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Variation in the concentration of antibiotics in tissue during oral antibiotic treatments in farmed salmonids

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ABSTRACT

Despite numerous control efforts, piscirickettsiosis remains the main cause of mortality due to infectious agents in Chilean salmonid aquaculture. In-feed treatments with antibiotics are commonly used to control this disease; however, a high proportion of these treatments are ineffective. The inconsistent treatment results have prompted a search for the cause of failure. Antimicrobial resistance has been proposed as one of the reasons for treatment failure, but recent studies place the proportion of resistant isolates well below the observed failure level. Another source of treatment failure may be the inadequate dosage of antimicrobials. We describe the tissue concentration in fish mid- and post-treatment for two commonly used antibiotics: oxytetracycline and florfenicol. We used mixed-effects linear models to assess the variation of concentration of antibiotics in the tissue of fish and evaluate the factors that may be associated with this variation. We found that most of the variation in antibiotic tissue concentration occurred between individuals in the same pen, and the second largest source of variation was between treatment events. Among the factors associated with antibiotic tissue concentrations, the weight of fish was highly significant. Other factors associated with antibiotic concentration in fish were species, water temperature, and days since the start of treatment. We discuss several hypotheses for the variation in our data and suggest future research to improve the concentration of antimicrobials in fish tissue during in-feed treatments.

1. Introduction

In 1989, piscirickettsiosis was described for the first time in Chile (Bravo and Campos, 1989). Since then, this disease, caused by *Piscirickettsia salmonis* (Fryer et al., 1992), has reached industry-wide distribution, affecting all species of salmonids produced in Chile, and is now the main cause of mortality for this industry during the seawater grow-out phase (Sernapesca, 2016). Although the disease has been described in other salmonid industries (Brocklebank et al., 1992; Brosnahan et al., 2016; Corbeil et al., 2005; Grant et al., 1996; Jones et al., 1998; Olsen et al., 1997; Rodger and Drinan, 1993), it does not occur on the same scale it does in Chilean salmon aquaculture. Numerous efforts, private and public (Sernapesca, 2012), have been made to prevent and control this disease. Vaccines against *P. salmonis* have been available since 1998, but to date no product has been able to protect fish from disease for the complete seawater grow-out period. The most frequent control measure for piscirickettsiosis is treatment with in-feed antimicrobials (Jakob et al., 2014), but the success of these treatments has been inconsistent, and often treatments fail to stop the progression of disease (Jakob et al., 2014; Rozas and Enríquez, 2014).

The reasons for treatment failure are still poorly understood. Assuming that the disease has been correctly diagnosed, likely causes for treatment failure include antimicrobial resistance (AMR) and inadequate dosing of antimicrobials. The identification of AMR-conferring genes in quinolone-resistant *P. salmonis* isolates (Henríquez et al., 2014) and other bacteria present in sediment (Buschmann et al., 2012) may explain the failure of some of these treatments. However, a recent study shows that *P. salmonis* remains largely susceptible to florfenicol and oxytetracycline (Henríquez et al., 2015), the two most widely used antimicrobials in Chile (Sernapesca, 2017). On the other hand, the doses currently used for antimicrobials were established on the basis of laboratory experiments with small populations of healthy individuals in a controlled environment where the uptake of medicated feed of each individual was carefully manipulated. However, in fish

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populations under field conditions individuals consume an uneven amount of feed that may result in varying tissue concentration levels and treatment efficacy (Coyne et al., 2004a).

The objectives of this study were to assess the variation in the concentration of antimicrobials in tissue of fish raised commercially, and evaluate factors that may be associated with this variation.

