MONITORING EFFECTIVENESS OF EMAMECTIN BENZOATE FOR THE TREATMENT OF SEA LICE (LEPEOPHTHEIRUS SALMONIS) ON FARMED ATLANTIC SALMON (SALMO SALAR)

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MASTER OF SCIENCE

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Faculty of Veterinary Medicine
University of Prince Edward Island

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Charlottetown, PE, Canada
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We, the undersigned, certify that **Patti G. Jones DVM**, candidate for the degree of **Master of Science**, has presented his/her thesis with the following title:

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and that the thesis is acceptable in form and content, and that a satisfactory knowledge of the field covered by the thesis was demonstrated by the candidate through an oral examination held on October 25, 2012.

Examiners:

Dr. Jeff Davidson _____

Dr. Crawford Revie _____

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Date: October 25, 2012

Abstract

Resistance development to parasiticides has been a problem in both terrestrial and aquatic environments. A major challenge exists in early detection of resistance emergence due to the low frequency at which resistant organisms can occur initially within a population and the difficulty in detecting these organisms. Emamectin benzoate is an avermectin compound which has been used effectively in the treatment of sea lice on farmed salmonids around the world. The main purpose of this thesis was to examine field collected sea lice abundance data and treatment records to estimate changes in the effectiveness of emamectin benzoate for treatment of sea lice (Lepeophtheirus salmonis) infestations on farmed Atlantic salmon (Salmo salar).

The objectives of this thesis were: (1) to review and describe methods for the evaluation of sea lice treatment effectiveness, (2) to evaluate and establish if changes in effectiveness of emamectin benzoate for the treatment of sea lice (L. salmonis) on farmed Atlantic salmon in New Brunswick, Canada, had occurred from 2004 to 2008, and (3) to determine if there was evidence of differences in the temporal development of resistance to emamectin benzoate across L. salmonis life stages using data collected in both Canada and Scotland.

Field collected data from New Brunswick salmon farms (2004 to 2008) were examined for temporal and spatial changes in treatment effectiveness in populations of L. salmonis. Data analysis was carried out in two parts: examination of trends in treatment effectiveness and L. salmonis abundance and an examination of multiple factors influencing post-treatment L. salmonis abundance and treatment outcome through the use of statistical models (linear and logistic regression). A reduction in treatment effectiveness was found from 2004 to 2008 and treatment effectiveness was found to vary by location.

Further analysis was carried out to examine for possible differences in treatment effectiveness between different sea lice life stages. Life stages were categorized into two groups: adult females (gravid and non-gravid) and other mobile stages (pre-adult female, pre-adult male, and adult male). Sea lice abundance records following emamectin benzoate treatments from the west coast of Scotland (2002 to 2006) and from New Brunswick, Canada (2004 to 2008) were examined. Differences in treatment effectiveness were found between the two groups by year and location. Changes in sea lice sensitivity to emamectin benzoate were not synchronized in all life stages. A difference in the rates of resistance development between locations was noted, with resistance developing more rapidly in New Brunswick than in Scotland.

In summary, this research examined methods for the evaluation of emamectin benzoate effectiveness for the control of sea lice. A reduction in emamectin benzoate effectiveness occurred over time and varied by location. In addition, resistance development was not synchronized in all sea lice life stages. These methods could help aid in the detection of resistance development in parasites. However, no single method is likely to suffice for monitoring changes in sea lice sensitivity. In future, coordinated analyses from both laboratory and field studies will likely yield the best results.

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List of Abbreviations

ABC transporter protein	ATP-binding cassette transport protein
ABMA	Aquaculture Bay Management Areas
AChE	Acetylcholinesterase
AMRL	Administrative maximum residue limits
ANOVA	Analysis of variance
EC_{50}	Effective concentration
EDR	Emergency Drug Release
PCR	Polymerase chain reaction
PMRA	Pest Management Regulatory Agency
VDD	Veterinary Drug Directorate

Chapter 1 General Introduction

Sea lice are ectoparasitic marine copepods of the family Caligidae that are found worldwide on a variety of aquatic organisms. They feed on the mucus, skin and blood of fish resulting in varying levels of damage to the host. While low numbers of sea lice cause only minimal damage to the host, high numbers can result in severe effects and even death of the host fish. Although large numbers of sea lice have been associated with commercial fish farming around the world, as early as 1940 there were reports of sea lice causing severe damage and mortality in wild fish (White 1940). Under intensive salmon farming conditions, sea lice numbers can increase rapidly and cause serious problems as a result of the high density of available hosts (Murray & Peeler 2005, Robertsen 2011).

1.1 Background

Sea lice are the most common parasitic copepod known to infest fish in the marine environment. The species of interest to marine and brackish water cultured fish originate from the family Caligidae (Johnson et al. 2004). The two genera of interest to salmon aquaculture are Caligus and Lepeophtheirus (Pike & Wadsworth 1999) with over 250 known species of Caligus and over 150 species of Lepeophtheirus documented (Walter & Boxshall 2012). Lepeophtheirus spp. and Caligus spp. are found on Salmonidae of the genera Salmo (North Atlantic salmon and trout), Salvelinus (trout and charr), and Oncorhynchus (Pacific salmon) (Pike & Wadsworth 1999, Costello 2006). However, Caligus species are thought to be more generalist in host selection and can be found on a variety of fish (Pike & Wadsworth 1999).

Lepeophtheirus salmonis (Krøyer 1837) is considered to be a specialist, generally only being found on salmonids. However, in British Columbia, Canada, this parasite has been found on the threespine stickleback (Jones et al. 2006). Jones et al. (2006) confirmed that early stages of L. salmonis can exist on the threespine stickleback, which may act as a temporary host for L. salmonis.

Of particular importance to salmon farming are L. salmonis and several Caligus species. Lepeophtheirus salmonis has been the most problematic species of sea lice on farmed Atlantic salmon (Salmo salar L.) in the northern hemisphere (Scotland, Norway, Ireland, the east and west coasts of Canada, and the United States), since severe infestations with this species can result in disease of the host fish leading to economic losses for fish farmers (Pike & Wadsworth 1999, Johnson et al. 2004). In Chile, the species known to be the most problematic is Caligus rogercresseyi (Boxshall & Bravo 2000), while on the east coast of Canada and Scotland, Caligus elongatus (von Nordmann 1832) can be present in significant numbers. Caligus elongatus, while being commonly found on farmed salmon in these regions, is usually not of great concern to the health and welfare of fish. In British Columbia, L. salmonis is the species of most concern on farmed fish although outbreaks resulting in disease are uncommon, while the secondary species of interest is Caligus clemensi (Parker & Margolis 1964) (Johnson et al. 2004). The majority of current literature has involved L. salmonis and to a lesser extent Caligus species, with the majority of that literature pertinent to C. elongatus (Boxaspen 2006).

1.2 Sea lice biology

This review will mainly pertain to L. salmonis and, to a much lesser extent, C. elongatus. Sea lice have a multiple stage life cycle with free-swimming or planktonic and parasitic phases. Adult female lice produce strings of eggs which hatch into first stage nauplii. There are two free-living planktonic naupliar stages and one free-swimming copepodid stage. The copepodid is the infectious stage which locates and attaches to a fish thus beginning the parasitic phase. Once on the fish, there are four attached juvenile stages, known as chalimus, followed by the mobile stages consisting of two pre-adult and one adult. Each of the life stages is separated by a moult (Johnson & Albright 1991a, Johnson & Albright 1991b, Schram 1993). Copepodids moult into first stage chalimus and attach to the host by a frontal filament, while later pre-adult and adult stages move over the surface of the fish feeding on epidermal tissue (Heuch et al. 2000, Boxaspen 2006). The sexes can be distinguished visually once the chalimus IV stage is reached and sexual maturation occurs in the later parts of the second pre-adult stage (Pike & Wadsworth 1999). Caligus elongatus has an eight-stage life cycle (Piasecki 1996), while L. salmonis consists of a ten-stage life cycle (Johnson & Albright 1991a); C. elongatus lacks the pre-adult stage of the life cycle and matures from the last chalimus stage (IV) directly into an adult (Piasecki 1996).

Nauplii and free-living copepodids are non-feeding and live off internal reserves derived from the remainder of the yolk sac. As this energy supply is limited, it is important that the copepodid find a host fish and attach prior to this source depleting. Nauplii have limited ability to swim (Bron et al. 1993), but potentially cover great distances by moving primarily with the current (McKibben & Hay 2004, Costello 2006).

The copepodid, however, is a more active swimmer than the naupliar stages which aids it in locating a host. Once the copepodid finds and attaches to a fish, it begins feeding as this stage has developed mouthparts and a functional gut (Pike & Wadsworth 1999). Heuch et al. (1995) found that copepodids may find hosts by moving through the water column and rising to the surface during the day and sinking down and spreading out through the deeper layers during the night. This positive phototaxis has also been witnessed in laboratory studies on sea lice (Hogans & Trudeau 1989, Pike et al. 1993). Caged salmon are known to rise to the surface to feed during the day but otherwise remain lower in the cage during day light hours, while rising closer to the surface during the night (Pike & Wadsworth 1999). Heuch et al. (1995) postulated that this movement through the water column facilitates several copepodid-fish interactions throughout a 24 hour period. Copepodids are also influenced by salinity; their survival is reduced at salinities lower than 29% and they will actively avoid salinities below 27% (Bricknell et al. 2006).

Adult female sea lice produce paired egg strings. The estimated number of eggs per string is variable. Heuch et al. (2000) described finding approximately 55 to 704 eggs per string while Schram (1993) reported observing 180 to 300 eggs per string. The length and number of eggs is dependent on age of the adult female sea louse, season and temperature. Adult females can produce multiple pairs of egg strings (Heuch et al. 2000, Mustafa et al. 2000). Heuch et al. (2000) found that adult females could produce as many as 11 pairs of egg strings with adult females surviving up to a maximum of 191 days at 7.2 °C under laboratory conditions. In addition, the number of eggs produced varied with temperature, with more eggs produced at a lower temperature (7.1 °C)

versus a higher temperature (12.2 °C). However, a higher proportion of non-viable eggs was found at the lower temperature. Mustafa et al. (2000) found that up to ten pairs of egg strings per female (mean of six pairs) could be produced under laboratory conditions at 10 °C with adult females living for up to 210 days. Number and viability of eggs has been shown to increase with age of the adult female louse and each successive pair of egg strings. The first pair of egg strings produced by a female will have fewer eggs than subsequent rounds of egg strings (Schram 1993, Heuch et al. 2000).

Generation time of L. salmonis on Atlantic salmon held at 10 °C in the laboratory was determined to be 40 days for males (i.e. 400 degree days) and 52 days for females (Johnson & Albright 1991b). However, generation times have been found to vary considerably (Pike & Wadsworth 1999). Females mature more slowly than males and sea lice generation time can be affected by temperature, with a shorter generation time occurring with increasing temperatures (Johnson & Albright 1991b).

A notable difference between L. salmonis and C. elongatus is size; L. salmonis adults are much larger than C. elongatus adults. Adult female and adult male L. salmonis differ in length with adult females being significantly longer than adult males, while there is minimal difference in length between the adult male and female C. elongatus (Johnson & Albright 1991a, Piasecki 1996). There is considerable variation in the size of adult L. salmonis depending on season, water temperature, location, and whether the lice are parasitizing farmed or wild fish (Tully & Whelan 1993). Tully & Whelan reported that sea lice on wild salmon were found to be considerable larger than those found on farmed salmon. Typically L. salmonis adult females tend to be larger

with decreasing water temperature or during winter months (Ritchie et al. 1993). The adult female L. salmonis is approximately 10 mm long whilst the male is approximately 5 mm (Johnson & Albright 1991a). A detailed description of the life cycle and developmental stages of L. salmonis has been published by Johnson & Albright (1991a).

1.3 Host-parasite relationship

1.3.1 Impacts on host

In the northern Atlantic Ocean, L. salmonis tend to cause the most damage to fish, most likely due to their size and more aggressive feeding (Pike & Wadsworth 1999). While low numbers of sea lice on fish tend not to cause significant problems for the host fish, high numbers that are beyond the host's ability to compensate can result in severe and, sometimes, life threatening issues (Pike & Wadsworth 1999). The quantity of sea lice required to cause serious damage to the host depends on size of the fish, species of fish, and species of copepod, but as few as five adult L. salmonis have been known to cause significant pathology on smolts (Wooten et al. 1982). Jones & Hargreaves (2009) estimated the threshold of lethal infection as 7.5 L. salmonis g^{-1} in juvenile pink salmon (Oncorhynchus gorbuscha). Fish infested with significant numbers of sea lice can display behaviours caused by irritation from the presence of sea lice. Fish may jump out of the water (i.e. flashing) more frequently or rub along the nets. Rubbing along nets may cause further external damage to the fish (Stone et al. 2002).

Direct damage to the host fish is caused by the feeding activity of the sea louse. Chalimus stage sea lice cause slight damage around the area of attachment of the frontal filament, while the mobile, and more aggressive, pre-adult and adult stages can cause extensive tissue injury or destruction. Such aggressive feeding results in disruption of

the mucus layer followed by damage to the skin and subsequent loss of scales. With more severe damage, the lice will feed on blood and there may be exposure of underlying muscle or cartilage. The head is a particularly vulnerable area owing to the lack of scales and thinness of the skin. Fish with severe sea lice damage on the head penetrating to the underlying cartilage are called "whiteheads" due to exposed cartilage (Pike & Wadsworth 1999).

Indirect damage, such as osmoregulatory failure, can result from severe wounds to the skin and accompanying loss of scales leading to the fish's inability to maintain water and electrolyte balance within the environment (Pike & Wadsworth 1999). Consequently, host blood aberrations can occur, including a stress response leading to elevations in cortisol and glucose (Bowers et al. 2000), anemia, reduced lymphocytes, and ion imbalances (Dawson et al. 1999). Subsequently, such changes, along with physical disruptions of the skin, leave the host prone to secondary infections, such as vibriosis or furunculosis (Tully & Nolan 2002).

Host infection can result in decreased feeding activity leading to reduced growth and specific growth rates. Dawson et al. (1999) found that pre-adult L. salmonis seemed to affect Atlantic salmon most severely resulting in significantly decreased food consumption compared to fish primarily infested with chalimus or adult sea lice. These fish were experimentally infected with sea lice, therefore, the sea lice were at similar stages in their life cycle at each of three sampling times (12, 21, 30 days post-infection). However, the total number of sea lice present decreased with time (i.e. there were fewer adult than pre-adult sea lice). Although food consumption decreased with the presence of pre-adults, specific growth rates of the fish were not affected. When the sea lice

moulted from pre-adults to adults, feed consumption increased and skin pathology improved.

Susceptibility of salmonids to L. salmonis varies by fish species, with Atlantic salmon having a tendency to be more susceptible to infestation compared to some Pacific salmon species (Fast et al. 2002, Jones et al. 2006). Species differences have been associated with variations in skin, mucus, production of mucosal enzymes, and the ability of certain species to raise an inflammatory response to the sea lice (Wagner et al. 2008). Atlantic salmon were found to have a thinner epidermal layer compared to rainbow trout or coho salmon (Fast et al. 2002). The study by Fast et al. concluded that coho salmon seem to have an innate ability to reject L. salmonis infestations, which may be related to the mucus biochemistry of the host.

1.3.2 Impact on aquaculture

There are a number of potential economic impacts as a result of sea lice populations that are difficult to control. Major costs are incurred with the purchase of parasiticides and equipment, human resources for monitoring and treatment administration, along with reductions in fish growth and feed conversion efficiency (Costello 2009). Sea lice were determined to be the most commercially limiting parasite in salmon aquaculture in Europe, along with North and South America (Costello 2009). Costello (2009) estimated the costs associated with sea lice infestations as equivalent to approximately 6% of the total production value.

In 2009, the world production of Atlantic salmon was estimated at over 1.4 million tonnes. The largest producer of Atlantic salmon in the world was Norway, which produced over 860 000 tonnes of salmon in 2009, followed by Chile with over 230 000

tonnes. Production in the United Kingdom was estimated at over 130 000 tonnes, whereas Canada produced approximately 100 000 tonnes of Atlantic salmon in 2009 (Food and Agriculture Organization of the United Nations 2011).

Not all countries where salmonids are produced are affected by sea lice. Costello (2009) speculates that limited production may impede the opportunity for sea lice epidemics to develop. Countries with lower production, such as Iceland, Russia, and France, tend not to have problems with sea lice infestations. Countries where Pacific salmon species are primarily cultured, such as coho salmon production in Japan and Chile, do not have significant problems with sea lice as coho salmon tends to be more resistant to infestations (Johnson et al. 2004). In addition, coho salmon production usually involves only one year in marine cages which may affect sea lice abundance since sea lice levels tend to be higher during the second year of production (Costello 2009).

1.4 Treatment and control measures for sea lice infestations

There are several types of methods for control of sea lice: biological, management practices, and chemical. An example of biological control is the use of wrasse, a type of fish which are kept in ocean cages along with the farmed fish species. Wrasse will feed on sea lice infesting farmed fish (Treasurer 1993). Large numbers of wrasse have been regularly stocked on salmon farms in Norway (Treasurer 2002). Management practices, such as fallowing and year class separation, assist in keeping sea lice numbers low by reducing the exposure of young fish to lice sourced from older fish. Site location is an example of a management practice or environmental control where

farms are located in areas that are not conducive to a thriving sea lice population (i.e. areas with lower salinity or strong currents and flushing effects).

1.4.1 Therapeutic options

There are several therapeutic options available for treatment of sea lice. The options available to practitioners will vary by country since licensing regulations are different for every country. In Canada, most aquaculture therapeutants delivered through feed are regulated by Health Canada's Veterinary Drug Directorate (VDD) under the Canadian Food and Drugs Act and Regulations. When treatments are administered as a bath treatment to control external parasites in the aquatic environment, they are considered pesticides and are regulated under the Canadian Pest Control Products Act and Regulations. The Pest Management Regulatory Agency (PMRA) approves pesticides under the Pest Control Products Act. If a new drug is not marketed or registered for use in Canada, a veterinarian can submit a request through the Emergency Drug Release (EDR) program, administered by VDD, for use in a veterinary medical emergency. If a pesticide which is not approved by Health Canada is requested for emergency use by a practitioner or government body in Canada, an emergency release permit may be approved by PMRA under the Pest Control Products Act. Azamethiphos (Salmosan®), an organophosphate, and deltamethrin (AlphaMax®), a pyrethroid, are examples of products that have been approved temporarily in the recent past by PMRA for limited use in the Bay of Fundy, New Brunswick, Canada. Two drugs are currently approved by VDD for use in Canada: teflubenzuron (Calicide®), a chitin synthesis inhibitor, and emamectin benzoate (SLICE®) which is a type of avermectin.

1.4.2 Avermectins

Avermectins are a class of macrocyclic lactones which were produced from a culture of Streptomyces avermilitis (Lasota & Dybas 1991). They have been used in human and veterinary medicine in addition to agricultural use for control of insects. Avermectins work by interfering with nerve transmission; they bind irreversibly to glutamate-gated chloride channels resulting in increased neuron cell membrane permeability to chloride ions at inhibitory synapses (i.e. hyperpolarisation) causing paralysis and death (Wolstenholme & Rogers 2005). As with other anthelmintic products used in terrestrial medicine, avermectins have been plagued with tolerance or resistance problems, which have been well documented in nematodes (Jackson & Coop 2000, Le Jambre et al. 2000, Prichard 2001, Wolstenholme et al. 2004).

1.4.2.1 Emamectin benzoate

Emamectin benzoate (4"-deoxy-4" epimethylaminoavermectin B_1) is an avermectin product and is the active chemical in SLICE®. A 0.2% pre-mix was developed for use in aquaculture by Schering-Plough Animal Health (now Merck) under the trade name SLICE®. SLICE® is fully registered for use on salmon farms in Canada through the VDD. This product is administered in feed to the fish at a dose of 50 $\mu g\ kg^{-1}\ day^{-1}$ over the course of seven days and was found to be effective against all stages of sea lice found on host fish (Stone et al. 1999, Stone et al. 2000c). When fed to fish, emamectin benzoate is absorbed in the gut and distributed throughout the tissues of the fish. The focus of this research project was to evaluate changes in the effectiveness of emamectin benzoate because this product has been a popular choice for the treatment of

sea lice on farmed salmon and resistance development has become a concern in several areas (Horsberg 2012).

Sevatdal et al. (2005a) determined the elimination half-life of emamectin benzoate in mucus on Atlantic salmon to be 11.3 days and that concentrations of emamectin benzoate were no longer detectable in plasma at day 77. Fish in the study by Sevatdal et al. were housed on a commercial salmon farm in Norway where water temperatures ranged from 15-19 °C. The highest concentration of emamectin benzoate occurred at the end of treatment administration (day 7) (Sevatdal et al. 2005a) or shortly after the cessation of medicated feed administration (Whyte et al. 2011). Sevatdal et al. (2005a) found concentrations of emamectin benzoate had a tendency to be higher in mucus than in blood or muscle. Kim-Kang et al. (2004) found emamectin benzoate levels to be below 85 ppb in skin and muscle samples of Atlantic salmon fed the product at the prescribed dose. Whyte et al. (2011) found concentrations of emamectin benzoate to be higher in skin than in muscle (approximately 4 times higher) when emamectin benzoate was given at the prescribed dose. Whyte et al. (2011) also found residue levels in skin to be higher than in previous studies (Kim-Kang et al. 2004, Sevatdal et al. 2005a). Health Canada (VDD) has set administrative maximum residue limits (AMRL) for fish destined for human consumption of 1000 ppb in skin and 100 ppb in muscle (Health Canada 2012). Whyte et al. (2011) concluded that residue limits of this magnitude were not observed in muscle samples for the prescribed dose from day 5 onward in the post-treatment period, while skin samples did not attain the AMRL at any point during the same period.

