribution of antibiotic in populations and whether antibiotic tissue concentrations correlate with treatment success under field conditions.

Acknowledgements

This research was undertaken, in part, thanks to funding from the Canada Excellence Research Chair Program. We would like to thank William Chalmers for editorial assistance in the preparation of the manuscript, and our industry partners for insightful discussions and access to their data.

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