2. Material and methods

Using data on antibiotic treatments conducted between June 2010 and February 2016, supplied by the aquaculture industry, we assessed the variation in concentration of antibiotic in tissue obtained from skin-on muscle samples taken from fish at the mid-point (day 7) and the end (day 14) of oral treatments with oxytetracycline or florfenicol against piscirickettsiosis. During the sampling, additional data such as the species, weight of fish, days since the start of treatment, antibiotic used, prescribed dose, and identification of pen and farm were also recorded. Each farm in our study was treated one or more times, and one or two pens were sampled during each treatment. The tissue samples obtained from 5 to 10 fish from each pen were tested for their respective antibiotics concentrations using high-performance liquid chromatography (HPLC) (Hormazabal et al., 1993; Reveurs and Díaz, 1994) and the results were reported in parts per billion (ppb). The florfenicol levels reported included the amine breakdown product and the active form of the drug. A more detailed summary of the sample collection and methodology was published in Price et al. (2018), where the probability of individual fish having tissue concentrations above certain thresholds was evaluated.

Descriptive statistics and graphics were used to summarize the variation of tissue concentration by antibiotic product, species, and farm. To analyze the factors associated with tissue concentration, mixed-effects linear models were fitted for each antibiotic separately. Farm, treatment event, and pen were included as random effects, while days since the start of treatment (7 or 14), water temperature in Celsius degrees (°C), fish weight in kg, species, and dose (mg/kg/day) were evaluated as fixed effects. In our database, the prescribed dose for oxytetracycline was always 100 mg/kg/day, while florfenicol was dosed at 15, 20, 25, and 30 mg/kg/day.

For our regression models, we log-transformed the drug tissue concentrations to meet the model assumption of equal variances. To avoid errors in the computation of the natural logarithm when the values were reported as 0 (not detected) we added 30 ppb (the detection limit) to the observed concentrations.

A set of models with no fixed predictors (null models) was fitted to assess the variance at the different hierarchical levels. Subsequently, we included all fixed predictors (days since start of treatment, water temperature, fish weight, species, and dose) in our random effects models to assess which predictors were significantly associated with the concentrations of antibiotics in fish tissues. For our final model, we only retained factors with a P-value $< .05$ based on the likelihood ratio tests. All plots and statistical analyses were done in R 3.3.3 (R Core Team, 2017), and the package lme4 (Bates et al., 2015) was used to fit the mixed-effects models.

3. Results

Thirty-four farms contributed to our study, with samples obtained from 87 treatment events. During each treatment event 1 or 2 pens were sampled, for a total of 119 pens. Our analyses comprised a total of 2231 muscle samples obtained during treatments against piscirickettsiosis; 1403 samples were from Atlantic salmon treated with florfenicol, 767 samples were Atlantic salmon treated with oxytetracycline, while 224 samples were rainbow trout treated with florfenicol, and 61 were rainbow trout treated with oxytetracycline.

The concentration of antibiotics in tissue varied widely by species and product (Fig. 1). The average concentration of florfenicol was approximately 4000 ppb, but a difference by species was observed. The average concentration of florfenicol in tissue was 3021 ppb for Atlantic salmon and 8490 ppb for rainbow trout. For oxytetracycline, the average concentration was 2940 ppb, which was quite similar between species: 2970 ppb in Atlantic salmon and 2560 ppb in rainbow trout.

The distribution of concentrations was markedly right-skewed, and varied by species and antibiotic product (Fig. 1). We found that some individuals had extremely high concentrations. For example, 5% of the fish treated with oxytetracycline and florfenicol were above 12,352 ppb and 6523 ppb, respectively. A summary of the data, in terms of mean, median, minimum, maximum, and interquartile range, is provided in Table 1.

With the exception of the 30 mg/kg/day dose, the median concentration of florfenicol in tissue rose higher as the dose was increased (Fig. 2). Regarding the number of days since the start of treatment, we observed that for florfenicol, the median concentration of the product in tissue was higher during the middle of the treatment than at the end of treatment, while for oxytetracycline the trend was less evident and depended on the species (Fig. 3).

Trends were also observed with weight of fish and temperature. In our database, concentrations were generally lower in smaller than average size fish. In general, we also observed that tissue concentrations decreased as temperature increased (Fig. 4).

Undetectable levels of florfenicol were reported for 38 samples, while undetectable levels of oxytetracycline were reported in 10 samples. We dropped two florfenicol treatment events from our statistical analyses because weight of the fish was not recorded.