1.4.2.1.1 Review of studies evaluating emamectin benzoate

Several studies evaluated emamectin benzoate efficacy in both the laboratory and field trial situations. Stone et al. (1999), evaluated emamectin benzoate as a treatment for sea lice on Atlantic salmon in a controlled laboratory trial. The objective of this initial study was to select a dose of emamectin benzoate which would be effective against all stages of the sea louse found on fish. The results of the study showed a significant decrease in mean mobile sea lice following treatment. The lowest optimum dose was found to be 50 $\mu g\ kg^{-1}$ from the three doses evaluated (25, 50, 100 $\mu g\ kg^{-1}$).

A field trial evaluation of emamectin benzoate efficacy was performed by Stone et al. (2000c). Efficacy of emamectin benzoate was evaluated against both L. salmonis and C. elongatus on Atlantic salmon housed in pens at a commercial salmon farm. The fish used for the study were post-smolts in their first year at sea with trials run in August and September. A third study on second year at sea fish was performed in April when temperatures were lower. Fish were dosed with emamectin benzoate mixed in feed at 50 $\mu g\ kg^{-1}$ biomass per day for seven consecutive days. Emamectin benzoate was effective in the removal of both mobile and chalimus stages of L. salmonis and C. elongatus with numbers reduced by 68-98% on treated fish. Sea lice damage was observed to be less severe on treated fish. This study found that treatment at lower temperatures resulted in slower removal of mobile stages of lice. As in a previous study (Stone et al. 1999), there was a gradual increase in chalimus numbers on treated fish, but little increase in mobile lice, suggesting that chalimus on treated fish had impaired development into mature mobile stages. Authors noted an improved feeding response in fish following treatment

and suggested that improvements in growth may be a benefit to in-feed treatments since bath treatments require several days of starvation and cause stress to fish during treatment administration.

Stone et al. (2000b) evaluated the effectiveness of emamectin benzoate as a 0.2% aquaculture pre-mix (SLICE®) in a clinical situation on farmed fish in commercial pens. The trial examined the use of emamectin benzoate during autumn and winter months. A delay in efficacy was found in the winter trial compared to the autumn trial. Sea lice levels remained lower on treated fish than control fish with reductions of more than 90% up to day 64. The study concluded that temperature might influence the duration of efficacy.

A subsequent study (Stone et al. 2000a) to examine the duration of effect of emamectin benzoate (using the 0.2% pre-mix SLICE®) in Atlantic salmon held in tanks in a laboratory confirmed that it was highly effective in reducing existing sea lice burdens (greater than 90%), but also prevented development of newly recruited sea lice for up to 62 days following start of treatment.

Roy et al. (2000) examined the tolerance of Atlantic salmon and rainbow trout to emamectin benzoate. No adverse effects were found at doses up to 3.5 times the therapeutic dose and no mortalities occurred at seven times the therapeutic dose. Signs of emamectin benzoate toxicity in fish were lethargy, dark colouration, inappetance, and loss of coordination. At higher doses, emamectin benzoate is able to cross the blood brain barrier, resulting in neurological symptoms previously mentioned. A comparison of the toxicity of emamectin benzoate and ivermectin in fish was discussed by Sevatdal et al. (2005a) and Horsberg (2012); both papers concluded that symptoms and

mortalities occur more readily with ivermectin. The likely reason for these differences in toxicity symptoms is the ability of ivermectin to accumulate in the brain.

Ramstad et al. (2002) evaluated emamectin benzoate (using the 0.2% premix SLICE®) in study pens at four commercial Atlantic salmon farms in Norway. In the study by Ramstad, they elected not to use control groups for fish welfare and commercial reasons. Instead, they chose to evaluate efficacy by determining a reduction in sea lice levels by comparing post-treatment counts with pre-treatment counts. Subsequently, this type of evaluation has been used in several other studies (Gustafson et al. 2006, Lees et al. 2008b). In Ramstad et al. (2002), the fish were in their first year at sea and all treatments occurred during summer months. Sea lice levels on fish treated with emamectin benzoate were reduced by over 90% at 21 days following treatment initiation.

In Atlantic Canada, a field trial was performed to evaluate the efficacy of emamectin benzoate (using the 0.2% premix SLICE®) (Armstrong et al. 2000). The trial was performed at two commercial sea farming sites. Due to increasing numbers of sea lice on fish in the control groups, treatments with azamethiphos were required and baths with this agent were applied to each control pen at three different times. Treatment efficacy in this study was found to reach a maximum of 95% at days 28 or 29 of the study. The duration of efficacy was found to be approximately 43 days, after which sea lice began to slowly increase in number. Treated fish tended to have higher levels of copepodids.

1.4.2.2 Ivermectin

Ivermectin has been used worldwide in terrestrial animals since the early 1980's. Ivermectin (22, 23-dihydroavermectin) is a semisynthetic avermectin registered for use in veterinary and human medicine (Lasota & Dybas 1991). Its mode of action is similar to that for emamectin benzoate. In some countries, ivermectin has also been used in aquaculture as an extra-label prescription (Horsberg 2012).

Ivermectin has been used to treat sea lice on Atlantic salmon and is administered as an in-feed treatment. Ivermectin can be effective against mobile sea lice as well as chalimus stages (Johnson & Margolis 1993). Efficacy against chalimus stages is an important quality as this can extend the period of time between treatments (Roth et al. 1993). Johnson & Margolis (1993) determined that a dose of 0.05 mg kg^{-1} fed twice per week would be efficacious in the treatment of sea lice (L. salmonis) on Atlantic salmon.

There are some concerns with the use of ivermectin as a treatment for sea lice. Ivermectin has a narrow safety margin and can be toxic to salmon (Johnson et al. 1993b) which is in contrast to emamectin benzoate (Sevatdal et al. 2005a, Horsberg 2012). Johnson & Margolis (1993) found a significant increase in mortalities when increasing the dose from 0.1 mg kg^{-1} to 0.2 mg kg^{-1} (both administered every second day). Signs of toxicity in fish include a darkened colouration, loss of equilibrium, and decreased feeding activity. With a narrow margin of safety, one would have to be concerned with the possibility of inadvertent overdoses since there can be discrepancies between the prescribed dose and the actual dose attained by fish due to differences in feeding rates, poor drug distribution when mixing into feed, or inaccurate biomass estimation. Ivermectin likely remains present in fish tissues for extended periods of time since

efficacy has been found to be adequate at 28 days following treatment (0.05 mg kg^{-1} every third day for six doses) (Johnson & Margolis 1993). Ivermectin is known to bind to sediments and breakdown is dependent on light and temperature. Its potential for accumulating in sediments beneath cages is a concern for susceptible benthic organisms (Roth et al. 1993, Horsberg 2012).

1.4.3 Other treatment products

1.4.3.1 Chitin synthesis inhibitors

Teflubenzuron is a chitin synthesis inhibitor belonging to a group of compounds known as insect growth regulators. When a sea louse moults from one stage to another it produces a new chitinous exoskeleton. Chitin synthesis inhibitors disrupt the formation of a new exoskeleton (Horst & Walker 1995) during the moult between stages when demand for chitin is greatest. Teflubenzuron has been registered for use as Calicide® (Nutreco Aquaculture) and diflubenzuron has been manufactured and sold as Lepsidon® (Ewos). Calicide® or Ektobann® (Nutreco Aquaculture) are authorized for use in Scotland, Norway, Ireland, and the Faeroe Islands (Grant 2002) while Calicide® is available for use in Canada. Diflubenzuron has been used in Chile on a trial license (Grant 2002).

Chitin synthesis inhibitors are administered in feed to salmon for the treatment of sea lice. These products are effective against only moulting stages of sea lice (i.e. chalimus and pre-adult stages). This product is not overly effective against adult stages because they have a decreased requirement for synthesizing chitin. Chitin synthesis inhibitors are most useful when few adult sea lice are present on the fish and the entire site is treated simultaneously (Branson et al. 2000). Although available for use in many

countries, teflubenzuron has not been a popular option likely due to its limited efficacy, particularly against adult stages as it is unable to break the reproductive cycle, and lack of extended residual effect (Campbell et al. 2006).

1.4.3.2 Organophosphates

Azamethiphos is an organophosphate used for the treatment of sea lice. Azamethiphos is marketed as Salmosan® by Novartis Animal Health Ltd., and licensed for use in Scotland, Norway, Faeroe Islands, Canada (until 2002) and Chile (Grant 2002, Burridge et al. 2010). When emamectin benzoate treatment failures in New Brunswick became evident in 2009, Salmosan® was one treatment option explored and PMRA issued an emergency permit for this treatment (Chang et al. 2011). Azamethiphos is administered as a bath treatment at a concentration of 0.1 mg L^{-1} (Burridge et al. 2010). As an organophosphate, the mechanism of action is through the irreversible inhibition of acetylcholinesterase (AChE) activity. Acetylcholinesterase decreases the activity of the neurotransmitter acetylcholine (at cholinergic synapses), thereby blocking the nerve impulse at the synapse. The inhibition of acetylcholinesterase by an acetylcholinesterase inhibitor results in overstimulation of the nerve impulses leading to spastic paralysis and death of the sea louse.

Azamethiphos can be efficacious for the removal of pre-adults and adults but variable in the removal of chalimus stages (O'Halloran & Hogans 1996, Roth et al. 1996). Some populations of sea lice in different areas of the world (e.g. Norway and Scotland in the early to mid-1990's) developed resistance to organophosphates (Jones et al. 1992, Denholm et al. 2002) and this resistance may remain within the population for years after product use has ceased (Fallang et al. 2004). The persistence of this

resistance in a population may lead to rapid onset of failed treatments if the product was to be re-introduced into the area.

1.4.3.3 Pyrethroids

Cypermethrin (Excis®) has been used in Scotland, Ireland and the United States, while high-cis-cypermethrin (Betamax®) and deltamethrin (AlphaMax®) have been used in Norway. Synthetic pyrethroids (i.e. synthetic analogs of pyrethrins) were developed as insecticides and act by altering (i.e. opening) the sodium channels thereby increasing nerve impulses resulting in spastic paralysis and death. These products are administered as bath treatments.

With the development of a bioassay for deltamethrin, researchers were able to detect a decrease in sensitivity to deltamethrin in sea lice from an area with a history of treatment failures when compared with sea lice from other areas (Sevatdal & Horsberg 2003). In a subsequent study, Sevatdal et al. (2005b) found that reduced sensitivity to pyrethroids was occurring occasionally; this study was conducted on sea lice collected from Ireland, Scotland, and Norway, some areas of which had anecdotal reports of treatment failures.

1.4.3.4 Hydrogen peroxide

Hydrogen peroxide is a strong oxidizing agent administered as a bath for the treatment of sea lice. Hydrogen peroxide had been used in Norway, Canada, Faeroe Islands and Scotland for the treatment of sea lice (Treasurer & Grant 1997) and has been relied upon more recently in New Brunswick, Canada, and Chile (Treasurer & Bravo 2010). Hydrogen peroxide has been marketed under several names which include Salartect 350® or Salartect 500® (Brentage UK), as well as Paramove 35® or Paramove

50® (Solvay Interox). As it does not persist in the environment, hydrogen peroxide is rapidly converted to oxygen and water, this compound generates fewer concerns regarding potential ecological effects compared to other bath agents (Roth et al. 1993). The mechanism of action of hydrogen peroxide on sea lice is not well understood, but has been speculated to involve a mechanical paralysis resulting from the liberation of oxygen in the gut and hemolymph causing the lice to float up and off the fish (Thomassen 1993, Grant 2002).

Hydrogen peroxide can be effective for the removal of adult and pre-adult stages, but the effect on chalimus stages can vary (Johnson et al. 1993a, Thomassen 1993). Hydrogen peroxide can be toxic to fish and this is most notable with increasing water temperatures, exposure time (Johnson et al. 1993a, Thomassen 1993), and increasing dose (Grant 2002). Hydrogen peroxide treatments are not recommended at water temperatures above 14°C (Thomassen 1993). However, in Scotland, treatments with hydrogen peroxide have been safely performed in the summer at temperatures up to 15°C by reducing the treatment time (Treasurer & Grant 1997).

There has been a longstanding question about whether sea lice can recover following exposure to hydrogen peroxide and potentially re-infect fish (Hodneland et al. 1993, Treasurer & Grant 1997, McAndrew et al. 1998). While Thomassen et al. (1993) did not report finding that lice would reattach after a bath treatment with hydrogen peroxide, Treasurer and Grant (1997) felt that this possibility might be underestimated. Even at treatment protocols high enough to cause mortalities of the host fish, high numbers of removed sea lice recovered following exposure (Johnson et al. 1993a). McAndrew et al. (1998) examined the ability of sea lice previously exposed to hydrogen

peroxide in a laboratory situation and determined that all mobile stages of L. salmonis were capable of re-infecting salmon after treatment with hydrogen peroxide. This finding is in contrast to Treasurer and Grant (1997) who found that, in a field situation, treated sea lice did not reattach to salmon, although the detached lice might have moved away from the farm with tidal currents.

1.5 History of sea lice in Atlantic Canada

Farming of Atlantic salmon in the southwestern Bay of Fundy region of New Brunswick, Canada, began in 1978. According to Statistics Canada, the annual harvest of Atlantic salmon has ranged from 24 000 - 26 000 metric tonnes for the years 2008 to 2010 (Fisheries and Oceans Canada 2012). For the same years, production ranged from 4 000 - 7 000 metric tonnes for Nova Scotia.

During early production years, C. elongatus was the primary species found on salmon farms in the Bay of Fundy (Hogans & Trudeau 1989). In the fall of 1994, the first outbreak of L. salmonis involving multiple farms occurred (Hogans 1995, O'Halloran & Hogans 1996). At that time, a variety of treatments were attempted with varying success, such as hydrogen peroxide, cypermethrin, ivermectin, and deltamethrin (Hogans 1995, O'Halloran & Hogans 1996, Chang et al. 2011). In the autumn of 1995, a time-limited registration was granted for the use of the organophosphate, azamethiphos (Salmosan®) as a bath (O'Halloran & Hogans 1996). Emamectin benzoate (SLICE®) was introduced in New Brunswick in 1999 through Health Canada's Emergency Drug Release (EDR) program (Armstrong et al. 2000). For the next 9 to 10 years, this was the treatment of choice for several reasons: effectiveness against all life stages, prolonged efficacy post-treatment, and ease of administration in feed allowing for the simultaneous

treatment of multiple cages (Stone et al. 2000a, Stone et al. 2000c, Westcott et al. 2004). Reports of isolated treatment failures emerged in late 2008, initiating suspicions of emamectin benzoate resistance development. Starting in 2009, the industry and governing bodies had to consider other treatment options (Chang et al. 2011).

1.6 Monitoring clinical use of emamectin benzoate

Several studies have evaluated the clinical effectiveness of emamectin benzoate through examination of field-collected data in Scotland, United States (Maine), and Canada (British Columbia). Clinical trials are an ideal way to determine treatment efficacy, but often the use of medications in a true clinical situation can yield different results. The practice of monitoring clinical treatment response through the analysis of field-collected data remains a key factor in the detection of changes in treatment effectiveness. In this thesis, the term efficacy is used to refer to the measure of how a product works in a controlled trial or laboratory study, while effectiveness is used to refer to how well a treatment works in a clinical practice environment. Evaluations of treatment effectiveness usually involve a retrospective analysis.

Gustafson et al. (2006) examined the effectiveness of emamectin benzoate on farmed Atlantic salmon in the Cobscook Bay region of Maine. Treatment episodes were selected based on the following criteria: counts included a minimum of five fish from each of five cages on a site and were performed at least every other week for eight weeks post-treatment. A total of 19 treatments across 11 different farms from 2002 to 2005 were evaluated in the study.

The use of a control group is common practice in most study designs. However, when assessing clinical use of a product, use of a control group is not always an option

due to concerns for animal welfare. Gustafson et al. (2006) described effectiveness as percent effectiveness which is the proportion of pre-treatment sea lice removed by the treatment [Effectiveness = 100 – (average lice per fish post-treatment/average lice per fish at start of treatment)*100]. Most of the treatments in this study attained close to 100% efficacy at some point in the post-treatment evaluation period. Lepeophtheirus salmonis numbers, as well as total Caligus sp. populations (a specific species was not specified, but C. elongatus would be the most common in that region) were evaluated. Maximum percent effectiveness and time to maximum effectiveness were calculated for each of the following life stages: chalimus, non-gravid mobiles (pre-adults and non-gravid adults), and gravid females for L. salmonis. Caligus sp. was classified as total Caligus rather than individual life stages. Time to maximum effectiveness ranged from two to eight weeks following treatment initiation. Duration of treatment effect ranged from six to ten weeks. No determinations were made on the presence of a decline in treatment effect with time, although this might have been unlikely as all treatments appeared to be efficacious.

Lees et al. (2008b) examined the effectiveness of emamectin benzoate against sea lice (L. salmonis) on farmed Atlantic salmon in Scotland. This study examined the clinical use of emamectin benzoate from 2002 to 2006. The following factors were examined in relation to treatment effectiveness and post-treatment sea lice abundance: location, time (i.e. year and season), age of fish (i.e. first or second year of the production cycle), and use of strategic treatment (i.e. single site treatment or synchronized entire loch treatment). Sea lice counts were evaluated as a group of total mobiles, which included pre-adult male and female, adult male, non-gravid and gravid

female sea lice. Treatment episodes meeting the following inclusion criteria were examined:

1. Pre-treatment count within 16 days prior to the start of treatment;
2. At least three post-treatment evaluations in the 12 weeks following treatment or until another treatment administration occurred (if occurring within the 12 weeks);
3. Entire site required treatment (i.e. no partial site or single cage treatments). No mixed treatment episodes were included (i.e. part of site was treated with one treatment and the rest with another);
4. Site had to be treated with emamectin benzoate as medicated feed at a dose of 50 $\mu g\ kg^{-1}$ for seven consecutive days.

This study by Lees et al. (2008b) was performed in two sections: first, treatment effectiveness and trends, and second, regression models to examine factors involved with treatment outcome and post-treatment lice counts. The first section examined post-treatment mean abundance of sea lice and treatment effectiveness. Treatment effectiveness was determined to be a percentage of pre-treatment total mobile abundance = (mean post-treatment abundance/mean pre-treatment abundance * 100). A treatment episode was determined to be effective if values fell to less than 40% of the pre-treatment abundance at some point in the post-treatment evaluation. A general linear model was used to assess the importance of factors influencing post-treatment lice levels, such as region, year, season, presence of synchronized treatments, and stage in the salmon production cycle.

There were 185 emamectin benzoate treatment episodes available for review and 108 treatment episodes met the inclusion criteria. In all years except 2006, the post-treatment lice levels fell to below 45% of pre-treatment levels within 27 days of treatment. In 2006, the maximum effectiveness achieved was 35%. Time to maximum effectiveness was reached 29-34 days post-treatment with the lowest lice levels in the range of 21 to 62 days, similar to Gustafson et al. (2006). In this study, Lees et al. (2008b) found that post-treatment lice levels began to rise around the ninth week following treatment initiation. Duration of treatment effect has been reported as long as five months in the Broughton Archipelago region of British Columbia (Saksida et al. 2007). In the linear regression model, post-treatment abundance was highest following winter (Nov-Jan) and spring (Feb-Apr) treatments, during the second year of the production cycle, and abundance varied by region. Increased sea lice abundance during the second year of the production cycle in Scotland has been noted in other studies (Revie et al. 2002b, Lees et al. 2008c). In summary, the authors determined that not all treatments evaluated were equally effective. This study showed that the effectiveness of emamectin benzoate varied between geographical region, time, and stage of fish production cycle.

A follow-up short communication (Lees et al. 2008a), using the same data (Lees et al. 2008b), developed a logistic regression model to determine factors associated with ineffective treatment episodes. These data excluded the year 2002 as there were no ineffective treatments for that particular year. There were a total of 73 treatment episodes included in this model. Results indicated that ineffective treatment episodes were more likely to occur in the winter months and in the year 2006. Interestingly, pre-

treatment lice abundance was included as a forced covariate and was found to be non-significant, indicating that pre-treatment lice levels did not affect treatment outcome (i.e. success or failure). The mean pre-treatment abundance ranged from approximately 4 to 15 mobile lice per fish. If pre-treatment sea lice abundance was found to be excessively high, then this finding may not hold true. At excessively high levels, fish may show signs of stress and decreased food consumption, thereby not ingest the emamectin benzoate in feed. Several other factors were included as predictor variables and were found to be non-significant: production year, region, and whether or not the treatment was part of a loch-wide intervention. Sensitivity analysis was run to assess the impact of changing the effective treatment criteria of 40%. Separate models were run where the 40% cut-off was changed to 20% and 50% and only the statistically significant factors previously mentioned, year and season, remained.

In 2010, Saksida et al., reviewed emamectin benzoate treatment data from British Columbia, Canada. SLICE® is the only product used for treatment of sea lice in British Columbia. Inclusion criteria were similar to that of previous studies conducted in Scotland (Lees et al. 2008a, 2008b). This study examined treatment episodes administered on farms in British Columbia between 2003 and 2008. Three years had treatment episodes meeting the inclusion criteria, 2003 (n=5), 2007 (n=11) and 2008 (n=9). One of the study objectives was to determine if there was a decline in treatment effectiveness over time and, as it happened, the episodes included the first year and the last two years of the study. The majority of treatments were administered in the autumn or winter months and during the second year of production. A number of farms did not need to treat for sea lice during this time; the proportion varied from 18% in 2004 to

58% in 2007. The frequency of annual treatments remained fairly constant in British Columbia between 2003 and 2008. No significant differences were found between pre-treatment lice counts across years. Treatments for all years were found to be effective, resulting in the reduction of sea lice counts to 3 mobile L. salmonis or less for at least three months following treatment. For all years, sea lice levels had fallen to below 20% of pre-treatment levels by one month post-treatment and, for the remaining follow-up period, the majority of sea lice levels remained at 10% or below the pre-treatment levels. The authors concluded that there was no evidence of a decline in treatment effectiveness on salmon farms in British Columbia from 2003 to 2008.

