2019 EMB Resistance Bioassay: Cedar Coast Field Station

by

Christian Carson, Robert Humenny

at

Cedar Coast Field Station

Abstract:

In 2018, resistance to the sea lice pesticide emamectin benzoate (EMB) was reported to have developed on multiple open-net pen salmon farms in Clayoquot Sound, British Columbia. In BC, EMB is the primary pesticide used by salmon farms to control their sea lice populations. Without EMB or alternative treatments, sea lice management can become impaired. Ineffective management can lead to a dramatic increase in sea lice infestations on farms and ultimately endanger wild salmon populations. Substantial resistance to EMB has developed throughout many salmon farming regions around the world, whereas British Columbia is one of the exceptions. Acute instances of EMB resistance have occurred in several farming regions of BC since 2015, but widespread resistance has not been observed. Cedar Coast Field Station conducted a bioassay to determine a baseline for sea lice sensitivity to EMB. When compared to bioassays in the future, this baseline could be used to determine whether sea lice have developed resistance to EMB. This research aims to determine how sensitive "wild-type" sea lice are to EMB treatments. Motile stage sea lice of both sexes with the exception of adult females were collected off adult wild salmon caught by sport fisherman in Clayoquot Sound. These sea lice were exposed to various concentrations of EMB. We recorded the response of sea lice after 24 hours of submersion in each concentration. We used a generalized linear fixed-effect model to analyze our data. This analysis provides no evidence of resistance being detected, as resistance can only be described across several bioassays. We calculated EC_{50} values for males and females of each life stage. PAL II males were sensitive to an EC_{50} of 119.6 ppb, PAL II females were sensitive to an EC_{50} of 46.3, and adult males were sensitive to an EC_{50} of 152.2. Future bioassays should be performed to determine if resistance to EMB has developed in "farm-type" sea lice parasitizing wild juvenile salmon throughout Clayoquot Sound.

Introduction:

The salmon louse (*Lepeoptheirus salmonis*) is a naturally occurring crustacean ectoparasite of wild salmon. In high numbers, sea lice can negatively affect salmon survival, especially in juvenile fish (Bateman et al., 2016). The pathological effects that sea lice have on juvenile salmon can negatively impact their populations, reducing the economic productivity of both wild and farmed stocks (Bateman et al., 2016; Peacock, 2015). Sea lice transmission is influenced by many factors, notably: temperature, salinity, light, host presence and abundance (Brooks, 2009; Costello, 2006; Stien, Bjørn, Heuch, & Elston, 2005). Open net-pen salmon farms are a major threat to wild juvenile salmon populations as they travel along their migration routes (Dill et al., 2011). The effects of salmon aquaculture, coupled with the effects of overfishing and climate

change, suggest wild salmon stocks of the Pacific Northwest are vulnerable to collapse if trends continue (Costello, 2009).

Salmon aquaculture increases the transmission of sea lice to juvenile wild salmon as they migrate past salmon farms (Krkošek, Lewis, & Volpe, 2005). In a farmless environment, juvenile salmon rarely are parasitized by sea lice because of the gaps in seasonal migrations of juvenile and adult salmon, which reduces interaction between the generations (Costello, 2009). Salmon farms operate year-round, with farms holding up to 500,000 salmon. Juvenile wild salmon are exposed to unnatural levels of sea lice infestation when they pass near salmon farms (Krkošek et al., 2005). Juvenile salmon have not yet developed competent immune and osmoregulatory systems, as well as lack the scales and body mass necessary to resist sea lice infestations (Sackville, Tang, Nendick, Farrell, & Brauner, 2011). Sea lice infestations have risen in regions of the ocean where salmon farms are present (Costello, 2009). As a result, there has been a global increase in the use of pesticides in an attempt to reduce sea lice impacts on both wild and farmed salmon populations (Burridge, Weis, Cabello, Pizarro, & Bostick, 2010).