In our final florfenicol regression model, only number of days since the start of treatment ($P < .01$), fish weight in kg ($P < .01$), and species ($P < .01$) were significant fixed predictors; however, we also retained water temperature ($P = .13$) to control for its small confounding effect (Table 2). Our final oxytetracycline model only had two significant predictors: log transformed fish weight ($P < .01$) and water temperature ($P = .03$) (Table 3).

For both florfenicol and oxytetracycline our null models indicated that most of the variance in the antibiotic tissue concentrations was at the fish and treatment event levels. A summary of the random effects for our models, with and without fixed predictors, is presented in Table 4.

4. Discussion

We assessed the concentration of antibiotics in tissue during in-feed treatments against piscirickettsiosis. This is important because an association between therapeutant concentration and treatment efficacy has already been established for several fish diseases and antibiotics (Gaunt et al., 2004; Soto et al., 2013; Vik-Mo et al., 2005). Tissue concentration of antibiotics, when delivered via feed, depends on feed consumption, the rate of absorption, metabolism, and the excretion of the product by the animal. Ideally, when antibiotics are administered to treat a fish population, all fish within the population would have similar and adequate levels of drug in their target tissues. However, our analysis of 87 treatments on 34 farms suggests that not all fish within pens have similar levels of antibiotics at the end of their in-feed treatments. We found a wide range of concentrations of antibiotics in muscle tissue for both rainbow trout and Atlantic salmon, especially for fish treated with florfenicol (Fig. 1). The range of tissue levels for fish treated with florfenicol was from 0 (not detected) to 38,417 ppb. The range of tissue concentration for fish treated with OTC was from 0 (not detected) to 14,747 ppb.

Having different concentrations of antibiotics in a population is a concern because, on the one hand, a proportion of the population may be exposed to sub-therapeutic antibiotic concentrations, increasing the risk for the development of AMR (Andersson and Hughes, 2014). On the other hand, another sub-group of the population may be receiving excessive amounts of antibiotic and may be at risk of toxic effects from the

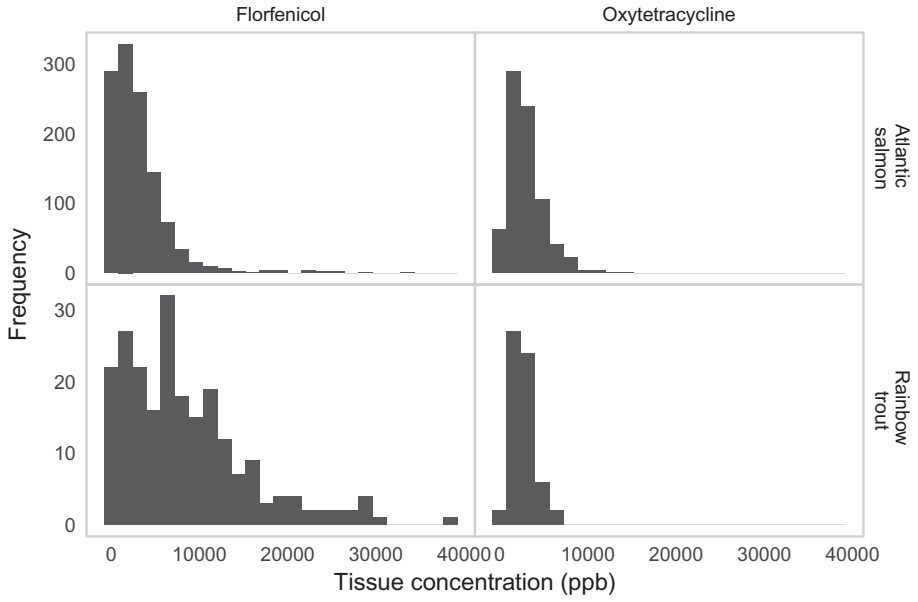

Fig. 1. Distribution of tissue concentration (ppb) by species and product.

Table 1
Summary statistics of tissue concentration (ppb) by species and product.