1.7 Resistance development in parasiticides

Two terms requiring clarification are the difference between tolerance and resistance development. Drug tolerance refers more to unconfirmed instances of an organism surviving a treatment with a product. This trait is not necessarily heritable and may be a transient property. Drug resistance refers to genetically based development of an organism's ability to survive following treatment. These traits are heritable to the next generation. The above terminology was adapted from principles used in weed science pertaining to herbicide resistance (Technology Notes 1998).

Resistance development to macrocyclic lactones used in the treatment of parasites in terrestrial animals has been well documented and appears to be a consequence of repeated use in populations (Prichard & Roulet 2007, Wolstenholme & Kaplan 2012). A variety of mechanisms can result in resistance development of an organism to a treatment product. The mechanism of resistance will usually involve decreased absorption or uptake of a product, or changes in how the product is

metabolised by the organism (Wolstenholme et al. 2004). It is likely that the genes conferring resistance already exist in the population and resistance emergence is inevitable when treatments inadvertently select for these resistance factors. In essence, those parasites with genes conferring resistance have a selective advantage for survival over more sensitive parasites. The rate at which resistance develops can depend on a multitude of factors such as: potency of resistance mechanisms, frequency of chemical/pesticide use, and biology of the parasite (Denholm et al. 2002).

The development of treatment resistance in sea lice has become problematic for intensive salmon farming regions around the world. Resistance, or at least tolerance, has been noted in sea lice toward organophosphates, dichlorvos and azamethiphos, in Scotland and Norway (Jones et al. 1992, Roth et al. 1996, Devine et al. 2000). Resistance in sea lice to azamethiphos has been documented at a molecular level in adult female sea lice from Norway, but was less evident in Canada (Fallang et al. 2004). Reduced sensitivity through use of bioassays to deltamethrin has been documented in a population of sea lice in Norway with a history of treatment failures (Sevatdal & Horsberg 2003). A reduction in sea lice (C. rogercresseyi) sensitivity to emamectin benzoate in Chile was documented using bioassays (Bravo et al. 2008).

Previous studies have also noted some life stage differences in resistance development. Roth et al. (1996) found differences in the sensitivity patterns of different life stages with the use of azamethiphos. They found that adult females were less sensitive while pre-adult males appeared to be the most sensitive. Treasurer et al. (2000) also found that gravid females were less sensitive to hydrogen peroxide than other mobile stages of sea lice.

Often the mechanism of resistance development is unknown and this can delay detection of resistance. Bioassays can aid in the detection of changes in sensitivity patterns when the resistance mechanism is unknown (Denholm et al. 2002). While a useful diagnostic tool, they are often labour intensive and require multiple replicates to ensure that results are accurate (Westcott et al. 2008). Bioassays are used to measure the concentration of a product required to achieve a predetermined effect on an organism and have been used as a method of resistance detection. The sensitivity of that organism to the product of interest is determined using an EC_{50} value which is the concentration that immobilizes 50% of the target organisms. For a detailed explanation of the methods used for laboratory bioassays with sea lice, see Westcott et al. (2008). With the confirmation of changes in sensitivity patterns, further testing is required. Use of molecular tests, such as PCR (polymerase chain reaction), which could detect gene level resistance, would be ideal. However, tests at the molecular level require detailed information on the mechanism of resistance.

1.7.1 Resistance development in avermectins (emamectin benzoate)

Resistance to macrocyclic lactones have been observed (Prichard & Roulet 2007). Le Jambre et al. (2000) found that resistance to ivermectin in Haemonchus contortus, a pathogenic nematode of ruminants, showed strong indications of resistance development being sex-linked or sex-influenced, but only at the adult stage. They noted that in adult stages, males appeared to be more susceptible to the drug than females. Although an interesting finding for resistance development research, results found in nematodes may be difficult to extrapolate to arthropods, including sea lice.

P-glycoproteins have been associated with resistance development in nematodes, including H. contortus, and may be an accurate marker for resistance emergence (Prichard 2001). P-glycoprotein is an ATP-binding cassette (ABC) transporter protein which moves a variety of substances across cell membranes. Avermectins are good substrates for P-glycoprotein which act by removing avermectin from the cell through the transporter protein. This removal decreases the drug's access to the target sites and, thus, decreases concentration at the receptors. Macrocyclic lactones each have different affinity as a substrate for P-glycoprotein (Lespine et al. 2007). It has been suggested that emamectin benzoate may be a better substrate for P-glycoproteins and more readily transported than ivermectin (Igboeli et al. 2012). An increase in P-glycoprotein is thought to result in more rapid removal of the drug from nematodes (Wolstenholme et al. 2004). Prichard and Roulet (2007) reviewed resistance development in macrocyclic lactones and suggested that increased expression of ABC transporters may be the primary mechanism responsible for resistance emergence. Overexpression of mRNA for P-glycoprotein has been associated with emamectin benzoate exposure and has been linked to possible resistance development in sea lice to emamectin benzoate (Igboeli et al. 2012).

1.8 Evaluations of sea lice populations on farmed salmon

Revie et al. (2002b) reviewed historical sea lice (L. salmonis) counts in Scotland from 1996 to 2000. Abundance data were analysed in association with several key factors: year, stock type, region, coastal exposure, and level of treatment. Mean abundance of L. salmonis varied by year and was found to be significantly higher on fish during the second year of the production cycle. No significant differences were found in

sea lice numbers on farmed salmon between geographical regions or between farm locations (e.g. closer to the shore versus those with more coastal exposure). Temperature was not found to be a significant factor in sea lice abundance, which differs from what was found by Boxaspen (1997). Intensity of treatment did appear to have a major impact on the level of sea lice found on fish; the authors found evidence of a six week treatment intervention cycle.

Subsequent to the above study, Revie et al. (2003) expanded on epidemiological factors affecting L. salmonis populations on farmed Atlantic salmon in Scotland using general linear models. Factors influencing mobile sea lice abundance, both within management control and those outside farm management control were examined. Sea lice populations were split into six month periods resulting in three divisions of populations: the last half of the first year in ocean net pens, the first half of the second year, and the second half of the second year. This allowed for evaluation of factors at different times during the production cycle. The first six month period of the first year in net pens was not examined as sea lice levels are generally quite low during this period of time. Significant factors in the model within management control were: treatment level, treatment type, and cage volume; while non-significant factors included stocking density, biomass, strategic treatment, neighbours with poor management practices, and length of fallow period. Factors outside of farm management control found to be significant were current speed and flushing; while non-significant factors were water temperature and current pattern. It is important to note that there were likely other variables, such as salinity, which may have influenced mobile sea lice abundance, but these could not be investigated due to inadequate availability of data. The inclusion of

sea lice abundance from the preceding six months was found to increase the variation explained by the model. The most unexplained variation occurred during the last six months of production where the only factor of significance was treatment type.

Furthermore, Revie et al. (2003) found that temperature was not a significant factor affecting sea lice abundance, while other studies have found the opposite to be true (Johnson & Albright 1991b, Boxaspen 1997, Tucker et al. 2000). The temperature range in that study was 6.0-13.2 °C. Many of these studies were laboratory-based experiments and this may emphasize that differences can be found between laboratory and field results. Boxaspen (1997) found that sea lice abundance variations were correlated with temperature resulting in increased abundance at higher temperatures (> 6°C). This was a study where small cages containing salmon were placed at four sites (i.e. one in the middle of a salmon farm, one at a fallowed salmon farm site, and two at a salmon farm with one closer to inshore and the other offshore from the farm). The study covered most months of the year and temperatures in Norway can range from 2.5-19.5 °C. There was a significant increase in lice abundance from 1993-1994 to 1994-1995 (i.e. greater than double) and this corresponded with a change in temperature. However, the difference in temperature was small; 1993-1994 was found to be 0.68 °C lower than long-term average, while in 1994-1995 it was 0.38 °C higher than the average. This study concluded that the differences in abundance levels may have been due to changes in temperature. With such a small difference in temperature, the possibility of other unmeasured variables influencing sea lice abundance, such as other fish as reservoirs for infection and water current/flushing speed, could not be ruled out.

In addition, Revie et al. (2003) found no association between length of fallow period and sea lice abundance. The fallow period was dichotomized into short or long periods, with long periods being in the 70-80 day range while the short periods were approximately 40 days. This finding is in contrast to a previous study which evaluated length of fallow period. Bron et al. (1993) concluded that the use of longer fallow periods was more effective than short fallow periods. However, this evaluation used a small data set from two Scottish sites, one with a long fallow period and one with a short period. While neither study determined the appropriate length of a fallow period, the use of such practices remains an important factor in control of sea lice levels especially during the early months after smolt transfer.

In 2003, Heuch et al. compared epidemiological patterns of sea lice infestation between Scotland and Norway. Some of the more interesting differences were related to farming practices. In Norway, cages were larger and deeper than in Scotland where pen depth did not change throughout production. Scotland tended to have single year class sites, while Norway had a mix of farms, with only 38% of sites holding fish of a single generation. In Scotland, farms tended to be stocked with fish over a period of less than three months (spring), while Norway stocked fish at various times between April and November. Norway was less likely to practice fallowing, while Scotland typically fallowed their sites between two and four months. Scotland tended towards higher lice abundance, higher infection pressure, and more treatment applications. Cleaner fish were used by 47% of Norwegian farms. Norway (2.5-6.4°C) has lower winter temperatures than Scotland (5.5-9.2°C), as well as an overall wider range of

temperatures throughout the production cycle. Both locations were coldest in March and warmest in August.

Saksida et al. (2007) evaluated sea lice (L. salmonis and C. clemensi) abundance levels on farmed Atlantic salmon in the Broughton Archipelago from 2003 to 2005. They found the lowest levels occurred between June and September and increased through the autumn and winter months. For the full sampling period, higher numbers of mobile sea lice of L. salmonis and C. clemensi were found on second year fish compared to first year fish. There were no significant differences found in the inter-annual variations of mobile L. salmonis populations which were separated into year 1 and 2 classes.

In British Columbia, there was a mean of 1.6 treatments per production cycle and these treatments consisted of only emamectin benzoate (Saksida et al. 2007). The majority of treatments in British Columbia were applied during winter and spring (October to March), while in Scotland and Norway treatments tended to occur most frequently during summer and fall months. In British Columbia, the average farmed salmon population resided in seawater for almost nine months before requiring their first treatment. The number of treatments per production cycle was significantly less in British Columbia compared to those reported in Scotland and Norway. Revie et al. (2002a) found an average of 2.1 and 6.5 treatments per farm per year in year 1 and year 2 respectively in Scotland (1996-2000), while Heuch et al. (2003) found approximately two treatments per farm per year in year 2 populations in Norway (i.e. less than one third of treatments used in Scotland) (1997-1999). For the other studies performed in Scotland and Norway, there were a variety of treatments, none of which were

emamectin benzoate, as this was prior to the use of emamectin benzoate. Since bath treatments have little, if any residual effect, shorter periods of low level sea lice infestations would be expected to result in a higher frequency of treatment application, especially with high external infection pressure. The length of the "lice-free" period in British Columbia and the generation time for lice reproduction at ambient water temperatures suggests that re-infestation from within a farm following an effective site-wide treatment is unlikely. Clinical effects from sea lice infestations are rarely reported on farmed fish in British Columbia unlike other salmon farming areas (Hogans 1995, Armstrong et al. 2000, Johnson et al. 2004).

Lepeophtheirus salmonis abundance was generally lower in British Columbia (Saksida et al. 2007) than in similar reports from farm-based data in Scotland and Norway (Revie et al. 2002a, Revie et al. 2002b, Heuch et al. 2003). Regardless of production area, sea lice levels increase the longer the fish are exposed to seawater (Revie et al. 2002a, Revie et al. 2002b, Tully & Nolan 2002). The proportion of adult female L. salmonis was higher in the second year than in the first year of production in British Columbia (Saksida et al. 2007). In Europe, the highest proportion of gravid females on farmed salmon has been reported to occur in the winter months (Tully 1989).

There are many factors which may contribute to differences in sea lice abundance between British Columbia and other regions. Single year class sites are used in British Columbia (Saksida et al. 2007), similar to Scotland but different from Norway. The water temperatures in the Broughton Archipelago (6.0-13.2°C) are similar to those reported in Scotland (5.5-16.2 °C), while temperatures in Norway have a greater range (2.5-19.5 °C) (Heuch et al. 2003). There is a larger wild salmon population

in British Columbia than in Norway. Norway is considered to have the largest wild Atlantic salmon population in Europe (2-2.5 million) (Heuch & Mo. 2001, Irvine & Fukuwaka 2011), while the average annual catch for wild Pacific salmon in eastern North Pacific Ocean has been well over 100 million per year from 1990 until recent years (Irvine & Fukuwaka 2011). It is plausible that returning wild adult salmon may be a source of sea lice on farms located in regions with large salmon runs.

Lees et al. (2008c) reported a notable change in sea lice abundance (L. salmonis and C. elongatus), infestation patterns and ectoparasiticide use over the 11-year period studied (1996 to 2006, excluding 2001) in Scotland. The study divided sea lice counts into two time periods: 1996-2000 and 2002-2006. There was a significant drop in lice abundance from the first to second time period with a marked reduction in abundance through the summer months of the second production year. This reduction did vary by region, with the Western Isles region having significantly higher levels of lice than the North. Interestingly, this later time period corresponded to the introduction of the use of emamectin benzoate. After the introduction of emamectin benzoate, lice levels dropped and were similar to those observed in the Broughton Archipelago in British Columbia where this product was also in use.

Lees et al. (2008c) also found higher levels of C. elongatus on fish during their first year at sea compared to second year fish and, during the first year, these were at a similar level to mobile stages of L. salmonis. However, during the second year at sea, L. salmonis were around eight times more abundant in Scotland. This finding is in contrast to Saksida et al. (2007) who found no difference in abundance of C. clemensi on fish between first and second years at sea in British Columbia. The reason for this variation

could not be determined but species and location differences are two plausible explanations.

Heuch et al. (2009) reviewed Norwegian sea lice (L. salmonis) levels from 2004 to 2006 and examined factors influencing these levels. They found that salinity, mean fish weight, and treatment type were all significantly associated with L. salmonis abundance. Similar to Scotland and British Columbia, larger fish had a tendency towards higher L. salmonis abundance. Sea lice abundance was lower on sites with low to medium salinity (<26.8‰ S). Adult female sea lice abundance was significantly lower during times when emamectin benzoate was being administered, as opposed to other treatment options, which is in agreement with the findings of Lees et al. (2008c). Fish treated with emamectin benzoate appeared to have the lowest mean adult female lice abundance. A reason for this finding may be the long-lasting effect of this in-feed medicine (Stone et al. 2000a). Pyrethroid treatments are applied as bath treatments and do not have residual effects to provide the fish with protection much beyond treatment application. It is worth mentioning that in Norway, fish in the first year of production are generally treated with emamectin benzoate while larger, second year fish are treated with pyrethroids. Higher overall lice abundance during the second year of production may be a factor in higher lice numbers found on pyrethroid treated fish. Interestingly, in the study by Heuch et al. (2009), untreated fish had lower sea lice abundance than the pyrethroid-treated fish. Producers were only asked to administer treatment if fish had a total mean abundance above 0.3 adult female lice per fish. The low lice abundance recorded on such fish during the subsequent sampling period confirms that farmers were correct in their judgement that delousing treatments were not required.

There has been limited study of sea lice population trends on farmed Atlantic salmon in the Bay of Fundy, Canada. Hogans & Trudeau (1989) examined prevalence and intensity of sea lice on farmed salmon by regularly sampling three farm sites in the Bay of Fundy from 1988 to 1989. The predominant species at that time was C. elongatus accounting for 97.3% of sea lice observed during the study. Two other species were noted, L. salmonis and Caligus curtus (rarely found). Prevalence and intensity of sea lice were highest during late summer and fall when the water temperatures were the warmest (August through November). This peak was thought to be due to the five week generation time of C. elongatus since the highest number of chalimus stages was seen in the months of August and September. Even in the absence of treatment, there was a reduction in prevalence of sea lice during the colder months of December to February when water temperatures were lowest. Although only three sites were used in this study, researchers found some location differences where the site located in Lime Kiln Bay had the highest levels of sea lice infestation of all three sites (the other two sites were in Passamaquoddy Bay and near Campobello Island) (see Figure 2.1 on page 57). Water temperatures between all three sites were found to be similar; it was postulated that other environmental factors may have played a role, such as the enclosed nature of that area or the higher density of farm sites.

A subsequent examination of sea lice populations in the Bay of Fundy was instigated when there was a sudden increase in L. salmonis on farmed salmon beginning in the fall of 1994 (Hogans 1995). Again, infection intensity was positively correlated with water temperature. When water temperatures decreased through the winter months, the lice abundance levels decreased as well. They found that market size fish tended to

have more sea lice than smolts. At this time, both ivermectin and hydrogen peroxide treatments were in use as the heavy sea lice infestations were resulting in damage to the fish and subsequent mortalities. Prevalence and intensity of sea lice varied by location with the worst of the epidemic occurring in the Lime Kiln and Back Bay areas.

1.9 Thesis objectives

1.9.1 Overall goals

The overall goal of this research was to examine the effectiveness of the clinical use of emamectin benzoate against sea lice on farmed Atlantic salmon. Sea lice have developed resistance to this agent in various salmon farming regions around the world and, more recently, resistance emergence in Atlantic Canada was suspected.

1.9.2 Specific objectives

The focus of Chapter 2 was to examine the potentially changing emamectin benzoate effectiveness on L. salmonis. The objectives of this retrospective analysis were to establish if changes in effectiveness of emamectin benzoate were present from 2004 to 2008, to examine factors associated with treatment outcome (effective or ineffective), and to ascertain which variables influenced differences in post-treatment L. salmonis abundance. The area of interest was the Bay of Fundy in the southwestern region of New Brunswick, Canada.

The objectives of Chapter 3 were to determine if there was evidence of a temporal pattern to treatment responses that varied across sea lice life stages, and then to explore how these differences might be used to improve the monitoring of treatment effectiveness in a clinical or field setting. A simple and robust method for monitoring treatment effectiveness using field collected data would be beneficial as a tool which

could be used in conjunction with bioassays and molecular level tests for resistance detection. Two data sets from two salmon farming regions were examined: New Brunswick, Canada, and the west coast of Scotland.

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Chapter 2 Evaluation of emamectin benzoate effectiveness for treatment of sea lice (Lepeophtheirus salmonis) on farmed Atlantic salmon (Salmo salar) in the Bay of Fundy, Canada [1]

2.1 Abstract

Emamectin benzoate (an avermectin chemotherapeutant administered to fish as an in-feed treatment) has been used to treat sea lice (Lepeophtheirus salmonis) infestations on farmed Atlantic salmon (Salmo salar) in the Bay of Fundy, New Brunswick, Canada since 1999. This retrospective study examined the effectiveness of 114 emamectin benzoate treatment episodes from 2004 to 2008 across 54 commercial Atlantic salmon farms. Study objectives were to establish whether changes in the effectiveness of emamectin benzoate were present for this period, examine factors associated with treatment outcome, and determine variables which influenced differences in post-treatment L. salmonis abundance. The analysis was carried out in two parts: first, trends in treatment effectiveness and L. salmonis abundance were explored, and second, statistical modelling (linear and logistic regression) was used to examine the effects of multiple variables on post-treatment abundance and treatment outcome.

Post-treatment sea lice abundance increased in the later years examined. Mean abundance differed between locations in the Bay of Fundy, and higher numbers were found at farms closer to the mainland and lower levels were found in the areas around Grand Manan Island. Treatment effectiveness varied by geographical region and decreased over time. There was an increased risk for unsuccessful treatments in 2008

[1] Patti G. Jones, K. Larry Hammell, Ian R. Dohoo, Crawford W. Revie. Evaluation of emamectin benzoate effectiveness for treatment of sea lice (Lepeophtheirus salmonis) on farmed Atlantic salmon (Salmo salar) in the Bay of Fundy, Canada. Diseases of Aquatic Organisms.

and treatments applied during autumn months were more likely to be ineffective than those applied during summer months.

2.2 Introduction

Sea lice are aquatic ectoparasitic copepods of the family Caligidae, and feed on the mucus, skin, and blood of the host fish. While low level infestations of sea lice cause only minimal impacts to the host, high numbers can result in progressively worsening skin damage and even death of the host. As early as 1940, there were reports of high numbers of lice causing severe damage and mortality in wild fish (White 1940). Intensive salmon farming, particularly where fish farms are clustered in regions, can contribute to outbreaks of pathogens and, potentially, to clinical disease (Murray & Peeler 2005, Robertsen 2011).

Lack of adequate control over sea lice populations can result in a number of economic impacts on fish producers. In addition to mortalities related to sea lice infestation and the cost of parasiticides, other factors, such as increased personnel costs, reduction in food conversion efficiency, reduced fish growth, and mortalities secondary to treatment contribute to economic losses (Costello 2009).

There are two genera of sea lice commonly found on salmonids in marine and brackish waters: Lepeophtheirus and Caligus. Major species associated with salmon aquaculture along the east coast of Canada and the United States are Lepeophtheirus salmonis and Caligus elongatus (Boxaspen 2006). Lepeophtheirus salmonis is the more pathogenic species in the Bay of Fundy, New Brunswick, Canada, mostly due to its size and aggressive feeding in the later stages of the life cycle. Lepeophtheirus salmonis has a ten-stage life cycle with a moult between each stage. There is a free-swimming phase

consisting of two naupliar and one copepodid stage. The copepodid settles on the host fish to begin the attached phase, and will then moult through four chalimus, two pre-adult and one adult stage (Johnson & Albright 1991a, Schram 1993). Johnson & Albright (1991b) reported the generation time to be 40 days for adult males and 52 days for adult females at 10°C (time from egg to adult stage) under laboratory conditions.