One major pesticide used to control sea lice on farmed salmon is emamectin benzoate (EMB), commercially distributed as SLICE® (Merck, Canada) (Bateman et al., 2016). EMB is administered as an in-feed treatment which is ingested by salmon and becomes concentrated in their tissues. In most of the major salmon farming regions of the world, sea lice populations have developed biological resistance to EMB (Lam, Rosanowski, Walker, & St-Hilaire, 2020). Biological resistance in sea lice develops when individuals that are resistant to pesticides reproduce after each pesticide treatment. If the offspring of resistant sea lice inherit the resistance trait, they will also be less sensitive to the pesticide. If the same pesticide is used as a treatment for each generation, the following generations will be even less sensitive to the pesticide (Aaen,

Helgesen, Bakke, Kaur, & Horsberg, 2015). The longer this cycle continues, the more potential there is for the development of resistance. The evolution of biological resistance to a pesticide can develop rapidly when there is an overreliance on a single pesticide for treatment (Torrissen et al., 2013). The ease of use and relatively modest price of EMB has led to overuse in many regions around the globe (Igboeli, Fast, Heumann, & Burka, 2012). Salmon farms in Chile were among the first farms to have populations of sea lice that had developed resistance to EMB and were no longer effectively controlled through its use as a pesticide. Today Chile is on the brink of losing all control options, as sea lice resistance to EMB has developed to unprecedented levels (Bravo, Sevatdal, & Horsberg, 2010; Igboeli et al., 2012).

In British Columbia (BC), larger wild salmon populations are more likely to reduce the potential for the development of resistance to EMB by sea lice (Kreitzman et al., 2017). Adult Pacific salmon in offshore waters or on feeding grounds maintain populations of wild-type sea lice (Kreitzman et al., 2017). Wild-type sea lice have not been exposed to EMB and thus should not carry genes that would offer resistance. Adult Pacific salmon carry wild-type sea lice back to the nearshore environment during their migration, bringing sea lice to farmed salmon (Dill et al., 2011). When wild-type sea lice reproduce with farm-type sea lice, resistance to EMB becomes less prominent in the population. Thus, larger wild salmon populations contribute to EMB being an effective pesticide for the control of sea lice (Fisheries and Oceans Canada, 2018; Kreitzman et al., 2017).

Clayoquot Sound, located on the West coast of Vancouver Island, has a high density of salmon farms. The farms in this region have tested for sea lice resistance to EMB over the last decade with no indication of resistance until 2017 (Clayoquot Salmon Roundtable, 2018; Waddington,

2018). CERMAQ Canada Ltd., one of Canada's largest salmon aquaculture companies, reported evidence of sea lice resistance to EMB in Clayoquot Sound (Clayoquot Salmon Roundtable, 2018a; Waddington, 2018). In agreement with CERMAQ's findings regarding EMB resistance, independent monitoring by Cedar Coast Field Station (CCFS) in Clayoquot Sound, reported the highest sea lice levels ever recorded as of the springs of 2018 and 2019 (Bartlett, Simmerling, & Hunter, 2018; Morton & Wristen, 2018). Farmed salmon averaged between 11 and 50 sea lice per fish (DFO, 2019), and wild juvenile salmon averaged 8 sea lice per fish with a prevalence of 96% (Bartlett et al., 2018).

Due to a biological resistance to EMB on farms in Clayoquot Sound in 2018, the industry was issued an emergency drug release to use hydrogen peroxide bath treatments in order to control farm-type sea lice populations. The majority of the local salmon farming industry in Clayoquot Sound continued to use EMB on farms even though resistance had been detected (DFO, 2019). Considering the possibility of increased resistance both now and in the future, Cedar Coast Field Station is independently investigating whether wild-type sea lice in the Clayoquot Sound have developed resistance to EMB. The purpose of this study was to develop a procedure for EMB resistance bioassays in Clayoquot Sound and to collect data to establish a baseline EMB sensitivity in wild-type sea lice. This report lays out a baseline of EMB resistance in wild-type sea lice for the summer of 2019.