Product	Species	N°	Average (ppb)	Median (ppb)	Minimum (ppb)	Maximum (ppb)	25th percentile (ppb)	75th percentile (ppb)	Interquartile range (ppb)
Florfenicol	Atlantic salmon	1179	3021.4	2232.0	0.0	33104.6	833.4	4089.0	3255.6
	Rainbow trout	224	8490.0	6870.5	0.0	38417.0	3073.4	11695.0	8621.7
Oxytetra-cycline	Atlantic salmon	767	2970.5	2582.7	0.0	14,747.0	1624.5	3844.1	2219.6
	Rainbow trout	61	2560.8	2451.1	234.5	6642.7	1775.2	3023.3	1248.1
Total		2231	3540.4	2565.0	0.0	38417.0	1309.9	4402.9	3093.0

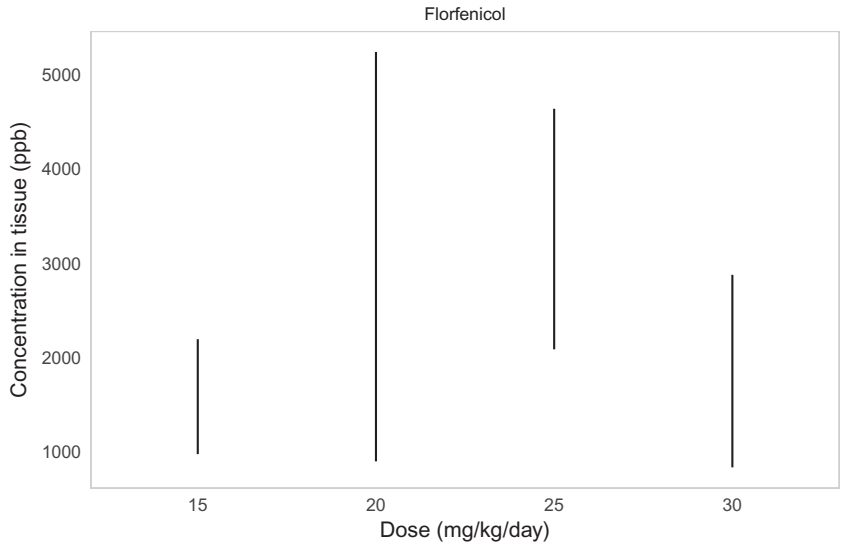

Fig. 2. Median florfenicol concentration (ppb) in tissue by prescribed dose, bars represent the interquartile range.

product. The individuals with high concentrations of antimicrobials in their tissues may also pose a food safety issue due to high residue levels in the meat.

Our variance component analyses suggested that for both florfenicol and oxytetracycline, most of the variation in the tissue concentration occurred between fish within the same pen and between treatments (Table 4, Fig. 5). Differences in antibiotic concentration in individual fish within pens are likely driven by several factors. For example, hierarchical behavior of fish within a population, which may lead to differences in feed consumption between individuals within the pen (Talbot et al., 1999), likely contributes to some of the variation in the antibiotic tissue concentration. It has been suggested that dominant fish generally consume proportionally larger amounts of feed than submissive fish by restricting sub-dominant individuals' access to food, and