Salmon farming is a two phase production system usually consisting of land-based, fresh-water hatchery and marine cage site phases. Fish are transferred from the hatchery as smolts to sea cages, which can occur anywhere from one to two years after egg hatching. Fish are harvested approximately 18 to 24 months after transfer to sea cages. In New Brunswick, smolts are normally transferred in the spring (April to June) or fall (November to December), and the majority of the fish are transferred during the spring months. Sea lice are a problem isolated to the marine stage of the production cycle.

Emamectin benzoate (SLICE®) is an avermectin chemotherapeutant administered to fish as an in-feed treatment (reviewed by Horsberg 2012). Emamectin benzoate was shown to be effective against L. salmonis on farmed Atlantic salmon in North America (Armstrong et al. 2000, Gustafson et al. 2006, Saksida et al. 2007). Several studies have examined the efficacy of emamectin benzoate in the United States (Maine), Scotland, and Canada (British Columbia) (Gustafson et al. 2006, Lees et al. 2008a, Lees et al. 2008b, Saksida et al. 2010). In Maine, all treatments that were applied appeared to be efficacious; however, the study did not examine for changes in effectiveness over the study period of 2002 to 2005 (Gustafson et al. 2006). Examination of emamectin benzoate use in British Columbia found no decrease in

effectiveness from 2003 to 2008 (Saksida et al. 2010). A decline in efficacy was reported in Scotland during the years examined, 2002 to 2006 (Lees et al. 2008a, Lees et al. 2008b). On the east coast of Canada, a recent investigation using laboratory bioassays found increases in EC_{50} values for emamectin benzoate in L. salmonis from southwestern New Brunswick collected in 2011 (Igboeli et al. 2012) compared with values from 2002-2005 (Westcott et al. 2008). A reduction in the sensitivity of Caligus rogercresseyi to emamectin benzoate based on laboratory bioassays has been documented in Chile (Bravo et al. 2008).

In New Brunswick, control of sea lice became a problem in the mid 1990's when there was a consistent increase in the abundance of sea lice found on farmed Atlantic salmon. Initially the majority of sea lice on farmed salmon were Caligus spp., but L. salmonis later became the predominant species and remains so to the present (Hogans & Trudeau 1989, Hogans 1995). In the fall of 1994, an outbreak of L. salmonis occurred on salmon farms in the Lime Kiln and Back Bay areas of southwestern New Brunswick (Hogans 1995). Around that time, a multitude of treatments were attempted with varying success, including hydrogen peroxide, ivermectin, and azamethiphos (Hogans 1995, O'Halloran & Hogans 1996). Emamectin benzoate was introduced in New Brunswick in 1999 through an emergency drug release (Armstrong et al. 2000). This product became the treatment of choice for several reasons: effectiveness against all life stages, prolonged effect, and ease of administration in feed (Stone et al. 2000a, Stone et al. 2000b). Because emamectin benzoate was an effective treatment, there was little incentive to seek approval for other sea lice treatment agents, resulting in this product being used for the majority of sea lice treatments. After almost a decade of use, isolated

treatment failures were observed in late 2008, resulting in suspicions about changes in sea lice sensitivity to emamectin benzoate. During 2009, it became evident that a more serious problem was occurring, which compelled industry and government to investigate other methods of treatment for sea lice (Chang et al. 2011).

The focus of the present study was to examine emamectin benzoate treatment effects on L. salmonis. The objectives of this retrospective analysis were: (1) to establish whether changes in effectiveness of emamectin benzoate were present from 2004 to 2008, (2) to examine factors associated with treatment outcome (effective or ineffective), and (3) to ascertain which variables influenced differences in post-treatment L. salmonis abundance. A cross-section of data obtained from health records collected by salmon farms in the Bay of Fundy region of southwestern New Brunswick, Canada, from 2004 to 2008 was examined.

2.3 Materials and methods

The Bay of Fundy is located on the east coast of Canada between New Brunswick and Nova Scotia and shares water with Cobscook Bay, Maine, USA (Figure 2.1). The area of interest for this study was along the New Brunswick coast of the Bay of Fundy where farming of Atlantic salmon represents a major aquaculture activity. A bay management system is used for the location and stocking of fish farms, called Aquaculture Bay Management Areas (ABMA). Figure 2.1 shows the eight ABMAs as they were established in 2006. In 2010, ABMA 4 was incorporated into ABMA 1, and for the purpose of this study that change was used for all years examined. Data were available from five ABMAs (Table 2.1): 1, Passamaquoddy Bay (including sites around Deer Island and Campobello Island); 2a, Lime Kiln Bay and Back Bay; 3a, which is

considered the “up shore region” heading east towards Saint John (including areas such as Beaver Harbour and Maces Bay); and 2b and 3b, which are both located on the eastern side of Grand Manan Island. These five ABMAs contained the majority of active salmon farms. There were no qualifying treatment episodes in ABMAs 5 or 6, but since only a few active salmon farms were present in these areas; this was not expected to have an impact on this study’s conclusions. For part of this analysis (i.e. statistical models), ABMAs 2b and 3b, which are located around Grand Manan Island, were grouped together because they are adjacent to each other and relatively far removed from the other ABMAs.

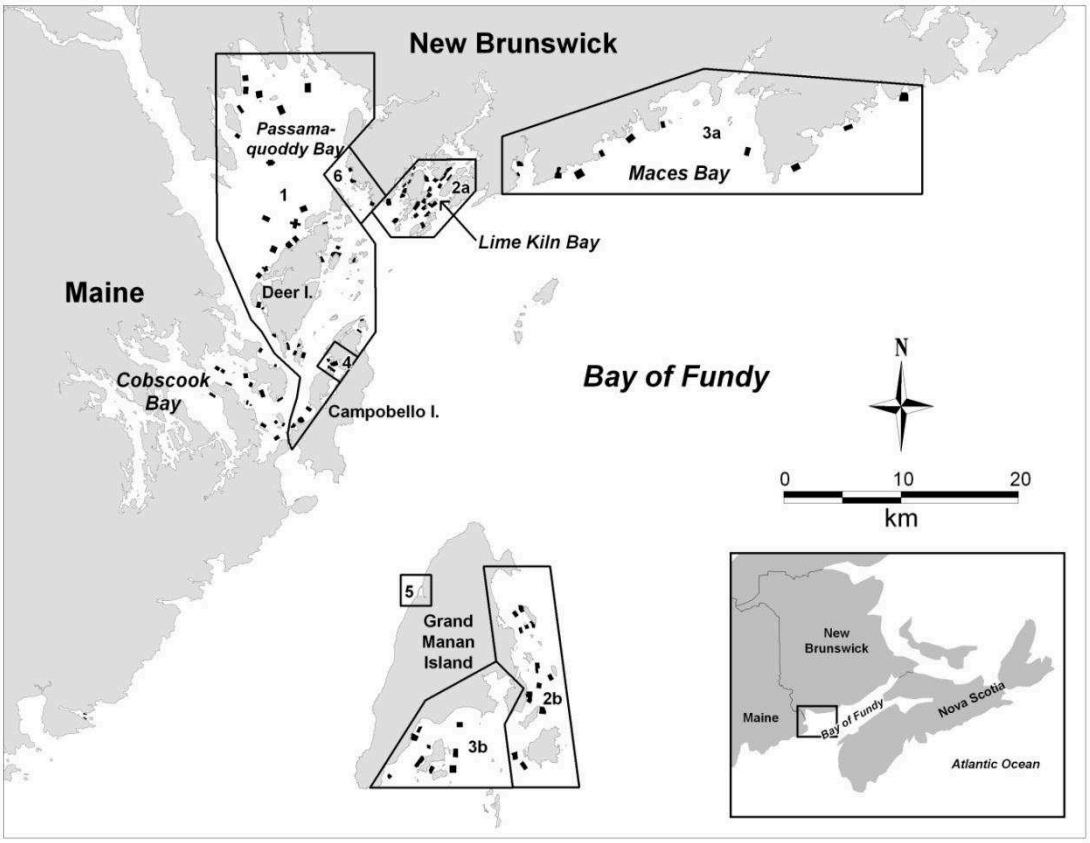

Figure 2.1 Aquaculture Bay Management Areas (ABMAs) for the southwestern New Brunswick Bay of Fundy salmon farming region (salmon farm leases for 2010 are indicated).

Note: Map kindly provided by Blythe Chang of the Department of Fisheries and Oceans Canada, St. Andrews Biological Station, St. Andrews, New Brunswick, Canada.

Table 2.1 Summary of qualifying emamectin benzoate treatment episodes that occurred in each Aquaculture Bay Management Area (ABMA) of the Bay of Fundy (2004-2008).

ABMA (Location)	Number of Qualifying Treatment Episodes	Effective Treatments (%)
1 (Passamaquoddy Bay & Deer/Campobello I.)	46	38 (82.6%)
2a (Lime Kiln, Back Bay)	26	16 (61.5%)
2b (Grand Manan Island)	7	7 (100.0%)
3a (Maces Bay)	20	18 (90.0%)
3b (Grand Manan Island)	15	15 (100.0%)

The data used in this study were extracted from records collected and maintained for sea lice management purposes by the veterinary services within Cooke Aquaculture Inc. (Blacks Harbour, New Brunswick) and Maritime Veterinary Services Ltd. (St. Andrews, New Brunswick). These records were associated with just over half of the fish farming sites and the majority of the companies operating in this region for the period under examination.

Regular sea lice counts are a routine part of salmon farming in this region, as they are for most salmon farming areas around the world, with counts typically occurring at least once every second week. A number of fish are opportunistically sampled by attracting them to the water surface with feed and capturing them with a hand net. Typically fish are anesthetized in a water bath with tricaine methanesulfonate added, and when sufficiently immobilized, the sea lice are counted and classified by using the following life stages: chalimus, pre-adult females, pre-adult males, adult males and adult females (gravid and non-gravid). However, pre-adult males and pre-adult females are often combined with adult males in records to form one category. In this particular study, we examined only L. salmonis as this is the more pathogenic and far more prevalent species on farmed Atlantic salmon in the Bay of Fundy (Hogans 1995).

The study design and methodology used in this investigation was similar to methods used in Lees et al. (2008a, 2008b) in Scotland. Historical sea lice count data and treatment records were examined from 2004 to 2008 and treatment episodes were selected based on specific study inclusion criteria. In order to be included, a treatment episode had to contain a pre-treatment count within 16 days of treatment being started, as well as a minimum of three post-treatment sea lice evaluations in the 12 weeks

following initiation of treatment. If there was more than one pre-treatment count available in the 16 day period, then the count closest to the date of treatment initiation was used. A specific sea lice count was only included when at least two cages were sampled and a minimum of five fish per cage were examined. Treatment of sea lice with emamectin benzoate involves the administration of medicated fish feed over the course of seven days. Any treatment that had a notation in the record regarding only partial site treatment or split site treatment was excluded from this analysis. For this analysis, pre-adult males, adult males, pre-adult females and adult females (gravid and non-gravid) L. salmonis were aggregated into a single group called 'total mobiles'.

The analysis was split into two parts: first, description of treatment efficacy and trends in L. salmonis abundance, and second, statistical modelling (linear and logistic regression) to examine the effects of multiple variables on post-treatment mean mobile L. salmonis abundance and treatment outcome. For the former, treatment trends were summarized at the farm level and examined by year in two ways: mean abundance of total mobiles before and after treatment, and treatment effectiveness as a percentage of the pre-treatment abundance. Pre-treatment mean abundance of mobile L. salmonis varied by year and this variance was examined by use of an ANOVA procedure. For comparisons of means, a post-ANOVA multiple comparisons procedure was performed using the Bonferroni method. Treatment effectiveness was determined by the following equation: Treatment effectiveness = (post-treatment mean abundance/ pre-treatment mean abundance) * 100%. A treatment was considered effective if post-treatment mean abundance fell to less than 40% of the pre-treatment mean abundance at any point during the post-treatment period. This value has been used in previous articles as a cut-

off point upon which to base treatment success with emamectin benzoate (Lees et al. 2008a, Lees et al. 2008b, Saksida et al. 2010).

The second part of the analysis involved the building of two statistical models: a linear model for post-treatment mean mobile L. salmonis abundance, and a logistic model of treatment effectiveness. Predictor variables used in the building of the statistical models were year, pre-treatment mean mobile L. salmonis abundance, location of farm (ABMA), season, month, season2 (see below), age of fish, and season of smolt transfer, as well as the week of count during the post-treatment period. Fish were classified into first or second production year based on the age of fish since smolt transfer to ocean cages. Fish were classified as first year fish if they had been at sea for less than 365 days and as second year fish if they were at sea for more than 365 days. Fish were also classified into groups based on the season of smolt transfer, either spring or autumn. Given that temperature and season can be important factors in relation to sea lice abundance, the variables month, season and season2 were created to examine their potential impact on post-treatment L. salmonis abundance. The month of treatment application was used as a predictor, along with a season variable created by categorizing months into the following groups: spring (April-June, 4-9 °C), summer (July-September, 11-14 °C), autumn (October-November, 8-11 °C), and winter (December-March, 1-7 °C). Water temperatures tend to be highest in August-September (12-14 °C) and lowest in February-March (1-2 °C). Thirdly, a variable, called season2, was created where season was dichotomized based on months where water temperatures were generally above or below 10 °C. Water temperatures were generally above 10 °C from July to October and below 10 °C from November to June. For the variable week, the baseline

value was week 2, corresponding to days 7 to 13 after the start of treatment. Week 1 was eliminated from this part of the analysis because there was usually no noticeable treatment effect at this point (often there was an increase in L. salmonis abundance, as noted in Figure 2.2). As mentioned above, for both statistical models, the Grand Manan Island ABMAs 2b and 3b were grouped together (Figure 2.1).

For the linear model, upon initial examination of the outcome variable (mean abundance of total mobiles), the data was found to be positively or right-skewed. A Box-Cox analysis produced a lambda value of 0.087. This led to logarithmic transformation of the data by [ln(mean + 0.1)] to help improve our statistical assumptions of normality of residuals and homoscedasticity. Pre-treatment abundance was also logarithmically transformed. Predictor variables were initially screened unconditionally, with those having a p-value <0.15 retained for model building. The linearity of the relationship between pre-treatment and post-treatment (log) counts was evaluated using a locally weighted scatterplot smoothing algorithm (Lowess) and found to be acceptable. Initially, both treatments and farms were considered random effects, but little unexplained variation was found at the farm level; therefore, a two level model (sampling weeks within treatment episodes) was constructed. A Toeplitz covariance structure to account for correlations among counts up to six weeks apart was applied at the lowest (week) level. Model building was carried out manually with interactions among key variables evaluated as part of the process. Residuals at both the week and treatment episode level were checked for normality and homoscedasticity.

The second model was a logistic regression model used to evaluate factors associated with treatment success or failure. Success was defined as a minimum of a

60% reduction in L. salmonis burdens at any point in the post-treatment period. The outcome variable was treatment success or failure. The predictor variables considered were year, month, season, season2, location (ABMA), fish age, season of smolt transfer, and pre-treatment mean L. salmonis abundance (log transformed). A multilevel logistic model using treatments nested within farms was initially assessed but there was little unexplained variation between farms; therefore, a simple logistic regression model was used.

A similar model building process to the linear mixed model was used to create the logistic regression model. The fit of the final model was evaluated using Hosmer-Lemeshow goodness-of-fit test. Specific observations not fitting the model or having undue influence on the model were evaluated by generating Pearson and deviance residuals and any outlying values were examined. Influence of outliers on the model was evaluated by generating leverage and delta-beta values.

Software programs used to analyze data were Microsoft Excel 2007 (Microsoft Canada Co., 1950 Meadowvale Blvd., Mississauga, Ontario, Canada) and Stata/IC 12 (StataCorp LP, 4905 Lakeway Drive, College Station, Texas, USA). Microsoft Excel 2007 was used to manage and format the data, and to create the mean abundance and efficacy graphs. Stata 12 was used to perform the trend and efficacy analysis along with the statistical models.

2.4 Results

After excluding treatment episodes based on study inclusion criteria, 114 treatment episodes across 54 farms remained in this analysis. There was a mean of 40 fish examined per sample (range 10-240) and 4.3 cages selected per sample date (range 1-19).

2.4.1 Trends in abundance and effectiveness

The pre-treatment mean abundance of mobile L. salmonis varied over the five years examined. In 2004, pre-treatment mean abundance was 0.7 mobiles per fish and increased annually to around ten times this value by 2007 and 2008 (Figure 2.2 & Table 2.2). Based on an ANOVA procedure, year had significant ($p < 0.01$) influence on pre-treatment abundance. Bonferroni multiple-comparisons determined that there were significant differences in pre-treatment means between years 2004 and 2007 ($p=0.001$), 2004 and 2008 ($p=0.001$), as well as between 2005 and 2008 ($p=0.035$). The difference between 2005 and 2007 was marginally significant at $p=0.056$.

Table 2.3 lists the number of treatments per year as well as classifying treatments by success or failure. If the post-treatment mean abundance of L. salmonis fell to less than 40% of the pre-treatment value at any point during the follow-up period, then an individual treatment episode was deemed effective. Effectiveness could not be calculated for episodes where pre-treatment abundance was zero and this occurred in two treatment episodes, one from 2004 and another from 2008. Both of the treatments resulted in very low L. salmonis abundance in the follow-up period, with a range of 0-0.07 mobiles per fish in all weeks; therefore, these treatments were classified as successful.

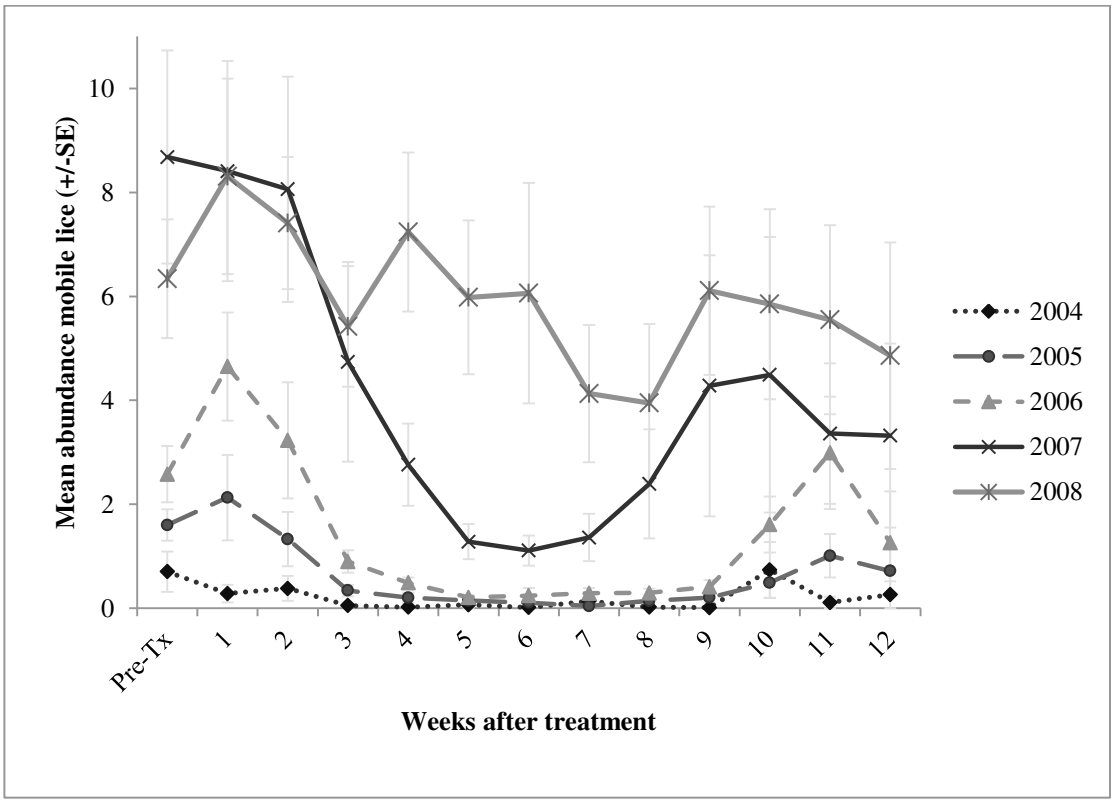

Figure 2.2 Mean abundance (+/- SE) of mobile Lepeophtheirus salmonis pre- and post-treatment with emamectin benzoate on farmed Atlantic salmon in New Brunswick, Canada (2004-2008).

Table 2.2: Mean abundance (+/- SE) of mobile Lepeophtheirus salmonis pre- and post-treatment with emamectin benzoate on farmed Atlantic salmon in New Brunswick, Canada (2004-2008).

Week	2004	2005	2006	2007	2008
Pre-Tx	0.70 (0.39)	1.60 (0.30)	2.58 (0.54)	8.68 (2.05)	6.34 (1.14)
1	0.28 (0.17)	2.13 (0.82)	4.65 (1.04)	8.41 (2.12)	8.31 (1.88)
2	0.38 (0.24)	1.33 (0.52)	3.23 (1.12)	8.06 (2.17)	7.41 (1.27)
3	0.05 (0.03)	0.34 (0.11)	0.90 (0.21)	4.74 (1.92)	5.42 (1.16)
4	0.02 (0.02)	0.20 (0.10)	0.49 (0.10)	2.76 (0.79)	7.24 (1.53)
5	0.07 (0.06)	0.15 (0.07)	0.21 (0.05)	1.28 (0.34)	5.98 (1.48)
6	0.01 (0.01)	0.11 (0.05)	0.24 (0.15)	1.11 (0.29)	6.06 (2.12)
7	0.14 (0.08)	0.04 (0.03)	0.29 (0.10)	1.36 (0.46)	4.13 (1.32)
8	0.02 (0.02)	0.14 (0.06)	0.30 (0.08)	2.39 (1.05)	3.95 (1.52)
9	0.01 (0.01)	0.21 (0.09)	0.41 (0.13)	4.28 (2.51)	6.11 (1.62)
10	0.74 (0.54)	0.49 (0.29)	1.61 (0.54)	4.49 (2.65)	5.85 (1.83)
11	0.11 (0.11)	1.01 (0.42)	2.99 (1.08)	3.36 (1.35)	5.55 (1.82)
12	0.26 (0.26)	0.72 (0.46)	1.26 (0.99)	3.32 (1.77)	4.86 (2.18)

Table 2.3 Summary of qualifying emamectin benzoate treatment episodes by year and number of treatments which were effective (percentage of effective treatments) or ineffective.