Methods

Our methodology and analysis are analogous to a previous bioassay done by Bateman et al., in 2016 at the Salmon Coast Field Station in the Broughton Archipelago, BC. The team conducted a bioassay on *Lepeophtheirus salmonis* from wild juvenile salmon in the Broughton Archipelago to test for salmon louse resistance to EMB. Here we use a similar bioassay protocol and

statistical analysis to determine the effective baseline concentration of EMB that resulted in 50% survival of salmon louse (EC_{50}) (Bateman et al., 2016).

Sea lice collection

Sea lice *(Lepeophtheirus salmonis*) were collected from adult Coho salmon (*Oncorhynchus kisutch),* and Chinook salmon (*Oncorhynchus tshawytscha*) caught by sport fishers in Clayoquot Sound during July and August of 2019. We assume that the sea lice we collected were wild-type as the adult fish were migrating back towards their spawning grounds from the open ocean at the time of collection. Both male and female pre-adult stage 1 (PAL I) and pre-adult stage 2 (PAL II) as well as male adult sea lice were collected. Sea lice were stored in 500ml containers of seawater and kept at 12 °C, in a small cooler with ice packs as a means of transport to the station. At the station, we changed the seawater in the containers and placed them with their lids off in a large styrofoam cooler, which was also kept at 12 °C. The water the sea lice were stored in was kept oxygenated with aquarium bubblers and its temperature was monitored every 4 hours to make sure it was between 10°C and 12°C. The sea lice were left for 20 hours to ensure they were suitable for the trial. We removed dead or moribund sea lice while monitoring the temperature to ensure a healthy trial population.

Preparation

We created a stock solution of EMB by mixing 100mg of EMB in its powdered form with 1000ml of methanol and left it for 24 hours in order for the EMB to dissolve fully. We kept the stock solution in a cooler at 12°C to avoid degradation. From the stock solution, we created a working solution at 1000ppb EMB (2ml of stock solution and 198ml of seawater) before every trial. Then, we diluted the working solution with seawater to produce six EMB concentrations at

0ppb (control), 31ppb, 63ppb, 125ppb, 250ppb, and 500ppb. From these concentrations, six trial baths were prepared using Petri dishes. Each dish contained 40ml of each concentration.

Trials

Only healthy sea lice were used from our collection to perform the bioassay. For which, we conducted a total of eight trials. We performed all our trials 20 hours after collection, following the approach outlined in "Sea lice resistance to chemotherapeutants: A handbook in resistance management with different concentrations" (SEARCH Consortium, 2006). Using a dissection microscope, we organized sea lice by sex and life stage and evenly dispersed them across the six pre-selected concentrations. Sea lice were placed in their designated concentration bath and were exposed to the treatment for 24 hours. Each collection day, we collected as many suitable sea lice as possible and used all the healthy sea lice collected from this sample for each trial. Due to day to day variation in sample size from our collections, the number of sea lice used in our bioassay also varied from trial to trial. After the trial started, we measured the temperature of the baths every 4 hours and changed the ice packs to ensure the sea lice were kept at a temperature between 10°C and 12°C. The lids were kept off the petri dish baths in order to allow for oxygen exchange. After the trial, we assessed sea lice under the microscope to see what their condition was in response to the EMB concentration administered: dead, moribund or alive. Sea lice that suctioned to the dish and swam normally when disturbed were classified as alive. Sea lice that were still alive but no longer suctioned to the dish and did not swim when disturbed or in small current were classified as moribund. Sea lice that showed no signs of life we classified as dead.