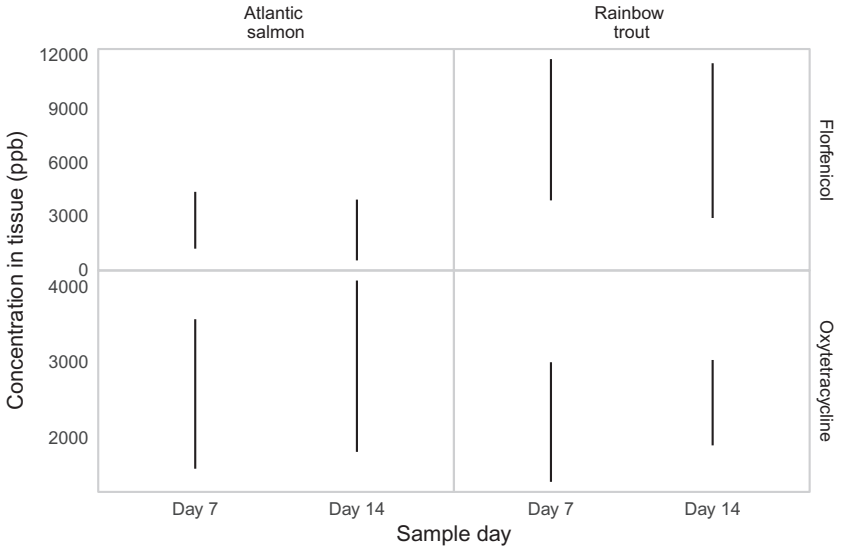

Fig. 3. Median concentration of antibiotic in tissue at days 7 and 14 after treatment start, bars represent the interquartile range.

this behavior can be exacerbated when feed availability is restricted in quantity and delivered in a confined area (Ruzzante, 1994).

Although we did not have specific information on the behavior of the fish in our study, our findings showed that weight, which largely explains dominance in salmonid populations (Symons, 1968), was significantly associated with florfenicol and oxytetracycline tissue concentrations. Including this predictor in our model explained some of the individual fish level variation in the data. For both products, larger fish had higher concentrations of antibiotics in tissue, and this association was consistent with what we expected, based on the hierarchical behavior of these species. It is also possible the large fish consumed proportionally the same amount of food as the smaller fish but did not metabolize or excrete the product at the same rate as smaller fish.

Several mitigation strategies to reduce the effect of hierarchical behavior of fish are used by farmers to minimize the competition for food within the pen. Dividing the population by size (i.e. grading) and delivering the daily ration in only one or two meals are two commonly used strategies. A consequence of delivering medicated feed only once or twice daily for products like florfenicol, is that, with a half-life of 12 h at 8 °C (Martinsen and Horsberg, 1995) therapeutic concentrations of this antibiotic might be difficult to maintain for extended periods of time, specially, if fish miss a meal. Thus, frequency of feeding should be considered when dosing with antibiotics. A newly-introduced feeding method, which may reduce the size variation in a pen is the use of micro-rations; however, this practice was not commonly used prior to our study. We were not able to evaluate the effect of feeding frequency on tissue concentration of antibiotics because we did not have detailed information regarding feeding frequency during treatments. The recent

Table 2
Coefficients, standard error (SE), P-value, and 95% confidence intervals for florfenicol model (fixed terms only).

Term	Coefficient	SE	P-value	(95% Conf. intervals)
(Intercept)	8.10	0.82		(6.5–9.78)
Sample day			< 0.01	
Day 7	(reference)			
Day 14	−0.50	0.07		(−0.63 to −0.37)
Fish weight (kg)	0.19	0.04	< 0.01	(0.11–0.28)
Species			< 0.01	
Atlantic salmon	(reference)			
Rainbow trout	1.48	0.36		(0.79–2.20)
Temperature (°C)	−0.09	0.06	0.13	(−0.22–0.02)

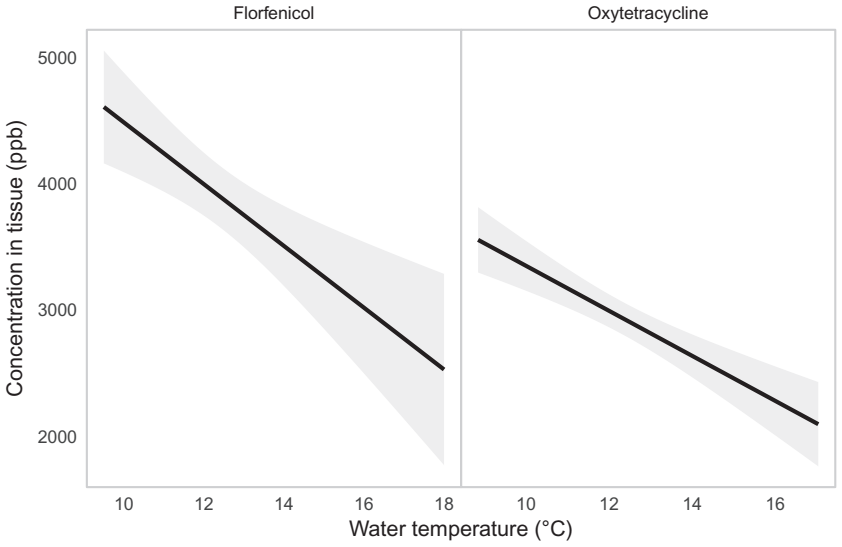

Fig. 4. Univariate regression line between water temperature and concentration of antibiotics in tissue.