Year	Number of Qualifying Treatment Episodes	Number of Effective Treatments (<40% of pre-treatment mobile L. salmonis abundance)	Number of Ineffective Treatments
2004	7	7 (100.0%)	0
2005	20	20 (100.0%)	0
2006	23	22 (95.6%)	1
2007	31	28 (90.3%)	3
2008	33	17 (51.5%)	16
Total Treatment Episodes	114	94 (82.5%)	20

Treatment effectiveness declined through the years examined. In 2004 and 2005, all treatments evaluated were deemed effective, while through 2006 to 2008, the number of ineffective treatments progressively increased each year. In 2006, the first ineffective treatment was noted and two other treatment episodes were marginally effective, where the maximum effectiveness was 36.9% and 33.7% of the mean pre-treatment abundance. Maximum effectiveness was determined using the lowest percentage attained during the post-treatment period. Overall, 94 of 114 treatment episodes or 82% were classified as effective for all the years examined. The major change occurred between 2007 and 2008 when the percentage of successful treatments decreased from 90% to 51%.

In 2007, 28 of 31 treatments were deemed effective in this study, though only 20 treatments reached a post-treatment abundance level of <1 mobile per fish. In 2008, 17 of the 33 treatments were considered effective based on the cut-off point of 40%; however, only 14 of these treatments reached post-treatment abundance of <1 mobile per fish.

Figure 2.3 demonstrates the effectiveness of emamectin benzoate summarized by year as a percentage of pre-treatment mean abundance of L. salmonis. All years, on average, exhibited overall treatment success, except for 2008. However, the maximum level of effectiveness attained decreased with each subsequent year. In 2004, post-treatment mean abundance fell to as low as 0.9% of pre-treatment levels during the follow-up period, while in 2005, maximum effectiveness was 6.8% of the pre-treatment level and this effectiveness continued to erode over the next three years (15.3% for 2006, 22.6% for 2007 and 75.7% for 2008).

Duration of treatment effect varied between treatment episodes, but in this study it appeared to last approximately nine to ten weeks during the early years (Figure 2.3). In 2004 and 2005, the trends were typical for an effective treatment with emamectin benzoate when the percentage of pre-treatment levels decreased to less than 20% by week 3 and remained relatively low for the following seven weeks. In 2006, there was an expected drop in mobile L. salmonis numbers following treatment, but effectiveness values did not stay low for as long as those seen in 2004 and 2005 as evidenced by an increase which began around week 8, after two to three weeks of minimum values. By 2007, there appeared to be a steady rise in values beginning around week 6 or 7 after treatment. The dramatic drop previously observed in the first few weeks following treatment was absent in 2008. Although just over 50% of treatment episodes were deemed effective, treatments as a whole for that year were classified as ineffective, this is evident in the failure to drop below 75% in 2008 (Figure 2.3). Overall, duration of treatment effect appeared to decrease with time.

Time to maximum effectiveness varied by treatment episode and year. In the early years, maximum treatment effect was generally attained between weeks 4 and 6 following the start of treatment (Figure 2.3). The time to maximum effect was extended to week 7 in the final year of the study (2008).

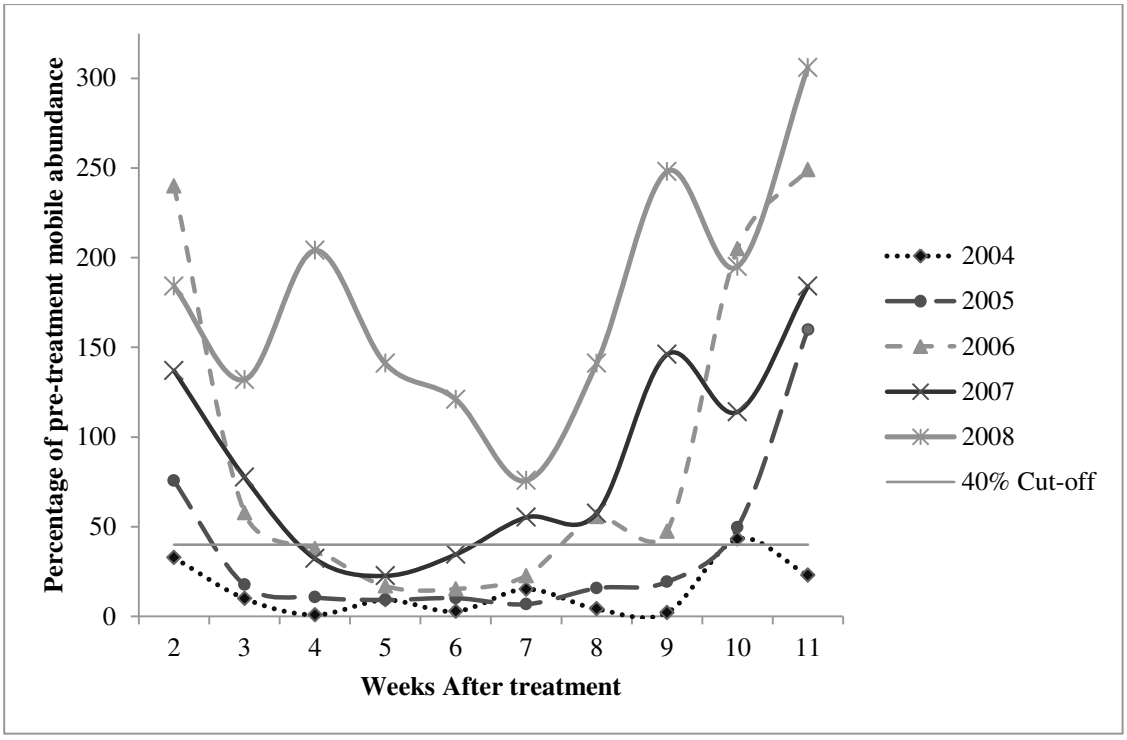

Figure 2.3 Trends in treatment effectiveness (as a percentage of pre-treatment mean abundance of mobile Lepeophtheirus salmonis) of emamectin benzoate treatments against L. salmonis on farmed Atlantic salmon in New Brunswick, Canada (2004-2008).

2.4.2 Statistical modelling of post-treatment sea lice abundance

The linear mixed model was evaluated initially using a three level model with weeks nested within treatments and treatments nested within farms. There were 114 treatments spread over 54 farms resulting in a mean of 2.1 treatments per farm (range was 1 to 5). We determined that the use of farm as a random effect was not necessary given there was little unexplained variation at the farm level, likely resulting from the fact that there were so few treatments per farm. As a result, a two level model was used with weeks nested within treatments. Treatment episodes were treated as random effects. In the two level model, there was little unexplained variation at the treatment level as all the variation was explained by both the fixed parameters and the unexplained variation by the covariance pattern (Toeplitz correlation structure) at the lowest level (week). The covariance estimates confirmed that there was a high level of correlation among counts collected close together (one to two weeks apart), but that this declined steadily and there was no remaining correlation once counts were more than six weeks apart.

Results of the linear mixed model are shown in Table 2.4 as the log transformed values. Post-treatment mean L. salmonis abundance increased during the later years of the study with significant differences found between 2004 and 2007, and between 2004 and 2008. No significant differences were found between post-treatment L. salmonis levels in ABMA 1 (Passamaquoddy Bay) and 2a (Lime Kiln Bay) or 1 and 3a (Maces Bay). Significantly lower levels of L. salmonis abundance were found at sites near Grand Manan Island (ABMAs 2b and 3b) compared to sites in Passamaquoddy Bay (ABMA 1).

Pre-treatment mean L. salmonis abundance was a significant predictor in the level of abundance during the post-treatment period. For every increase in the pre-treatment abundance of 1 natural log value (equivalent to an increase by a factor of 2.7 times), there was a corresponding increase in the post-treatment log-values of 0.44 (equivalent to an increase by a factor of ~1.5 times).

There was a significant interaction between age of fish and season of smolt transfer indicating that the effects of fall transfer were different in second year fish compared to first year fish. Ultimately, fish in their second year and transferred to seawater in the fall were more likely to have higher post-treatment L. salmonis abundance than second year fish which were transferred in the spring.

Table 2.4 Results of the linear mixed regression model of mean mobile Lepeophtheirus salmonis abundance (log transformed) following treatment with emamectin benzoate at Atlantic salmon farms in New Brunswick, Canada, for 2004-2008. (Baseline values are year=2004, bay code=Passamaquoddy, season of transfer=spring, fish age= <1 year, week=2).

Variable	Category	Coefficient (log transformed)	SE	p-value	95% Confidence Interval	
					Lower	Upper
Year	2005	0.10	0.32	0.759	-0.53	0.73
	2006	0.30	0.32	0.354	-0.33	0.93
	2007	0.86	0.33	0.010	0.21	1.51
	2008	1.60	0.34	0.000	0.94	2.26
Bay code (ABMA)	Lime Kiln (2a)	-0.41	0.24	0.087	-0.87	0.06
	Grand Manan (2b & 3b)	-1.09	0.18	0.000	-1.45	-0.73
	Maces Bay (3a)	-0.38	0.20	0.061	-0.78	0.02
Week	3	-0.66	0.07	0.000	-0.81	-0.52
	4	-0.90	0.09	0.000	-1.08	-0.72
	5	-1.15	0.10	0.000	-1.36	-0.95
	6	-1.22	0.12	0.000	-1.46	-0.97
	7	-1.23	0.13	0.000	-1.48	-0.98
	8	-1.07	0.14	0.000	-1.34	-0.80
	9	-0.80	0.15	0.000	-1.09	-0.51
	10	-0.46	0.15	0.000	-0.76	-0.16
	11	-0.26	0.16	0.111	-0.57	0.06
	12	-0.07	0.18	0.686	-0.43	0.28
Season of transfer	Autumn	0.16	0.28	0.572	-0.39	0.70
Fish age	>365 days	0.28	0.16	0.076	-0.03	0.60
Fish age x season of transfer interaction		0.84	0.32	0.009	0.21	1.47
Pre-treatment abundance (log transformed)		0.44	0.06	0.000	0.32	0.55
Constant		-0.42	0.33	0.200	-1.06	0.22
Random Effects Parameters		**Estimate**	**SE**		**95% Conf Interval**	
Treatment: Identity	Variance (constant)	1.26×10^{-15}				
Residual:	Covariance1	0.76	0.07		0.62	0.91
Toeplitz (6)	Covariance2	0.61	0.07		0.48	0.74
	Covariance3	0.47	0.06		0.35	0.58
	Covariance4	0.30	0.05		0.20	0.39
	Covariance5	0.22	0.04		0.14	0.29
	Covariance6	0.10	0.03		0.05	0.15
	Variance (e)	0.98	0.07		0.84	1.14

2.4.3 Statistical modelling of treatment outcome

A logistic regression model examined factors involved with treatment outcome, classified as either effective or ineffective treatments (Table 2.5). Given that there were no ineffective treatment episodes found in either 2004 or 2005, those years were dropped from this component of the analysis. The spring season was also dropped as it contained only four treatment episodes (one in 2006 and three in 2008), none of which were classified as ineffective. Based on the exclusion of the episodes from these years and season, a total of 83 treatment episodes were evaluated, which led to a mean of 1.8 treatments per farming site.

Location of treatment was not significant in the full model. However, the model was unable to estimate the effect of predictor variables on treatment failure in ABMAs 2b or 3b (Grand Manan Island) as there were no failed treatments in the data set, even in 2008. There appeared to be no significant differences in treatment outcome between ABMAs 1, 2a, and 3a. All possible interactions between variables were evaluated during the model building process and none were found to be significant. Fit of the model to the data was evaluated by use of the Hosmer-Lemeshow goodness-of-fit test; there was no evidence of lack of fit. All potentially influential observations or outliers were assessed and examined, and none were found to have undue influence on the model.

In summary, this model showed that treatments applied in 2008 had an increase in the odds of failure by 37 times over a treatment applied in the year 2006. Season was also a significant variable ($p<0.01$); treatments administered during autumn (October-

November) had an odds of failure approximately seven times that of treatments applied during summer months (July-September).

Table 2.5 Results of logistic regression analysis of factors associated with ineffective emamectin benzoate treatments at salmon farms in New Brunswick, Canada, for 2006-2008. (Baseline value is year 2006 in the summer season).

Variable	Category	Coefficient	SE	p-value	Odds Ratio	95% Confidence Interval	
						Lower	Upper
Year	2007	1.02	1.23	0.407	2.78	0.25	31.22
	2008	3.63	1.18	0.002	37.64	3.72	380.22
Season	Autumn	1.97	0.74	0.008	7.18	1.67	30.92
Pre-treatment abundance [ln (x+0.1)]		-0.24	0.26	0.357	0.79	0.47	1.31
Constant		-3.91	1.15	0.001			

2.5 Discussion

There are challenges commonly encountered when using historical production data, some of which were reviewed by Lees et al. (2008a). The challenges encountered in the current study were that numerous individuals carried out the sea lice counts (lack of consistency), pre-treatment counts occurred any time within 16 day period prior to treatment initiation (true pre-treatment levels at treatment initiation may have been higher than indicated), and treatment episodes were excluded which did not meet the inclusion criteria. The use of a large data set helped to improve the statistical power of this study. The data set used in the present study was comparable in size to that used by Lees et al. (2008a, 2008b), but larger than those used in similar previously published studies (Gustafson et al. 2006, Saksida et al. 2010).

A concern with the use of historical data is the lack of control groups as discussed in Gustafson et al. (2006). Classic assessment of treatment efficacy involves a study design in which the effect of treatment is based on the differences between two groups randomized to treatment or control, such as attempted by Campbell et al. (2006). In the treatment of parasite populations in the aquatic environment where disease progression is best controlled while in the early stages, there are animal welfare concerns when leaving cages of fish untreated with a growing sea lice infestation. To assess the treatment effectiveness of emamectin benzoate in a clinical environment that was not amenable to the inclusion of untreated control cages, treatment effects had to be based on the change in sea lice populations after treatment compared to the pre-treatment sea lice assessments.

This study relied on sea lice count data recorded by the fish farmers whose routine management required frequent enumeration of sea lice on Atlantic salmon. Although fish are obtained using non-random samples (i.e. attracting fish to the surface with feed and then capturing them with a hand net) and sampling bias may have been introduced, there are no practical solutions for frequent random samples in the salmon farming environment. Sampling practices have been reviewed in a number of studies (Revie et al. 2005, Revie et al. 2007, Heuch et al. 2011) and can be an accurate method for detection of farm level sea lice infestations. In field observational studies of sea lice patterns, any selection bias associated with estimates of the true mean sea lice abundance is assumed to be present in similar levels across different treatment events and thus inconsequential to the interpretation of effectiveness.

In this study, pre-treatment L. salmonis abundance in the early years was lower than the pre-treatment abundance in similar studies. In Lees et al. (2008a), the pre-treatment mean L. salmonis abundance ranged from approximately 5 to 15 mobiles per fish, while in Saksida et al. (2010) the range was from approximately 4 to 7 mobiles per fish. In the present study, pre-treatment values were, on average, fewer than 3.0 mobiles per fish for 2004-2006, but in 2007 and 2008 they rose to 8.7 and 6.3 mobiles per fish respectively (Table 2.2). The reason for this change in pre-treatment L. salmonis abundance between 2004-2006 and 2007-2008 cannot be determined with this study design. This observation may be associated with increased L. salmonis tolerance for emamectin benzoate (unable to maintain sufficient control over populations), natural variation in the levels of L. salmonis found on farmed fish in that region, or a tendency by farm management to initiate treatment decisions differently. Lees et al. (2008a) also

observed differences in pre-treatment abundance between years with higher abundance occurring in 2003 and 2004.

During the early years of the present study, duration of treatment effect appeared to last for approximately nine to ten weeks following treatment initiation. Comparatively, Lees et al. (2008a) found the lowest L. salmonis abundance between days 21 and 62 (weeks 4 and 9), with levels generally remaining below pre-treatment levels for the full observation period (83 days). We found the length of treatment effect was reduced in later years. Development of tolerance in L. salmonis to this compound may have been a factor, but one cannot rule out other possible variables, such as increases in external infection pressure, seasonal effects, or lack of data in the later weeks of some treatment episodes. Recent laboratory bioassay evidence is suggestive of the development of decreased sensitivity to emamectin benzoate in L. salmonis from the Bay of Fundy over time (Igboeli et al. 2012). In British Columbia, an assessment of emamectin benzoate effectiveness found that acceptable levels of post-treatment abundance were maintained for at least three months following treatment, with L. salmonis levels remaining significantly below pre-treatment levels, as well as staying below the 3 mobile L. salmonis trigger level that is used in British Columbia (Saksida et al. 2010). The reason for this continued success of emamectin benzoate treatments in British Columbia is unknown, but may be influenced by the large populations of wild Pacific salmon which may act as a refuge for sea lice sensitive to emamectin benzoate. In addition, there are differences in how farms are distributed between these areas; farms in British Columbia are located over a larger area, while in southwestern New Brunswick, farms are more densely concentrated in a smaller region. These differences

may play a role in the variation of L. salmonis sensitivity between these two regions and warrants further investigation.

Results for time to maximum effectiveness were similar to those in other studies. In Lees et al. (2008a), the lowest levels were found around the days 28-34 (week 5) in the early years of the study, while Gustafson et al. (2006) found similar range of 21-28 days (week 4) for the maximum effect to be reached. Maximum effectiveness was, on average, attained between weeks 4 and 6 from 2004 to 2007. In 2008, maximum treatment effect was not evident until week 7 (75.7%). These trends were supported by clinical observations that L. salmonis were remaining on fish longer following treatment administration when suspicions of tolerance development first emerged (M. Moore, personal communication, 2011).

The major finding from the linear mixed model for variables associated with post-treatment mean abundance of L. salmonis was that treatment effect varied both temporally and spatially. Post-treatment abundance increased from 2004 to 2008. In addition, there were differences by location, and the areas farthest from the mainland at Grand Manan Island (ABMAs 2b and 3b) had the lowest L. salmonis burdens. Similar findings were reported by Lees et al. (2008a) in Scotland. Variables related to month or season of treatment application were non-significant. The majority of treatment episodes (97 of 114) in this analysis occurred between July and October; consequently there were insufficient treatment episodes spread throughout the year to support conclusions on the effects of season on post-treatment abundance. Only one treatment met the inclusion criteria during the winter season (December-March) and this was an effective treatment in 2004. One could speculate that we would expect to see overall lower L. salmonis

abundance around treatment episodes during the winter months as sea lice levels in New Brunswick tend to be lowest throughout the winter months (Chang et al. 2011). However, time to maximum treatment effect may be delayed in winter as was shown in an efficacy study on emamectin benzoate that found treatments applied during colder months took longer to reach maximum effect (Stone et al. 2000c). In comparison, Lees et al. (2008a) found some seasonal variations in Scottish data in which treatments applied during winter (Nov-Jan) and spring (Feb-Apr) had higher post-treatment abundance of L. salmonis.

The frequency of treatments in young versus older fish was almost equivalent with 59 treatments being administered to fish less than one year in sea cages and 55 treatments applied to fish having been in sea water for more than one year. In New Brunswick, the majority of smolts are transferred in spring as opposed to fall, and this was reflected in the data. Eighty-eight of the 114 treatments were applied to spring transferred fish. In our analysis, the quantity of post-treatment L. salmonis abundance in second year fish depended upon whether these fish were transferred in spring or fall. For example, in 2008, second year fish had a notable increase in modelled post-treatment abundance between spring and fall transfers which went from 1.9 to 5.3 mobile L. salmonis per fish (for the sixth week following treatment initiation in year 2008 in Passamaquoddy Bay). There may be other explanatory factors contributing to the significance of this interaction which have not been explored in this study, such as the use of emamectin benzoate in the fresh water hatchery phase prior to transfer, proximity of other farms treating for sea lice, variation in fish size, or the inability to administer treatments due to inclement weather. A linear regression model was also used by Lees et

al. (2008a) who similarly found that post-treatment L. salmonis abundance was higher in second year fish. In general, L. salmonis abundance can be higher during the second year of production at sea (Lees et al. 2008c, Saksida et al. 2007).

In the logistic model, pre-treatment L. salmonis abundance was forced into the model due to the potential of being a confounding variable. However, pre-treatment abundance was found to be non-significant, indicating that pre-treatment levels did not appear to be a determining variable for treatment outcome. If a treatment was going to be unsuccessful, it was going to happen regardless of L. salmonis abundance prior to treatment application. A similar result was found by Lees et al. (2008b) when they examined the Scottish data. Likewise, Lees et al. (2008b) found year and season to be significant variables in the logistic model with an increased risk of ineffective treatments occurring with time and the most marked increase was noted for the last year examined (2006) in the Scottish data. In New Brunswick, a notable increase in risk of failed treatments occurred in the last year of the study (2008). Furthermore, autumn treatments were at significant risk of failure compared with treatments applied during the summer months. Again, there were insufficient data available to evaluate the effects of season on treatments applied during winter or spring months.