Statistical analysis

Our data set consists of 289 sea lice from both sexes, spanning multiple life stages, which we collected from September 4th to September 18th in 2019. Our dependent variable was the binary

response of sea lice either 0 (alive) or 1 (dead or moribund), to our independent variable, the concentration of EMB which the sea lice were immersed for 24 hours. We fit binomial generalized linear models in R with a logit-link transformation (Bates, Maechler, Bolker, & Walker, 2015). Each of our models had all or some of the following fixed effects in varying combinations: EMB concentration, sex, and life stage (Table 2). We created seven models with varying combinations of fixed effects (Table 1). To find the best fitting model, we compared Akaike Information Criterion values and weights using the "MuMIn" and "Stargazer" packages (Barton, 2009; Hlavac, 2018) (Table 1). We analyzed our data and calculated the EC_{50} values for each sex and life stage of sea lice using the best fitting model. The EC_{50} is the concentration of EMB required to generate a 50% probability of a louse displaying a response of either moribundity or death. We then plotted the dose-response curves of females and males of varying life stages with their 95% confidence intervals using "ggplot2" (Fig. 1 & 2) (Wickham, 2016).

Results:

Our best-fitting model was Model 1, which had the fixed effects: concentration, louse sex, and louse stage (Table 1). Model 1 had the lowest AIC value and the highest AIC weight of our 7 models (Table 1). PAL II females displayed a greater sensitivity with an EC_{50} of 46.3ppb (Figure 1) when compared to the EC_{50} of 119.6 for PAL II males (Figure 2). Our sample size for both male and female PAL I sea lice was too small to make any accurate predictions regarding dose-response for this specific stage. Adult males were the least sensitive to EMB, with an EC_{50} of 152.2ppb (Figure 3). The EC_{50} results for all stages and sexes with 95% confidence intervals are summarized in (Table 3).

Table 1*:* All models with their fixed effects, estimated degrees freedom, AIC values, and AIC weights.

Model	Degrees of Freedom	AIC Value	AIC Weights
Model 1 - Concentration + Louse Sex +Louse Stage	5	268.969	8.343221e-01
Model 2 - Concentration + Louse Sex	3	273.426	8.988014e-02
Model 3 - Concentration + Louse Stage	4	273.769	7.572105e-02
Model 4 - Concentration	2	287.558	7.671899e-05
Model 5 - Louse Stage + Louse Sex	4	387.453	1.559108e-26
Model 6 - Louse Sex	2	388.251	1.046442e-26
Model 7 - Louse Stage	3	392.843	1.053356e-27

Table 2. EC_{50} values and their corresponding 95% confidence intervals calculated using Model 1. Our model calculated that less than 50% of female PAL1 sea lice would survive a trial with a 0ppb concentration of EMB due to an insufficient sample size.

Sex and Life Stage	Value (ppb)	Upper (ppb)	Lower (ppb)
Female PAL I	<0	45.2	<0
Female PAL II	46.3	88.7	0.8
Male PAL I	41.3	110.4	<0
Male PAL II	119.6	164.7	79.4
Adult Males	152.2	192.3	120.4