Table 3
Coefficients, standard error (SE), P-value, and 95% confidence intervals for oxytetracycline model (fixed terms only).

Term	Coefficient	SE	P-value	(95% Conf. intervals)
(Intercept)	5.67	0.84		(3.97–7.57)
Log fish weight (Kg)	0.46	0.08	< 0.01	(0.29–0.62)
Temperature (°C)	−0.10	0.05	0.03	(−0.19 to −0.01)

shift towards the use of micro-rations may make some of our findings hard to extrapolate to the current situation in Chile.

Another reason fish may not be eating evenly is that some individuals are sick. This source of variation was likely minimal in our study because our samples were collected from the sub-clinical populations during the first feed of the day. We believe, had we captured more sick fish in our sample, the range of antibiotic tissue concentrations would have been even greater than observed.

The second largest source of variation in our data was attributable to treatment effect. This finding was consistent with our visual assessment; the differences in concentration between treatments were larger than the differences within treatment (Fig. 5). Having a significant proportion of the variation at the treatment level suggests there are factors that vary from treatment to treatment. Factors that may vary over time on a farm, such as the dosage of the antibiotic, the preparation of the (medicated) feed, and frequency of feeding, could explain this variation.

One source of variation at the treatment level would be the dose of the medication incorporated into the feed. Although the dose was not statistically significant in our regression models it is possible that we lacked statistical power and variation in our dosing data to detect a difference. Approximately 90% of the samples were obtained from fish treated with the 20 mg/kg/day dose. It is also worth noting that the treatment with the highest dose (i.e. 30 mg/kg/day) had one of the lowest median concentrations. It is possible that, in this case, the dose was increased in response to a severe outbreak of piscirickettsiosis. When medicated feed is delivered under these conditions, it is likely that the population has a diminished appetite or goes off-feed at some point during the treatment period (Coyne et al., 2004a, 2004b; Rigos et al., 1999), resulting, paradoxically, in lower concentrations in fish treated with the highest dose.

This decrease in feed consumption during the course of a treatment may also explain why the concentration of florfenicol in tissue was higher on day 7 than on day 14 (Fig. 3). The difference between the antibiotic concentration in samples obtained at day 7, compared to samples from day 14, might also be related to a larger than expected biomass gain during the treatment. If the disease has no negative effect on the growth of the fish, differences between estimated and real biomass gain may arise and should increase in time, resulting in lower than expected tissue concentrations towards the end of the treatment. Regardless of the reason for the difference, the trend was very consistent and unlikely to be spurious. Future research should focus on determining the cause of this difference to take effective corrective actions.

As discussed previously, farmers modify the rate and frequency of feed delivery to minimize the competition for food between individuals. But feed characteristics, such as how the antibiotic is incorporated into the pellet, either by coating the product on the pellet surface (top-coating) or incorporating the product in the pre-mix or the oil, may also have an impact on the palatability of the feed, and the intake and absorption of the product. Researchers have found that top-coated antibiotics may be lost upon contact with the water. Leaching of the antibiotic from the medicated feed has been described as time and temperature dependent, and has been documented for oxytetracycline and oxolinic acid (Duis et al., 1995; Rigos et al., 1999), amoxicillin and sulphamethoxazole (Duis et al., 1995), and florfenicol (Yanong et al., 2005). It is also possible that the antibiotic product somehow interferes with the palatability of the feed, especially when it is top-coated, which may also lead to a decrease in feed consumption over time. However, the available literature indicates that florfenicol is palatable at doses as high as 100 mg/kg/day (Inglis et al., 1991).