This study found that ineffective treatments occurred in all bays except those around Grand Manan Island (ABMAs 2b and 3b). Grand Manan Island is located 32 km south of Blacks Harbour, New Brunswick. The closest mainland is the easternmost point of Maine in the United States, close to the town of Lubec, which is 15 km across the Grand Manan Channel (see Figure 2.1). Given the location, sea farms in this area would be more exposed to the open ocean than other locations closer to the New Brunswick

mainland. These fish are exposed to different environmental variables (farm density, tidal excursion, current speeds) which may have resulted in lower sea lice infection pressure than fish on farms close to the New Brunswick mainland (Chang et al. 2011). Farms in this region are physically farther away from farms closer to the mainland and perhaps the resistant sea lice had not migrated that far between farms, or resistance had not yet developed locally from repeated treatments leading to selection of emamectin resistant sea lice. There were more qualifying treatment episodes on farms located in bays closer to mainland New Brunswick (ABMA 1 + 2a = 72 treatments versus ABMA 2b + 3b = 22). Because there were fewer treatments applied around Grand Manan Island, there may have been decreased selection pressure (i.e. selection of resistant sea lice) in comparison with other areas. Increased frequency of pesticide application is one of the factors associated with parasiticide resistance development (Denholm et al. 2002).

The underlying cause of ineffective treatments cannot be determined from this analysis. In the available treatment records reviewed for this study, emamectin benzoate was found to account for > 95% of sea lice treatments applied from 2004 to 2008. Although resistance to emamectin benzoate could be the primary cause for treatment failure, other reasons for reduced treatment efficacy, such as poor feed ingestion by fish, improper application (not feeding for full seven days or missed treatment days due to inclement weather), or inappropriate concentration or distribution of the drug within the feed may all contribute to subtherapeutic dosing and potentially lead to isolated treatment failures. Treatment failures can lead to erroneous conclusions of resistance development. In an effort to substantiate suspicions of a decay in treatment effectiveness, this study employed epidemiological principles and a relatively large

sample size encompassing both time and location to investigate this issue. Evidence presented in this study shows a decline in treatment effectiveness with emamectin benzoate which is suggestive of resistance development.

Changes in susceptibility of sea lice to emamectin benzoate have been assessed by other methods, of which the most common approach is the bioassay. Bioassays have been used in New Brunswick, Canada (Westcott et al. 2008), as well as in other salmon farming regions around the world for monitoring sea lice sensitivity to therapeutic agents (Sevatdal & Horsberg 2003, Sevatdal et al. 2005, Bravo et al. 2008). Westcott et al. (2008) examined L. salmonis sensitivity to emamectin benzoate by bioassay from 2002 to early 2005 in samples collected in New Brunswick and found no changes between regions or over time by year. There were indications of decreased sensitivity of L. salmonis to emamectin benzoate during the fall and winter seasons. More recently, Igboeli et al. (2012) found increases in EC_{50} values from L. salmonis collected in 2011 compared with values obtained by Westcott et al. (2008) from 2002 to 2005 suggesting the development of emamectin benzoate resistance had occurred over time.

In conclusion, this analysis presents evidence of a reduction in emamectin benzoate treatment effectiveness over time and between geographical locations in New Brunswick, Canada, for the period of time examined (2004 to 2008). These results correspond with the clinical picture witnessed in the field of a decline in treatment effect which became a concern in 2008 (Chang et al. 2011). Further investigation is warranted to confirm the underlying cause of this decline in treatment effectiveness. In addition, more collaboration is needed between laboratory investigations of sea lice sensitivity to treatment agents and epidemiological analyses of treatment events and sea lice trends

which would allow for a more holistic understanding of the development of parasiticide resistance in the aquatic environment.

2.6 Acknowledgements

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Chapter 3 Detection of emamectin benzoate tolerance emergence in different life stages of sea lice (Lepeophtheirus salmonis) on farmed Atlantic salmon (Salmo salar L.) [2]

3.1 Abstract

Emamectin benzoate has been used to treat sea lice (Lepeophtheirus salmonis) infestations on farmed Atlantic salmon (Salmo salar). Recent evidence suggests a reduction in effectiveness in some locations. A major challenge in the detection of tolerance emergence can be the typically low proportion of resistant individuals in a population during the early phases. The objectives of this study were to develop a method for determining differences in temporal development of tolerance between sea lice life stages, and to explore how these differences might be used to improve monitoring of treatment effectiveness in a clinical setting.

This study examined two data sets based on records of sea lice abundance following emamectin benzoate treatments from the west coast of Scotland (2002 to 2006) and from New Brunswick, Canada (2004 to 2008). Life stages were categorized into two groups (adult females and the remaining mobile stages) to examine trends in mean abundance and treatment effectiveness. Differences between the two groups in emamectin benzoate effectiveness were found by year and location suggesting that an important part of monitoring drug resistance development in aquatic ecto-parasites may be the need to focus on key life stages.

[2] P.G. Jones, K.L. Hammell, G. Gettinby, C.W. Revie. Detection of emamectin benzoate tolerance emergence in different life stages of sea lice (Lepeophtheirus salmonis) on farmed Atlantic salmon (Salmo salar L.). Journal of Fish Diseases.

3.2 Introduction

The development of treatment resistance in sea lice has become problematic for salmon farming regions around the world. Increased tolerance has been noted in sea lice for dichlorvos and azamethiphos (organophosphates) in Scotland and Norway (Jones et al. 1992, Roth et al. 1996, Fallang et al. 2004). Resistance in sea lice to azamethiphos has been documented at a molecular level in adult female sea lice, Lepeophtheirus salmonis (Krøyer 1837), from Canada and Norway (Fallang et al. 2004). There have been anecdotal reports of pyrethroid treatment failures in Scotland (Sevatdal et al. 2005). Reduced sensitivity to deltamethrin through the use of bioassays has been documented in a population of sea lice in Norway with a history of treatment failures (Sevatdal & Horsberg 2003). Reductions in emamectin benzoate treatment effectiveness have been demonstrated through the use of field data collected in Scotland (Lees et al. 2008a, Lees et al. 2008b) and the Bay of Fundy region of Atlantic Canada (Jones et al. in press), but not in British Columbia, Canada (Saksida et al. 2010). A reduction in the sensitivity of sea lice to emamectin benzoate based on laboratory bioassays has been documented in Chile, although the predominant species of sea lice in that region is Caligus rogercresseyi (Boxshall & Bravo 2000) (Bravo et al. 2008).

Emamectin benzoate is an avermectin product which has been used to treat sea lice in many salmon farming regions around the world. This product is mixed with feed and administered to farmed salmon over the course of seven consecutive days. When effective, emamectin benzoate generally provides an extended period of protection against all stages of sea lice found on Atlantic salmon (Salmo salar L.) (Stone et al.

2000). Emamectin benzoate has been used in New Brunswick since 1999 and in Scotland since 2000.

Resistance development against products used in the treatment of parasites associated with terrestrial animals has been documented and can be a consequence of repeated use in populations (Wolstenholme et al. 2004). The mechanism of resistance development can occur through a number of alterations in the organism. Overall, resistance usually develops through the prevention of a drug reaching the molecular target or through inactivation of the drug or the drug's actions (Denholm et al. 2002, Wolstenholme et al. 2004). The rate at which resistance develops can depend on a multitude of factors, such as the resistance mechanism, impacts on the host from infestation, frequency of chemical/pesticide use, and biology of the parasite (Denholm et al. 2002).

Genes conferring resistance to a particular parasiticide likely already exist in the population and resistance emergence is inevitable since use of the treatment selects for these resistance factors. A major obstacle with early detection of resistance is during the preliminary stages when the genes conferring resistance occur at such low frequency in a population that detection can be challenging, if not impossible. Typically, by the time drug resistance becomes evident in a population through documentation of multiple treatment failures, the resistant alleles are already prevalent in the population.

Various methods have been used for the detection and confirmation of ecto-parasiticide resistance. Bioassays are a valuable tool for detection of reduced sensitivity in sea lice populations especially when the underlying resistance mechanism is unknown (Denholm et al. 2002), but they can be expensive to perform and usually require long

term monitoring of populations in order to detect differences. Due to the tendency for bioassays to use large numbers of viable sea lice, consume personnel time, and require experienced laboratory staff to set up and interpret the results, they have been used mostly for research and not extensively as a tool for resistance monitoring and detection (Westcott et al. 2008). Further research is being conducted to develop modified bioassays which may be more practical for field use. Reports of treatment failures have been another method of potential resistance detection, but these reports need to be interpreted with caution as there can be many reasons for treatment failures. More recently, the use of epidemiological methods using field collected data has been shown to be an effective method for monitoring of changes in treatment effectiveness (Gustafson et al. 2006, Lees et al. 2008a, Lees et al. 2008b).

Being aware of differences in resistance development patterns or trends between life stages or sexes may contribute valuable insight for early detection. In sea lice, differences in sensitivity patterns have been noted in previous studies where adult female lice were found to be less sensitive than other life stages to some bath treatment products (Roth et al. 1996, Treasurer et al. 2000). The reasons for this variability were not determined but may have to do with differences between adult female sea lice and the other mobile stages. Potentially, anatomical differences, such as a larger size or thicker cuticle, might result in adult female sea lice being more tolerant to bath treatments. In addition, there have been anecdotal reports from producers and veterinarians that adult female sea lice sometimes remained on salmon following treatment with emamectin benzoate, leading to suspicions of emerging tolerance (L. Hawkins, personal communication, 2012). Churcher & Basáñez (2009) suggested that

host selection is important when sampling. This may be relevant for many parasitic diseases where abundance and predilection of certain parasite life stages on the host may be evident. Sea lice abundance on the host fish can vary by age or size of fish, as well as time of year and sometimes site location (Saksida et al. 2007, Lees et al. 2008c).

The objectives of this study were to determine if there was evidence of a temporal pattern to treatment responses that varied across sea lice life stages, and then to explore how these differences might be used to better monitor treatment effectiveness in a clinical or field setting. A simple and robust method for monitoring of treatment effectiveness using field collected data would be beneficial as a tool to be used in conjunction with bioassays and other molecular level tests for resistance detection. In this analysis, we separated total mobile sea lice (L. salmonis) into two groups: adult females, which contained both non-gravid and gravid adult females, and other mobiles, consisting of pre-adult males and females as well as adult males. In this article, the use of the term sea lice can be taken to be synonymous with L. salmonis.

3.3 Materials and methods

In this particular study, we only examined emamectin benzoate treatment response of L. salmonis as this is the more pathogenic and more prevalent species on farmed Atlantic salmon in the North Atlantic Ocean. Two data sets were examined in this study: one from New Brunswick, Canada, encompassing the years 2004 to 2008 and the other from the west coast of Scotland covering the years 2002 to 2006, neither of which had previously been used to examine treatment effectiveness at the sea lice life stage level. The New Brunswick data was retrieved from a number of fish farming companies through their own veterinarians or private veterinarians contracted by the

company, whereas the Scottish data was retrieved from one company who owned and managed all sites.

Inclusion criteria used in this investigation were similar to methods used in Lees et al. (2008a, 2008b) in Scotland. For each data set, historical sea lice count data and treatment records were examined and treatment episodes were selected based on specific study inclusion criteria (Lees et al. 2008a, Lees et al. 2008b). In order to be included, each treatment episode had a pre-treatment count within 16 days prior to the start of treatment, and a minimum of three post-treatment sea lice evaluations during the 12 weeks following initiation of treatment. A specific sea lice count was only included when at least two cages were sampled with a minimum of five fish per cage examined. All data available on each sampling date were used in this analysis to allow for as large a sample size as possible. Any treatment that had a notation in the record regarding only partial site treatment or split site treatment was excluded from this analysis. A treatment episode was followed for 12 weeks after treatment initiation or until another treatment was applied.

A problem emerged when examining the historical data records in that sea lice count data were not uniformly collected and recorded across years in New Brunswick. For example, in the New Brunswick data, one sub-set was categorized into the following groups: chalimus stages, mobiles, and gravid females while another segment was organized into chalimus, pre-adults, adult males, non-gravid adult females, and gravid adult females. Given the different classification systems present, we chose to group the data in a way which would reduce the chance of error. Since sea lice counters likely varied in their ability to correctly classify the life stages, we felt that counters were

probably able to accurately distinguish between adult female sea lice and the rest of the mobile lice stages. Adult female sea lice are larger (approximately 10.0 mm in length) and often have egg strings attached, while the other mobile lice stages (adult male, pre-adult female, and pre-adult male) are of a similar size (2.9-5.4 mm in length) (Johnson & Albright 1991); therefore, we chose to group pre-adult males, pre-adult females, and adult males together. This group will be referred to as 'other mobiles'. The other group consisted of adult female lice, both gravid and non-gravid.

The analysis consisted of an evaluation of treatment trends which were categorized and examined by year, using two methods. First, mean abundance (mobile sea lice per fish per farm) for each treatment episode was calculated prior to treatment initiation and for each of the twelve weeks following the start of treatment for which data were available. These values were then averaged by year. Second, treatment effectiveness as a percentage of the pre-treatment abundance was evaluated. Treatment effectiveness was determined by the following equation: Treatment effectiveness = (post-treatment mean abundance / pre-treatment mean abundance) * 100%. Both the abundance and effectiveness were calculated separately for the two groups of life stages and summarized at the farm level.

In order to further explore differences in treatment effectiveness between the two groups, a theoretical model was examined based on the assumption that rates of resistance development were likely to vary by life stage. This involved tracking changes in values of treatment effectiveness within the two groups by year together with total mobile sea lice. Post-treatment weeks 2 through 9 were combined as this appeared to be

the range when treatment effect was most evident (duration of treatment effect). Treatment effectiveness was then summarized by year using the calculation listed above.

A multilevel mixed effects linear regression model was developed to formally evaluate statistical significance of treatment effect between adult female lice and the other mobile lice over time. The outcome variable for this model was percent effectiveness (percentage of pre-treatment abundance remaining in the post-treatment period). Given than this dependent variable was found to be right skewed, the data were logarithmically transformed [ln (x + 1)] resulting in a more normally distributed outcome variable. Predictor variables were screened for unconditional associations with the outcome variable and those with p-values <0.15 were kept for inclusion in the model during the model building process. Model building was carried out manually with interactions among key variables evaluated as part of the process. Residuals at both the week and treatment episode levels were checked for normality and homoscedasticity. Predictor variables evaluated during the model building process were year, life stage group (adult female or other mobile), location (New Brunswick or Scotland), pre-treatment mobile mean abundance, and pre-treatment chalimus mean abundance.

Software used to analyze the data was Microsoft Excel 2007 (Microsoft Canada Co., 1950 Meadowvale Blvd., Mississauga, Ontario, Canada) and Stata/IC 12 (StataCorp LP, 4905 Lakeway Drive, College Station, Texas, USA). Microsoft Excel 2007 was used to manage and format the data, and to create the mean abundance and efficacy graphs. Stata 12 was used to perform the trend and treatment effectiveness analysis along with the statistical models.

3.4 Results

After excluding treatment episodes based on the inclusion criteria, 114 treatment episodes remained in the New Brunswick data while 108 treatment episodes qualified in the Scottish data. In each data set, all major farming areas in each region were represented. There were 54 farms represented out of approximately 80-90 farms in New Brunswick. Data from Scotland were drawn from 66 farms owned by one company and treatment episodes meeting the inclusion criteria were associated with 47 farms. In each region, the number of active fish farming sites varied by year.

3.4.1 Trends in New Brunswick post-treatment sea lice abundance and treatment effectiveness

From 2004 to 2006 (Figure 3.1), satisfactory clearance of adult female lice was generally achieved during the post-treatment period with levels of less than 1 adult female per fish being observed beginning two weeks after the start of treatment. Week 1 was classified as days zero through six during which time treatment was still being administered. In 2007, adult female lice dropped to less than 1 adult female per fish for weeks 4 through 9 but began fluctuating around 1 louse for the remaining weeks. Generally treatment effect was starting to wane by week 9 or 10 and the data tended to be sparser in the later weeks of a treatment episode. In 2008, there was an increase in post-treatment abundance of adult female lice in all weeks compared to pre-treatment quantities. Overall abundance of other mobile lice tended to be higher compared with adult female lice (Figure 3.2). This finding was not surprising as the other mobile group consists of pre-adult male, adult male, and pre-adult female lice combined. Essentially in the early years, good clearance was achieved with abundance levels decreasing to less than 1 other mobile lice per fish at some point during the post-treatment period from

2004 to 2007. A change in this trend was noted in 2008 where the lowest level achieved was 2.4 other mobile lice per fish, although there was still a decrease from the pre-treatment abundance, in contrast to adult female lice in which no decrease was observed.

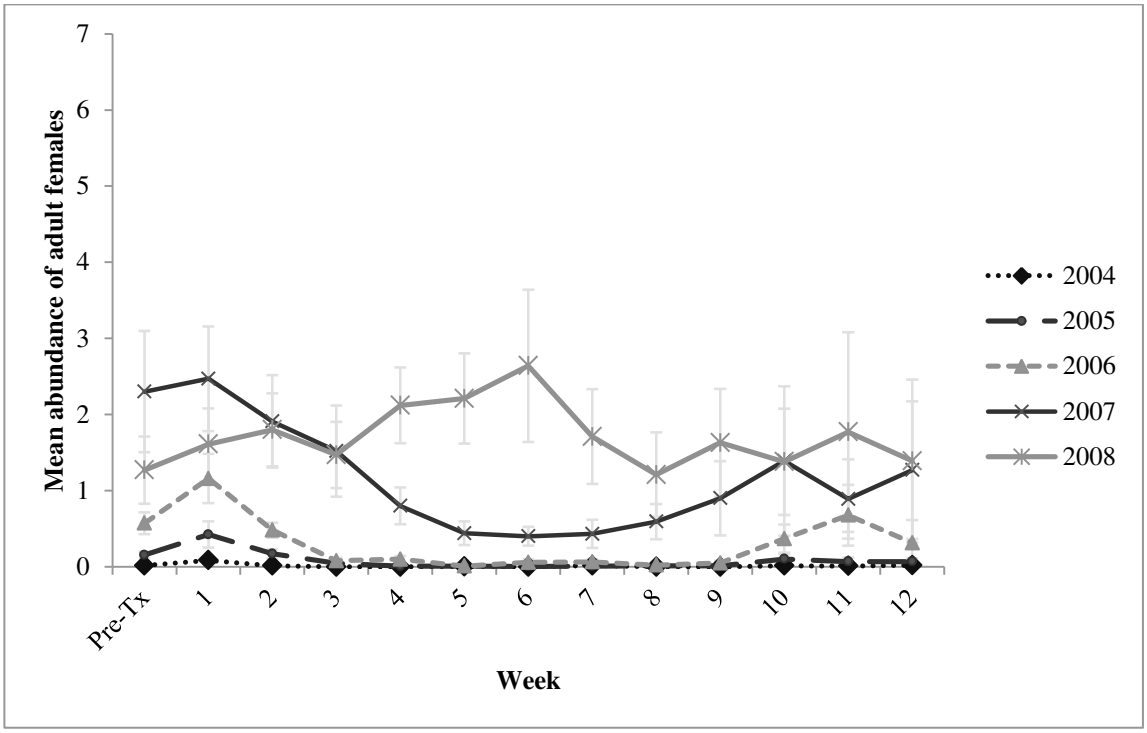

Figure 3.1 Mean abundance of adult female Lepeophtheirus salmonis pre-treatment and 1-12 weeks after commencement of treatment on Atlantic salmon farms in New Brunswick, Canada, from 2004 to 2008.

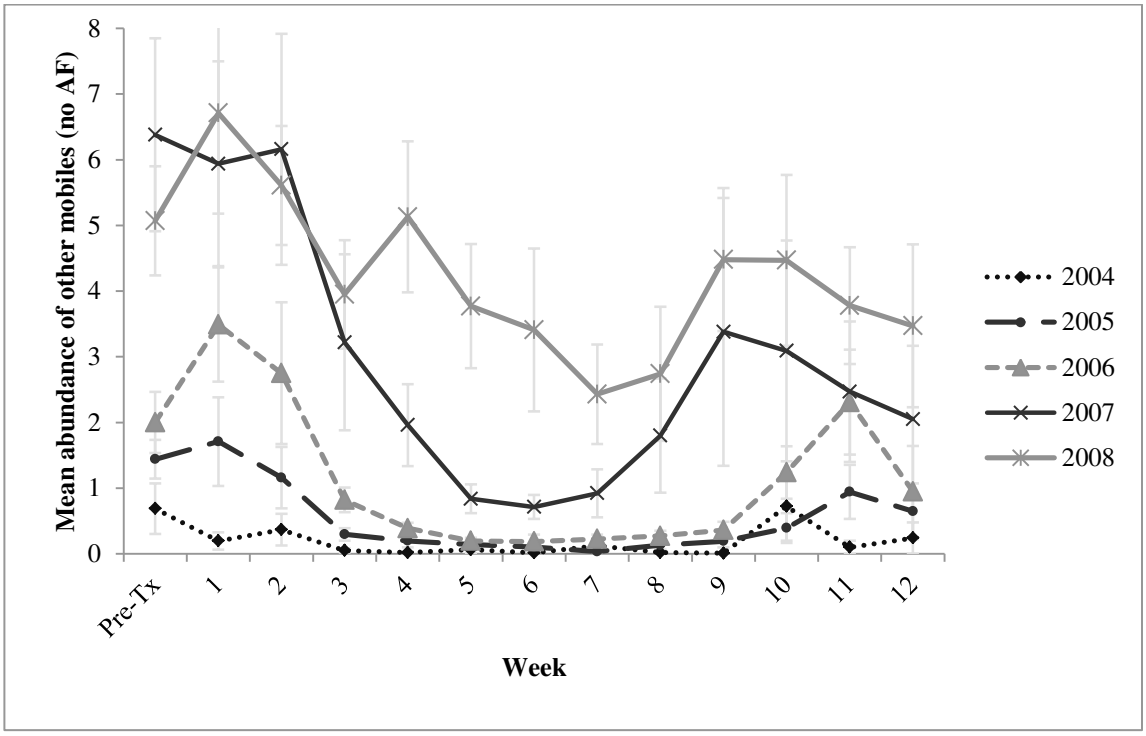

Figure 3.2 Mean abundance of mobile (except adult females) Lepeophtheirus salmonis, pre-treatment and 1-12 weeks after commencement of treatment on Atlantic salmon farms in New Brunswick, Canada, from 2004 to 2008.