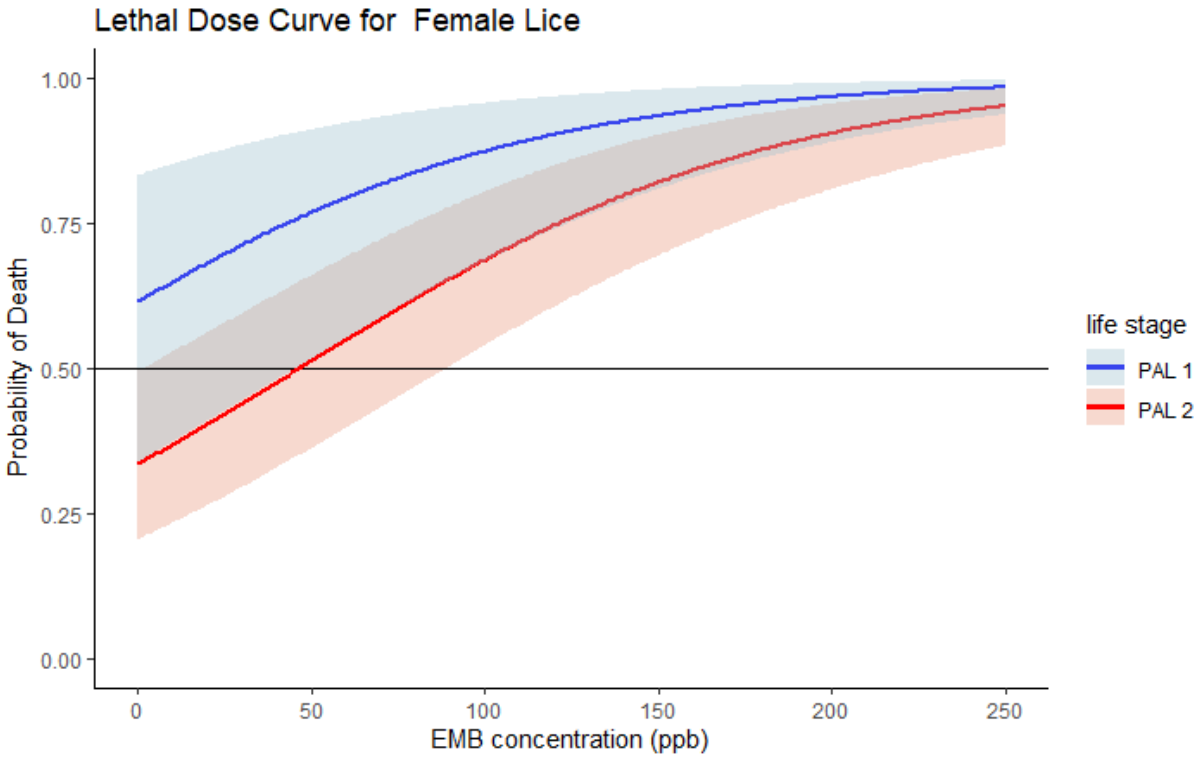

Figure 1. Dose-response curves for sea lice collected from wild adult salmon, showing the life stage-specific curves for female sea lice. The horizontal line indicates the EC_{50} of EMB needed to induce a response and the bars around the curves show the 95% confidence intervals for each stage.