Other factors that may affect variation in the concentration of antibiotics at the treatment level are environmental. In our study, we were only able to assess the effect of water temperature on antibiotic tissue concentrations. The poikilothermic nature of fish means they cannot regulate their body temperature and, therefore, water temperature has a significant impact on fish metabolism, including the metabolism of pharmaceutical products (e.g: Rigos et al., 2002). In our models, temperature had a negative coefficient, i.e., the concentration of antibiotics in tissue decreased as the temperature increased. This was expected, given that the general metabolism of the fish is faster at higher temperatures. We expected the effect of temperature to be more pronounced for products with a short half-life such as florfenicol, but despite the observed trend, this predictor was significantly associated with tissue concentrations only for oxytetracycline. In our florfenicol model, temperature was retained despite not being significant because we observed a small confounding effect with species.

In our analysis, pen-level variance was relatively small. This was not surprising considering that, within a farm, only a few factors are different between pens. The most notable difference between pens during an SRS outbreak is the mortality level. Price et al. (2016) found an association between mortality level and treatment success, but in our data mortality was not reported, so we could not assess this at the pen level. Further, our samples were collected in the morning during the first feeding of the day which likely selected for sub-clinical animals that were still on feed.

The variance in the data attributed to the farm level was also small. The only farm-level factor we could assess in our study was species. In our data, rainbow trout generally had higher concentrations of

Table 4
Comparison of random-effects variance and variance proportion by level for the florfenicol and oxytetracycline models without fixed predictors (null) and final models (final).

Model	Level	N	Null		Final	
			Variance	Proportion (%)	Variance	Proportion (%)
Florfenicol	Observation	1249	1.30	53.5	1.22	56.1
	Pen	99	0.14	5.9	0.16	7.6
	Treatment	52	0.73	30.0	0.57	26.0
	Farm	26	0.26	10.6	0.23	10.4
	Total		2.43		2.18	
Oxytetracycline	Observation	828	0.42	45.4	0.40	44.6
	Pen	62	0.07	7.9	0.07	7.6
	Treatment	33	0.40	44.0	0.43	47.9
	Farm	22	0.02	2.7	0.00	0.0
	Total		0.91		0.90	

Fig. 5. Median concentration of antibiotics in tissue by treatment event, species and product, bars represent the interquartile range.

antibiotic in their muscle tissue than Atlantic salmon. Interestingly, species was only significantly associated with tissue concentrations in our florfenicol model. Given that oxytetracycline was used in only three treatment events in trout, it is possible that this lack of significance was due to a lack of power.

Although only a few companies contributed data for this study, we feel these farms constitute a representative sample of the conditions under which in-feed treatments usually occur; however, given some of the limitations in the manner in which the samples were collected, we caution readers who may wish to extrapolate these results directly. We believe that, if anything, we have under-estimated the population-level variation in tissue concentrations after in-feed antibiotic treatments.

Another limitation of this study was the absence of the clinical status of the fish sampled, which prevented us from assessing the clinical outcome of the tissue concentrations. However, other authors have found an association between dose of medication administered (Gaunt et al., 2004; Soto et al., 2013) or tissue concentration (Vik-Mo et al., 2005) with total mortality after challenges in laboratory studies.

5. Conclusions

Although we could not explain a lot of the variation in the concentration of antibiotics in fish, our study confirms that in-feed antibiotics are not evenly distributed in fish on saltwater net pen sites. Our variance component analyses suggest that future studies should focus on management strategies that improve the distribution of antibiotics within pens of fish and between treatments. Increasing the amount of feed offered to the fish may partially solve the issue of the smaller individuals' reduced access to feed. However, this strategy will likely result in larger fish consuming an excess of food, increasing the risk of unwanted side effects in these individuals. Furthermore, the excess of food may result in waste of medicated feed into the environment increasing the risk of selecting for AMR genes in under-cage bacteria populations. Therefore, other solutions must be explored such as strategies to minimize competition for food between fish, as well as methods to optimize feed mixing and delivery.

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