3.4.2 Trends in Scotland post-treatment sea lice abundance and treatment effectiveness

Data from Scotland (Figures 3.3 & 3.4) were summarized in the same format as for New Brunswick data. In general, pre-treatment abundance levels were higher on Scottish salmon farms. Adequate removal of adult female lice (i.e. less than 2 adult females per fish) was found to occur in all years except 2006. In 2006, adult females decreased to less than 1 adult female per fish, but this finding did not happen until nine weeks after treatment initiation. In earlier years (2002 to 2004), maximum treatment effect was found by week 4 or 5. Other mobile lice were found to decrease to less than 1 other mobile per fish in all years assessed. Again, time to maximum effectiveness was delayed in the last year to approximately week 9.

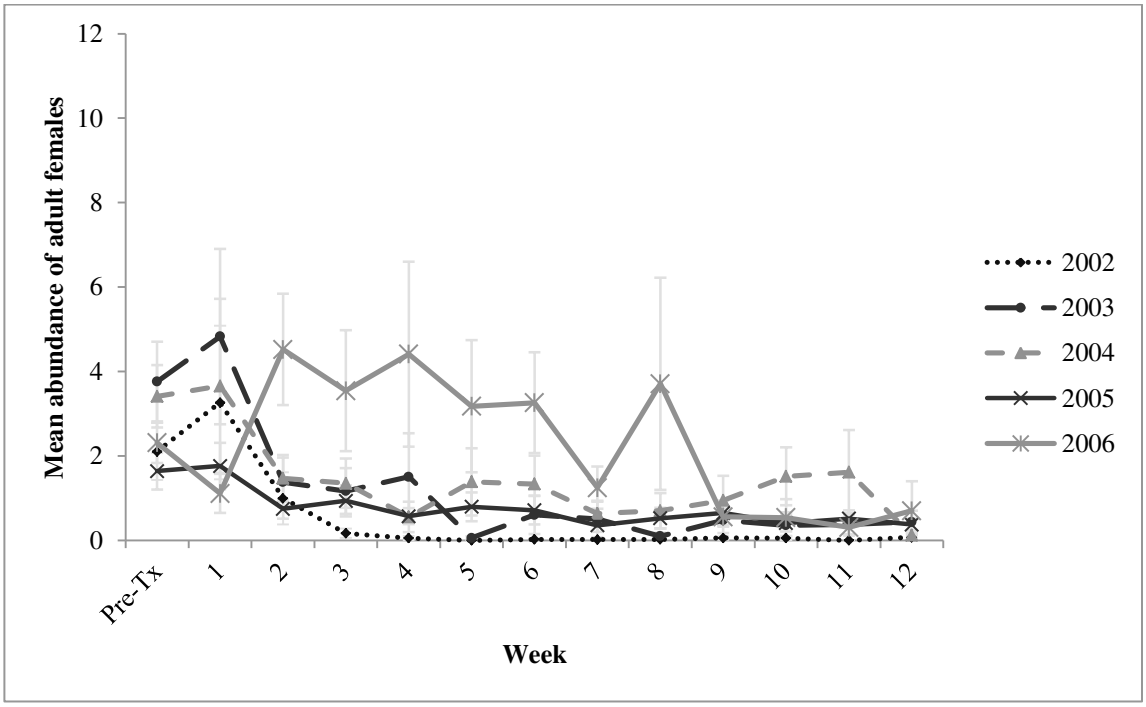

Figure 3.3 Mean abundance of adult female Lepeophtheirus salmonis, pre-treatment and 1-12 weeks after commencement of treatment on Atlantic salmon farms in Scotland from 2002 to 2006.

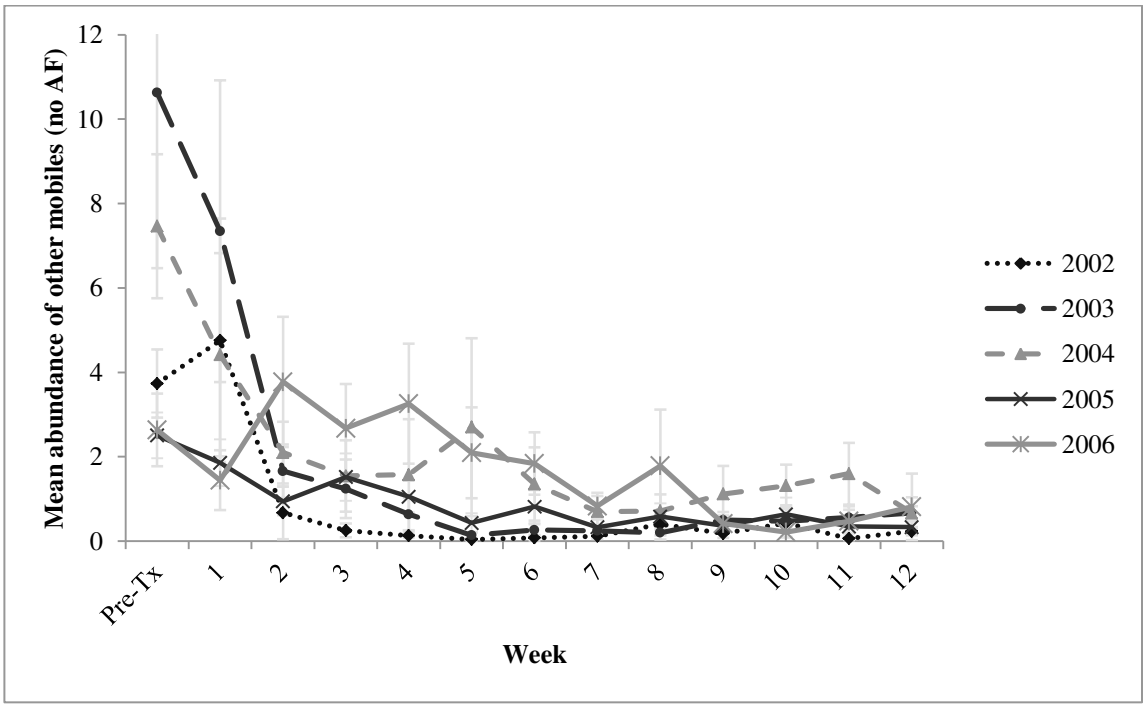

Figure 3.4 Mean abundance of mobile (except adult female) Lepeophtheirus salmonis, pre-treatment and 1-12 weeks after commencement of treatment on Atlantic salmon farms in Scotland from 2002 to 2006.

3.4.3 Theoretical model for evaluation of differences in tolerance emergence

A theoretic graph (Figure 3.5) was developed based on the premise that the pattern of resistance development would follow an exponential growth curve. We also speculated that tolerance development to emamectin benzoate in adult female lice would advance at a more rapid rate compared to other mobile stages. In New Brunswick, by 2008, there was a dramatic difference between the degree of treatment effect in adult female lice compared to other mobiles (Figure 3.6). Tolerance was sufficiently advanced that, compared to pre-treatment levels, there was an increase in the number of adult female lice present in the post-treatment period. Since effectiveness calculations were based on percent of pre-treatment abundance, anything over 100% constituted an increase in abundance from pre-treatment values.

An interesting finding from Figure 3.6 was that the traditional method of monitoring effectiveness, using total mobiles, basically tracked the effectiveness of the other mobile lice group. This was likely due to the larger number of other mobiles compared to adult females as a group, in other words, the majority of total mobiles consisted of other mobiles. In 2008, a marked difference arose between adult female and other mobile lice groups. By not tracking the effectiveness of emamectin benzoate on adult females, emergence of resistance in this stage may have been overlooked.

Summarized Scottish data is presented in Figure 3.7. Although differences between stages were not as dramatic in the Scottish data, an increase in values for adult female lice (i.e. decreased treatment effect) compared to the other stages was apparent. Figures 3.6 and 3.7 display effectiveness values against year (high values = poor treatment response). These curves appear to be more exponential in shape rather than

linear. Although they are not true rate of resistance development curves, they do indicate a decreased treatment response over time.

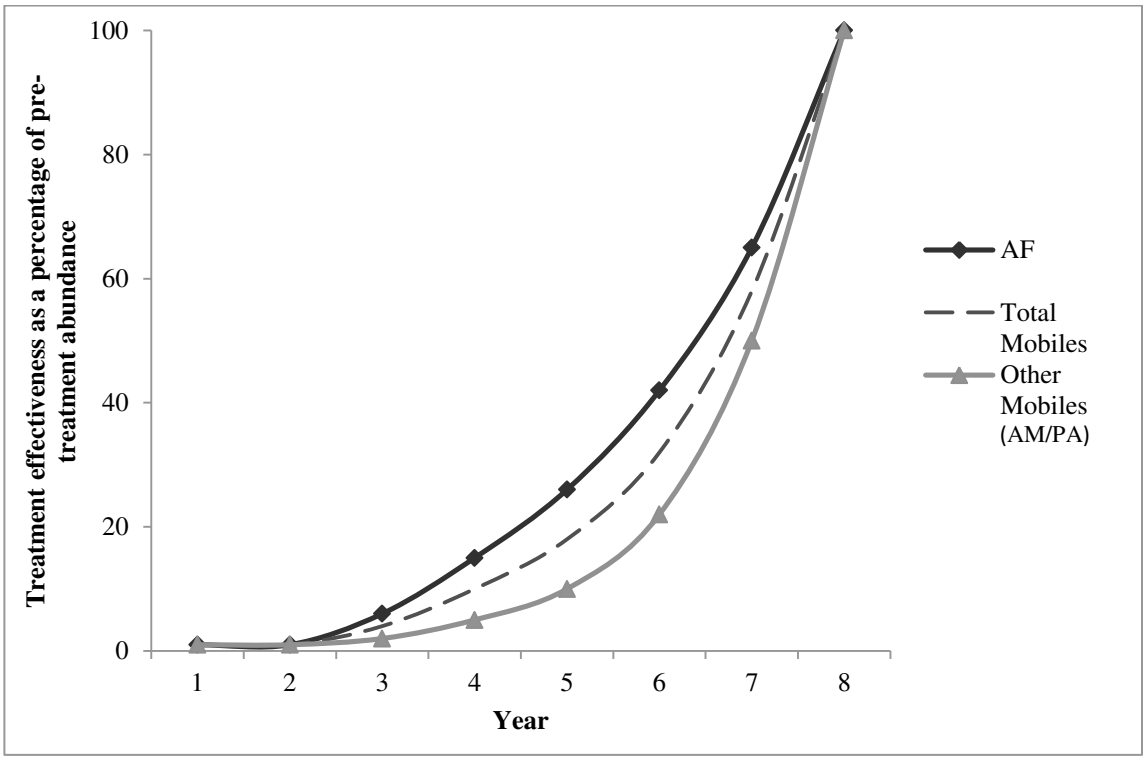

Figure 3.5 Theoretic graph comparing the values for emamectin benzoate treatment effectiveness (as a percentage of pre-treatment abundance) in groups of Lepeophtheirus salmonis life stages.

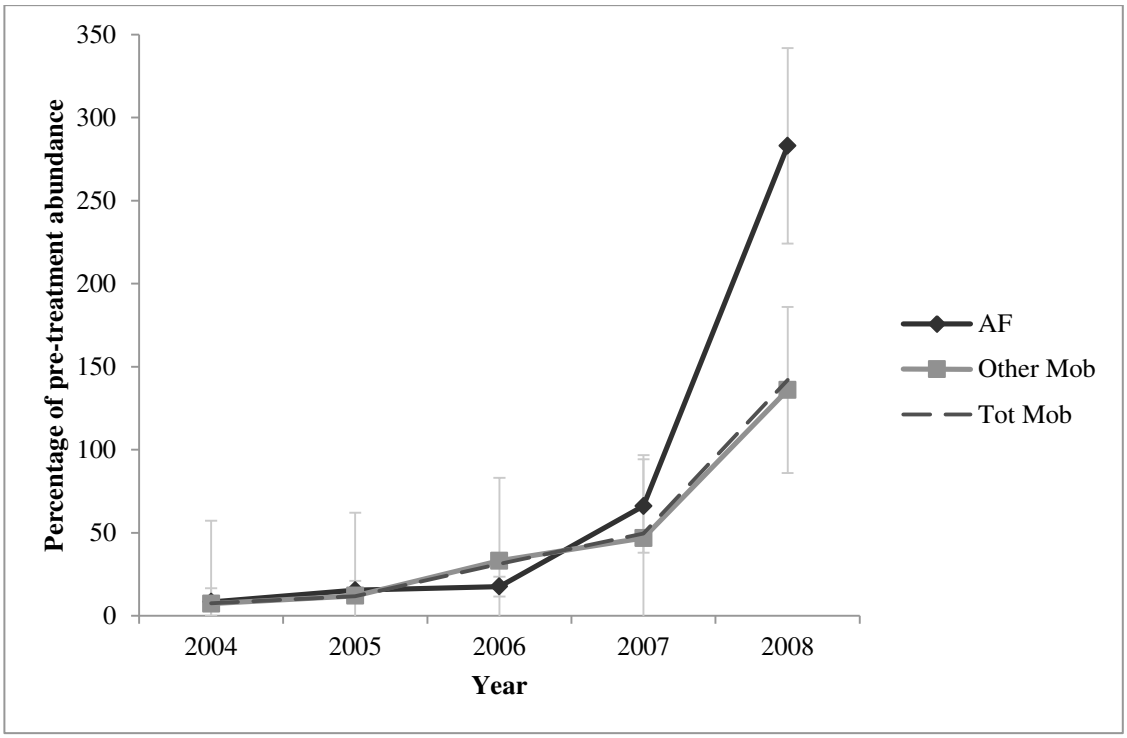

Figure 3.6 Effectiveness of emamectin benzoate (% of pre-treatment abundance) by year against Lepeophtheirus salmonis (total mobiles, adult females, and other mobiles except adult females) with post-treatment weeks 2-9 combined on farmed Atlantic salmon in the Bay of Fundy, New Brunswick, Canada.

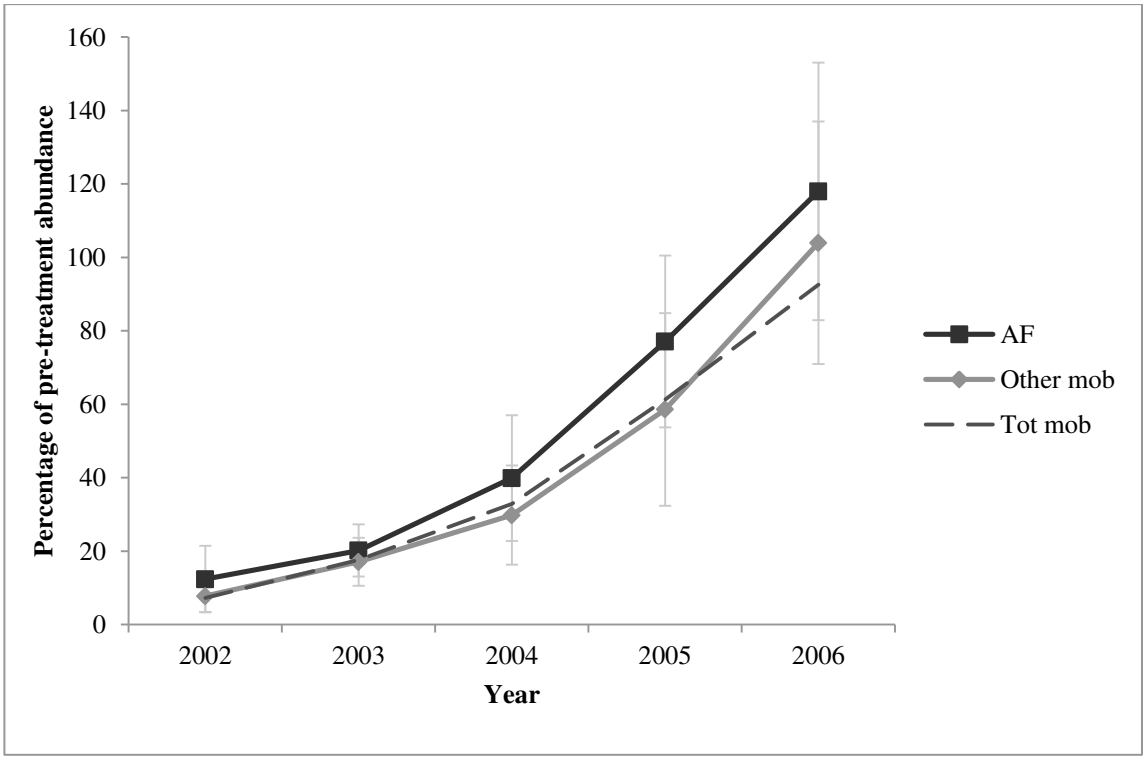

Figure 3.7 Effectiveness of emamectin benzoate (% of pre-treatment abundance) by year against Lepeophtheirus salmonis (total mobiles, adult females, and other mobiles except adult females) with post-treatment weeks 2-9 combined on farmed Atlantic salmon in Scotland.

3.4.4 Statistical model for evaluation of differences in treatment effect

For this model, treatment effectiveness values from both countries were combined into one data set to facilitate comparison of effectiveness values between locations as well as amongst years, and additionally, to improve statistical power of the analysis. The Scottish data had two treatment episodes removed as they were found to be outliers. The Scottish data set covered the years 2002 to 2006 while the data from New Brunswick included 2004 to 2008; previous analysis had shown the changes in treatment effectiveness were most evident in the final year of each set of data (Lees et al. 2008a, Lees et al. 2008b, Jones et al. in press). Therefore, for the purpose of coupled analysis, data sets were assigned a "year" value of one through five, representing a two year offset between locations in terms of actual calendar years.

The multilevel mixed effects linear regression was used for the examination of statistical differences between values of treatment effectiveness in the two groups (adult female and other mobile lice) (Table 3.1). A two level model was constructed with treatment episode as a random effect (i.e. effectiveness measurements within treatment episode). Significant variables remaining in the final model were location (Scotland or New Brunswick, Canada), year, and life stage group. Pre-treatment mean abundance of mobile lice was included as a variable (forced covariate) because of the strong association with post-treatment lice counts and the potential confounding effect due to associations with other variables in the model. There were significant interactions between year and life stage group as well as year and location.

A significant interaction between year and life stage group indicated that the value of treatment effectiveness in the two groups depended upon the year (Figure 3.8).

Higher values, indicating a reduced treatment effect, were found for adult females compared to other mobiles. Differences in effectiveness between adult female and other mobile lice appeared to be less dramatic in the Scottish data although the difference occurred more consistently over several years. In addition, treatment effect varied by location and year with values in Scotland being lower than those in New Brunswick (Figure 3.9). The trends in decreasing emamectin benzoate effectiveness (increase in values) appeared to follow a similar trajectory over the five years studied, though the speed of decline in effectiveness seemed to be more rapid in New Brunswick.

Model diagnostics included calculating residuals at both life stage group and treatment levels. Normal probability plots found residuals to be fairly normally distributed at both the individual group level as well as at the treatment level. Plots to evaluate homoscedasticity (residuals against predicted values) found no evidence of extreme differences in levels of variation.

Table 3.1 Results of the linear mixed model - evaluation of emamectin benzoate treatment effectiveness for the treatment of Lepeophtheirus salmonis on farmed Atlantic salmon in New Brunswick, Canada (2004-2008) and Scotland (2002-2006). (Baseline values are adult females in year 5 in New Brunswick, Canada, and pre-treatment level (ln (x+0.1) where the lowest x value was 0))

Variable	Category	Coefficient (log transformed)	SE	p-value	95% Confidence Interval	
					Lower	Upper
Stage	Other mobiles	-0.499	0.162	0.002	-0.817	-0.181
Year	1	-2.812	0.678	0.000	-4.140	-1.483
	2	-2.345	0.421	0.000	-3.170	-1.521
	3	-1.419	0.405	0.000	-2.212	-0.626
	4	-1.115	0.368	0.002	-1.837	-0.394
Stage * Year	Other mob*yr1	0.372	0.322	0.248	-0.259	1.002
	Other mob*yr2	0.496	0.231	0.032	0.043	0.949
	Other mob*yr3	0.544	0.224	0.015	0.106	0.983
	Other mob*yr4	0.366	0.214	0.086	-0.052	0.785
Location	Scotland	-0.749	0.427	0.080	-1.587	0.089
Location*Year	Scotland*yr1	0.407	0.855	0.634	-1.269	2.082
	Scotland*yr2	0.271	0.621	0.662	-0.945	1.488
	Scotland*yr3	-0.180	0.595	0.762	-1.347	0.986
	Scotland*yr4	0.192	0.557	0.730	-0.900	1.284
Pre-treatment abundance (log transformed)		0.008	0.079	0.923	-0.148	0.163
Constant		4.865	0.286	0.000	4.304	5.426
Random Effects Parameters		**Estimate**	**SE**		**95% Conf Interval**	
Treatment: Identity	Variance (constant)	1.574	0.183		1.253	1.977
	Variance (residual)	0.510	0.053		0.415	0.626
LR test vs. linear regression: chibar2(01) = 152.95 Prob >= chibar2 = 0.0000						

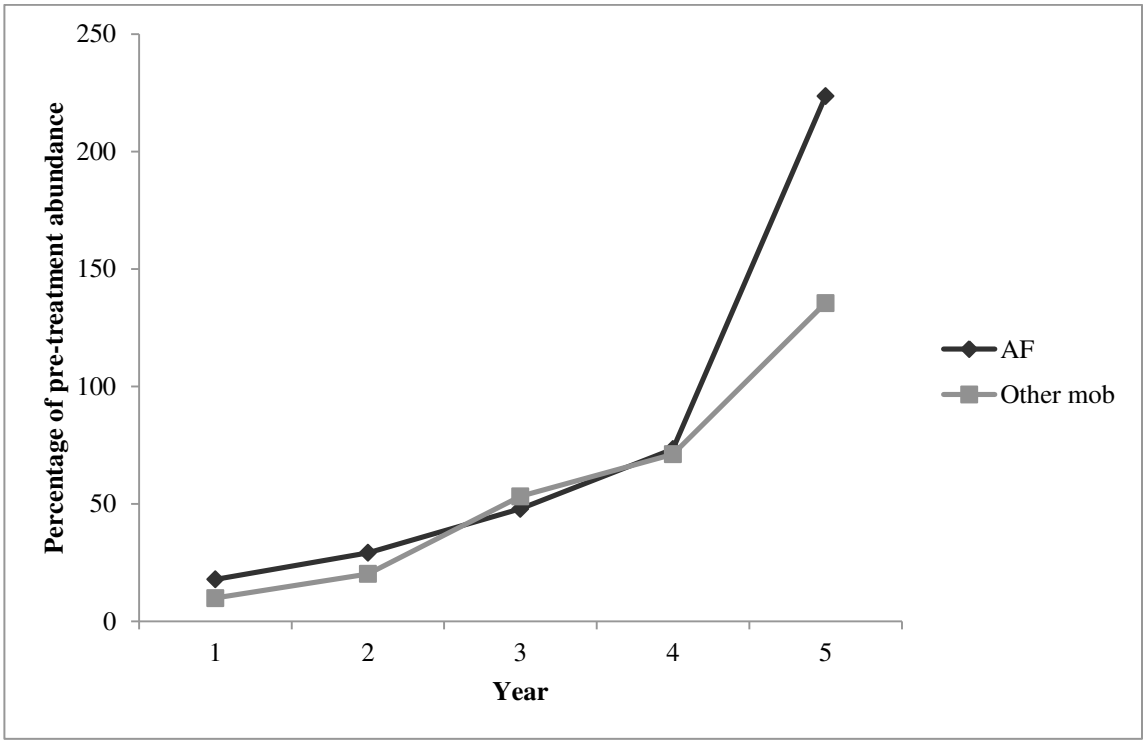

Figure 3.8 Interaction plot of life stage by year. Graph shows effectiveness (as a percentage of pre-treatment abundance) of emamectin benzoate in adult female and 'other mobile' Lepeophtheirus salmonis on farmed Atlantic salmon by year category for combined data from Scotland and New Brunswick, Canada.