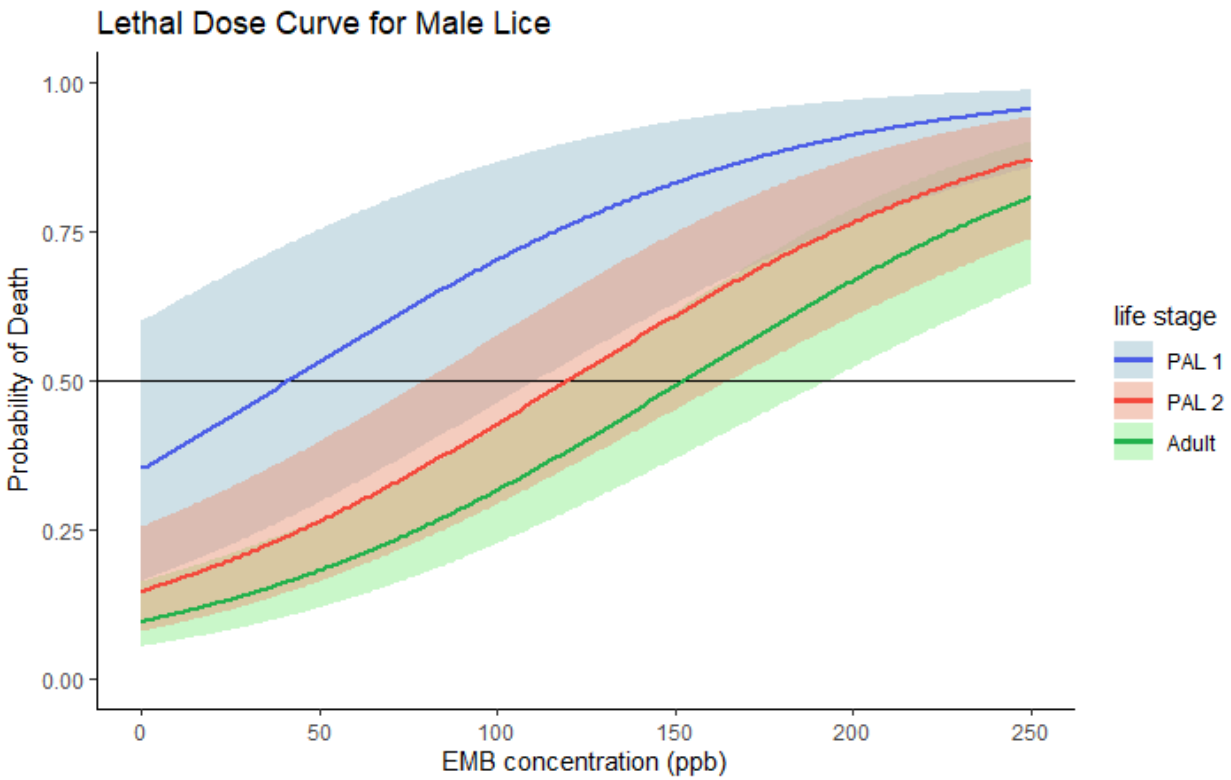

Figure 2. Dose-response curves for sea lice collected from wild adult salmon, showing the life stage-specific curves for male sea lice. The horizontal line indicates the EC_{50} of EMB needed to induce a response and the bars around the curves show the 95% confidence intervals for each stage.

Discussion:

Some of the results from our bioassay can be compared with the results from previous bioassays regarding sea lice EMB resistance (Marín et al., 2018). We found PAL II females to be significantly more sensitive to EMB than PAL II males which is consistent with the results from previous bioassays (Wescott, J.D., Stryhn, H., Burka, J. F., Hammell, 2008; Whyte, Westcott, Elmoslemany, Hammell, & Crawford, 2013). Sensitivity to EMB is related to the levels of P-glycoprotein (P-gp) mRNA expression. P-gp is an ATP binding cassette transporter is that known to be involved in drug resistance and known to interact with EMB (Igboeli et al., 2012). Male sea lice have been shown to express higher levels of P-gp than female sea lice and it has been suggested to cause the differences in EMB sensitivity between the sexes. The higher reproductive burden of female sea lice may also influence their sensitivity to EMB or their

ability to survive a trial (Igboeli, O. O., Purcell, S. L., Wottom, H., Poley, J., Burka, J. F., & Fast, 2013).

No conclusions can be made from our results regarding the EMB sensitivity of PAL I sea lice of either sex. PAL I males were not present in every concentration, and our sample sizes were too small for each sex. These shortcomings made it challenging to develop a useful model that explains the unusual result of a negative EC_{50} value for the female PAL I stage. Our inconsistent access to sea lice limited us in standardizing sea lice numbers across trials. This meant that the number of sea lice in an individual petri dish would be different between trials. Differing amounts of sea lice in petri dishes would result in differing rates of oxygen consumption between trials. This could potentially influence our results.

PAL II sea lice are the standard life stage used in bioassays to calculate EC_{50} values. PAL II sea lice are preferred for use in bioassays over all other stages because "PAL II" is a defined stage in the development of sea lice and all tests performed with this stage can be compared to one another (SEARCH Consortium, 2006). We decided to use all healthy motile sea lice from our collections, with the exception of adult females. We chose this procedure because we had limited access to motile sea lice and wanted to gain a better understanding of EC_{50} sensitivity across age and sex demographics. Other studies have also conducted tests across both PAL stages (I & II) of male and female sea lice and adult males (Marín et al., 2018; Whyte et al., 2013).

Our bioassay provides the first publicly available data regarding sea lice sensitivity to EMB in Clayoquot Sound. In order to confirm whether EMB resistance has developed in sea lice populations of Clayoquot sound, further bioassays must first be performed. EMB sensitivity determined by future bioassays, could then be compared to our results to determine if relative

resistance to EMB has developed. In turn, our bioassay results should not be interpreted as an indication of EMB resistance in wild-type sea lice of Clayoquot Sound. The purpose of this study was to develop a procedure for EMB resistance bioassays in Clayoquot Sound and to collect data to establish a baseline EMB sensitivity in wild-type sea lice. Through the dose-response curves, and the calculated EC_{50} values, we have successfully developed an EMB sensitivity baseline for wild-type sea lice of Clayoquot Sound. We have sent the sea lice we used for these EMB resistance trials to the Pacific Biological Station in Nanaimo for genetic analysis to see if resistance can be detected genetically within the population.

Recent years have shown a significant increase in sea lice infestations on both farms and wild juvenile salmon as well as lower returns in wild adult salmon (Bartlett et al., 2018). Farm audits from the Department of Fisheries and Oceans Canada has shown that farms are still using EMB in Clayoquot sound (DFO, 2019). Though the salmon farming industry continues its use of EMB, it does not allow public access to their data used in testing for sea lice EMB resistance. The continued use of EMB coupled with the increase in sea lice abundances on juvenile salmon suggests EMB resistance may have developed in Clayoquot sound (Bartlett et al., 2018; DFO, 2019). Management must enforce farms to use alternative chemotherapeutants or other methods of parasite control to reduce the transmission of sea lice to wild salmon (Bateman et al., 2016).

Future research by Cedar Coast Field Station aims to test for EMB resistance of farm type sea lice on wild juvenile salmon. Sensitivity to EMB treatments by farm-type sea lice populations should remain consistent over time if EMB is to be continued to be used in treatments to control lice levels. The low returns of adult salmon in 2018 should have limited the influx of wild-type sea lice into Clayoquot Sound's farm-type sea lice populations. This would theoretically result in sea lice populations with less sensitivity to EMB treatments. Our continued research on this

system allows us to test the wild-type sea lice hypothesis further, as Clayoquot Sound has limited wild salmon populations but is abundant with farmed salmon on farms.

The possibility of rapid development of EMB resistance should be taken seriously; resistance has developed over just a few years in other salmon farming countries throughout the world (Bateman et al., 2016). Thus, annual bioassays should be conducted using sea lice from both farms and wild juvenile salmon travelling through their migration routes. Annual bioassays would allow for early detection of the development of resistance and aid in the implementation of new management protocols. Along with annual monitoring, novel sea lice management methods should be developed and initiated in conjunction with current methods to prevent the development of EMB resistance in the future. With low returns of wild salmon across all of BC, the development of sea lice populations that are resistant to EMB treatments and therefore harder to manage poses an even greater threat to the recovery of the wild salmon populations of Clayoquot Sound.

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Appendix A.

Table 3: The fixed effects used in model 1 (m1). The left-most column signifies each fixed effect. Coefficients indicate how much each factor will affect the probability of death. Significant factors are indicated with asterisks; more asterisks indicate a higher level of significance. EMB concentration was shown to be the most significant factor in predicting the probability of a louse surviving its trial

	Coefficients	Standard Error	z value	Pr(>|z|)
(Intercept)	-1.158828	0.501374	-2.311	0.0208 *
EMB conc. ppb	0.014679	0.002052	7.152	8.55e-13 ***
louse sex Male	-1.075884	0.418198	-2.573	0.0101 *
louse stage PAL1	1.628953	0.564367	2.886	0.0039 **
louse stage PAL2	0.479231	0.381067	1.258	0.2085

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