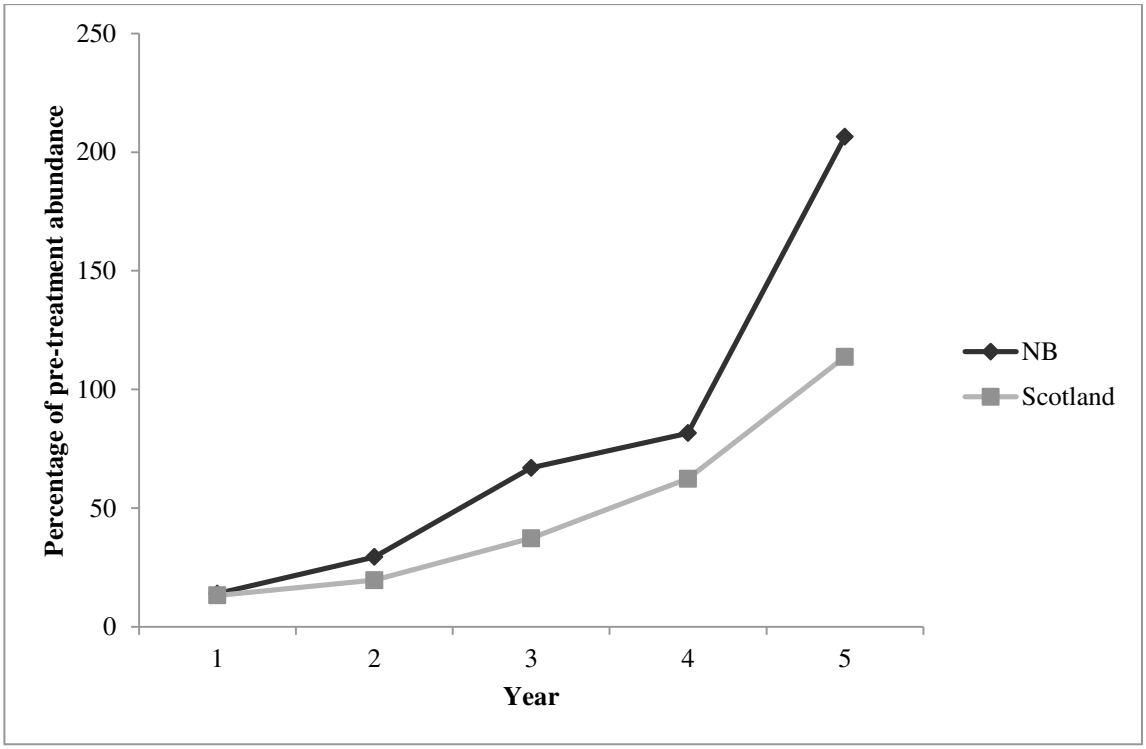

Figure 3.9 Interaction plot of location by year. Effectiveness (as a percentage of pre-treatment abundance) of emamectin benzoate in mobile Lepeophtheirus salmonis on farmed Atlantic salmon in New Brunswick, Canada, and Scotland by year category.

3.5 Discussion

Results of this analysis indicated a reduction in treatment effectiveness over time; however it does not confirm the presence of emamectin benzoate resistant sea lice populations. Confirmation of resistance development is normally through the identification of biochemical or biophysical properties known to convey an advantage to parasites which can be transferred genetically to offspring. Recent research has shown that increased expression of P-glycoprotein mRNA was found in field collected sea lice from 2011 compared to 2002 (Igboeli et al. 2012). The same study concluded that emamectin benzoate may be a substrate for P-glycoprotein and upregulation of this transporter protein may be linked with tolerance development. While there can be other contributing factors for isolated reductions in treatment effectiveness or treatment failures, we can speculate that at least some of the reductions in treatment effect over time were due to emamectin benzoate resistance development in populations of sea lice.

As mentioned previously, a major road block in the early detection of resistance development is the low frequency at which resistance initially starts in a population. Hastings (2001) discussed resistance development occurring in a logarithmic growth pattern. This type of pattern occurs in such a way that once resistance frequency is sufficiently high enough to detect, resistance is already rapidly spreading through the population. We expanded on this idea by postulating that resistance development might occur at different rates or to variable extents between sea lice life stages. If these life stages are monitored appropriately, then resistance development, or at least strong indications of emergence, could potentially be detected at an earlier stage. The current

study found that resistance appeared to be more advanced in adult female sea lice compared to a group containing the rest of the mobile stages.

Overall treatment effectiveness for each location was examined by plotting effectiveness values against time in years for each location. Scottish data exhibited lower, but steadier, increases over time while New Brunswick data displayed a more rapid increase. Although slope differences cannot be explained by a descriptive analysis such as this, we speculate that different use of sea lice treatment products may have contributed to differences in tolerance development. Review of available treatment records for New Brunswick showed that emamectin benzoate accounted for greater than 95% of all treatments during the study period. In Scotland, during the time period investigated, approximately half of the available treatment episodes reviewed indicated the use of emamectin benzoate (Lees et al. 2008b). We surmise that the use of several products in Scotland may have slowed the rate of parasiticide resistance development. If true, this supports the recommendation of alternating between two unrelated products in an effort to help delay resistance development (Denholm et al. 2002).

While our results showed that there was a more advanced decrease in treatment response of adult females (i.e. increase in effectiveness values) in 2008 in the New Brunswick data, while this was somewhat less evident in 2006 in the Scottish data. This study design could not confirm why there was such a fluctuation in effectiveness values. These data from Scotland were a mix of three different regions and differences may have occurred due to regional (within country) disparities in resistance development. Previous studies examining the same data set (Lees et al. 2008a, Lees et al. 2008b) found that emamectin benzoate treatment effectiveness on total mobiles varied by

region. It is possible that regions where treatment effect was still adequate could be obscuring data from areas where resistance was developing.

Tracking of individual life stages (rather than groups) may provide more detailed information since some of the individual stage information is obscured when the life stages are grouped. However, effective use of such monitoring methods requires that sea lice life stages are correctly categorized and recorded during sea lice counts. The granularity of New Brunswick data did not allow precise evaluation but the Scottish data contained classification of individual stages. One weakness of this study is the potential for inaccurate classification of sea lice life stages in these historical data. Companies collected and recorded data for their own in-house use hence there was no need to ensure that their data collection methods were consistent with other companies. Some variation in the accuracy of sea lice counts between companies was expected, but it was believed that any impact would have been minimized by the large sample sizes of both data sets. Many individuals performed regular sea lice monitoring and, while staff had received training, the experience level of the counter could vary. In the future, more attention to stage classification during collection of field data and routine sea lice counts would be needed for the individual stage method to be used.

This study did not consider the type of resistance mechanisms that occur in sea lice to emamectin benzoate. The genetic processes associated with resistance development may influence the rate at which resistance genes accumulate within a population (Jackson & Coop 2000). If a parasiticide can affect multiple parasite stages, then the parasite could potentially develop drug resistance in several different ways

should the resistance gene that protects one life stage from the actions of the drug fail to protect another (Churcher & Basáñez 2009).

Jackson & Coop (2000) have conveyed that there is a lack of simple robust techniques for field detection of emerging drug resistance. This study examined field collected sea lice treatment data for differences in treatment effectiveness between groups of life stages. In the past, development of resistance in different sexes or life stages could only be detected through the use of bioassays. A benefit to this type of trend analysis is that these types of data are already collected for monitoring of treatment effectiveness, therefore would not result in extra cost to the company or work for farm staff. However, practical use of these methods does not eliminate the need for other forms of treatment response monitoring, in particular the use of laboratory studies, such as bioassays.

When emamectin benzoate worked effectively in New Brunswick, Canada, bioassay results showed that pre-adult female lice were more sensitive to the drug than pre-adult male lice (Westcott et al. 2008). This seems contradictory to the findings in this study but there are several points to consider: the bioassay study examined sea lice from the Bay of Fundy from 2002 to 2005 and the bioassays only examined pre-adult male and pre-adult female lice. In a previous study on the effectiveness of emamectin benzoate in the Bay of Fundy from 2004 to 2008, results showed that treatments were effective for 2004 and 2005, which overlapped with the bioassay study (Jones et al. in press). This current study looks at life stages grouped by adult females and the remaining mobile stages (pre-adult males and females and adult males). There may be a change occurring in female sea lice during the moult from a pre-adult to an adult

resulting in differences in sensitivity patterns. Sex and life stage differences have been found in intestinal parasites of terrestrial animals (Le Jambre et al. 1995, Le Jambre et al. 2000). Sevatdal et al. (2005) reported that adult female lice were less sensitive to a pyrethroid product compared to pre-adult and adult male lice through the use of a bioassay. The same study also suggested that results of bioassays using pre-adult stages cannot necessarily be extrapolated to outcomes expected under field conditions where the removal of adult female lice is essential.

There can be several reasons for differences in results between bioassays and field collected data analysis. First, bioassays are performed in a laboratory where sea lice are immersed in a sea water bath containing a diluted pesticide. Sea lice absorb the pesticide through the cuticle and perhaps some of the product is ingested orally. In contrast, sea lice on farmed salmon are exposed to the product through ingestion of mucus and possibly skin of fish which have consumed the pesticide in medicated feed. Second, bioassays require separation of individual life stages and the stages of choice are evaluated, whereas the current study categorized life stages into two groups. If there were differences in the sensitivity of adult male lice compared to adult female lice, they may not have been detected since effects isolated to adult males may have been diminished by the presence of pre-adult stage lice. Third, sample location and time are both important factors to consider with the interpretation of either test. Resistance emergence appears to be a dynamic process and distinct changes can occur from year to year; therefore, comparing field results to bioassay results from different years may lead to contradictory findings. Treatment effectiveness can vary by location (Lees et al.

2008a, Lees et al. 2008b, Jones et al. in press) and, as a result, outcomes of both methods could vary depending on location.

In this study, the emergence of emamectin benzoate resistance development in sea lice appeared to be more advanced in adult female lice than in the other mobile lice combined. Taking this into consideration, perhaps a different approach should be taken when planning bioassays for monitoring drug sensitivity. In the past, pre-adults have been used exclusively by some laboratories for performing sea lice bioassays (Sevatdal & Horsberg 2003, Sevatdal et al. 2005). However, there should be some consideration given to targeting of life stages when selecting samples for bioassays or other laboratory tests for resistance (Westcott et al. 2008). Variability in both bioassays and treatment response analysis reflects the fact that resistance emergence is a dynamic process and prediction of changes in sea lice sensitivity is challenging. No single method is likely to suffice for monitoring sea lice sensitivity. Combining evidence from multiple sources for industry and policy decision makers will optimize treatment effectiveness over the longest term.

In conclusion, this analysis found differences in emamectin benzoate effectiveness between the two groups of life stages by year and location. Changes in sea lice sensitivity to emamectin benzoate were not necessarily synchronized in all life stages. A difference in the rates of resistance development between locations was noted, with resistance developing more rapidly in New Brunswick compared to Scotland. These results suggest that an important part of monitoring for drug resistance in aquatic ecto-parasites includes a focus on key life stages.

3.6 Acknowledgements

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Chapter 4 General Discussion

4.1 Main findings of thesis

The objective of Chapter 1 was to examine relevant literature and to determine the most appropriate epidemiological methods for evaluating the field use of emamectin benzoate in the treatment of sea lice (Lepeophtheirus salmonis). A broad range of scientific literature on the epidemiology and management of sea lice along with resistance development to parasiticides was reviewed. While resistance development to emamectin benzoate in sea lice is not novel, there have been few published reports and, in regard to salmon farming in Atlantic Canada, there is little documented evidence of treatment resistance; indeed the entire topic of sea lice epidemiology on the east coast of Canada over the past decade has been largely absent from the scientific literature. This is in contrast to the situation on the west coast of Canada or that seen for a number of European countries such as Ireland, Scotland, and Norway.

Chapter 2 examined and described the effectiveness of emamectin benzoate in the treatment of L. salmonis on farmed Atlantic salmon (Salmo salar) in New Brunswick. Trends in treatment effectiveness, as well as post-treatment sea lice abundance levels were examined. Factors associated with post-treatment sea lice abundance and treatment outcome (success or failure) were evaluated using appropriate statistical models. Temporal and spatial developments of tolerance to emamectin benzoate in populations of sea lice in the Bay of Fundy were found. This was in agreement with the clinical perspective in the field at the time when there were anecdotal reports of treatment failures. Since that time, emamectin benzoate has been found to be ineffective in many field situations and other treatment agents are now commonly used.

Chapter 3 further expanded the evaluation of changes in treatment effectiveness by examining two data sets for rates of change in separate sea lice life stages. One data set was from New Brunswick, Canada, while the other was from the west coast of Scotland. Due to differences in data collection methods, sea lice had to be grouped into two broad categories: adult female L. salmonis (gravid and non-gravid) and all the other mobile stages of L. salmonis. The preferred method would have been to examine each of the life stages separately but one of the limitations in using field data is that classifications may not be achievable after the event. It is difficult to identify some individual life stages in the field, particularly the differentiation between pre-adult female, adult male, and pre-adult male stages. In the end, differences in tolerance development between the two groups were found. This difference was more evident in New Brunswick than in Scotland. Resistance was found to have developed at different rates between the two data sets, with the New Brunswick data showing a faster rate of resistance development compared to Scotland. This was an interesting finding and, while the cause of this difference was not confirmed, the use of emamectin benzoate differed in these regions. In Scotland, a variety of treatment products were in use in addition to emamectin benzoate. In New Brunswick, emamectin benzoate was used in over 95% of the treatments applied during the study period. Denholm et al. (2002) listed the use of a single product as a key risk factor for the development of resistance.

This project used historical field-collected salmon production and health management data. There are benefits and drawbacks in the use of these types of historical data. Several concerns regarding historical data were mentioned in Chapter 2 and this topic was also discussed in Lees et al. (2008b). These concerns include issues

such as differences in experience level and training of sea lice counters, and the exclusion of treatment episodes not meeting certain inclusion criteria. Overall, many of these concerns were overcome or minimized by the use of a large data set. The data set analyzed for Chapter 2 was similar in size with Lees et al. (2008a, 2008b) and was substantially larger than those of Gustafson et al. (2006) and Saksida et al. (2010). Also with this project, there were concerns around the classification of sea lice life stages. This is important when examining individual life stages or groups of life stages for changes in treatment effectiveness. An ideal scenario would be to reliably monitor pre-adult male, pre-adult female, adult male, and adult female lice as individual stages.

We used epidemiological methods for monitoring changes in treatment effectiveness around the use of emamectin benzoate. This approach had been initially used in the evaluation of treatment effectiveness and then expanded to monitor for changes in treatment effect both spatially and temporally. This type of approach allows for the examination of large amounts of historical data.

Production data is often incomplete or unbalanced and these factors require consideration during the model selection process. For these reasons, hierarchical linear regression models were used in Chapters 2 and 3. A unique feature of the study in Chapter 2 was the use of a multilevel mixed model for the evaluation of the effects of multiple variables on post-treatment sea lice abundance. There has only been a series of two papers (Lees et al. 2008a; Lees et al. 2008b) which made use of statistical modelling to examine factors associated with treatment effectiveness. Chapter 2 expanded on previous work by using a multilevel model which accounted for the hierarchical structure of the data and the time ordering of the follow-up measurements. This type of

analysis allowed for the follow-up observations to be grouped into individual treatment episodes. This approach accounted for the fact that post-treatment observations within a treatment episode are not independent. A correlation structure (structure of the covariance matrix) was used to account for variability in the sea lice counts between weeks (repeated measures) within each treatment episode. A correlation structure is an extension of the linear mixed model. By use of the Toeplitz correlation structure, it was shown that subsequent counts were correlated within each episode but only up to a certain point (6 weeks apart) after which the correlation decreased significantly. This type of structure is not commonly found in published epidemiological literature and particularly not in aquatic epidemiology. Revie et al. (2002) examined the correlation between counts and found that the strongest correlations occurred at three weeks and six weeks apart. The counts three weeks apart were negatively correlated which would correspond to a treatment response of a reduction in sea lice abundance. The counts six weeks apart were positively correlated which could be explained by an increase in sea lice abundance following treatment. The majority of treatments included in that study were bath treatments which have an immediate and short-acting impact on sea lice populations.

4.2 Alternative approaches to analysis

Developing methods for the evaluation of treatment effectiveness without the use of a control group can be challenging. Some criticisms could be made over the use of pre-treatment sea lice levels as the baseline upon which to gauge the degree of change in sea lice abundance following treatment. The issue relates to the amount of variability which occurs with pre-treatment sea lice abundance. Problems were encountered with

treatment episodes which had very low pre-treatment abundance and thus presented difficulties in determining treatment effect. We used a window of 16 days to determine pre-treatment levels. Thus, if a count was done at 14 days, the sea lice levels could be significantly different by the time the treatment started. One alternative might be to calculate the mean of all the counts within the 16 day window which might give a more accurate assessment of sea lice abundance. The current study based effectiveness as a percentage of the pre-treatment abundance. A treatment was considered effective if it achieved a reduction to less than 40% of the pre-treatment lice level. An alternative method for determining treatment effectiveness might be to base treatment success on lice being reduced below a predetermined value (e.g. significantly less than a "trigger level", if such a regulation is in place for a particular region). Another alternative might be the development of a scoring system which would determine treatment effectiveness using a number of factors rather than a single calculation. An example might be to use the method employed in Chapter 2 along with an additional component of a score for the minimum level of abundance achieved (i.e. above or below a predetermined threshold).

In this study, treatment effectiveness was evaluated at the site level, meaning that all cages were averaged together to determine a site level mean for pre-treatment abundance as well as in each of the post-treatment weeks. Cage level analysis was not chosen since the same cages were often not evaluated at each follow-up point (i.e. would not be able to consistently match pre- and post-treatment counts for each cage). It would be interesting to carry out the same evaluation at cage level and determine if the results are comparable to the site level evaluations. Jiminez et al. (2012) evaluated the effectiveness of bath treatments in Norway at both a cage and site level. That study

calculated treatment effectiveness with the use of point estimates and confidence intervals at both the cage and site level. They found the quasi-Poisson method with a 90% confidence interval to be the most robust measurement of bath treatment effectiveness. Evaluation at the site level can result in overdispersion (i.e. variance being greater than the mean) given that lice abundance can vary between cages. The use of cage level treatment effect monitoring can minimize overdispersion. The proportion of acceptable treatments was larger at the site level compared to cage level when evaluated at the same confidence level (Jiminez et al. 2012). Jiminzez et al. demonstrated that there is benefit to the examination of site and cage level treatment effectiveness. They also showed that the use of confidence intervals rather than a point estimate may give more information to those responsible for the evaluation of treatment response. Chapter 2 examined emamectin benzoate effectiveness at a site level. Further investigation into cage level effectiveness would be a reasonable next step and could provide some interesting insights regarding heterogeneity of effectiveness across a site.

Chapter 3 evaluated two groups of sea lice life stages for changes in treatment effectiveness over time and geographical location. An alternative method to the one presented in Chapter 3 was also explored and this is presented in Appendix A. In general, the abundance of 'other mobile' lice tends to be higher in comparison to adult female lice making it difficult to evaluate for changes in absolute abundance between the two groups. In an effort to determine if resistance developed in adult female lice prior to other stages or if there were differences in the abundance of adult female lice over time, the proportion of the total mobile lice population which were adult females was calculated. The theory behind this calculation was that if resistance truly developed

in one stage before another then there should be an increase in the presence of that particular life stage as resistance emerged over time. We observed that there appeared to be an increase in the proportion of adult female lice over time which corresponded to decreases in effectiveness of emamectin benzoate. This finding may be relevant to future monitoring of resistance emergence in populations of sea lice and warrants further exploration. Further examination of the two data sets used in Chapter 3 by this method may provide further insight into early indicators for resistance emergence.

4.3 Future directions

A general epidemiological analysis of sea lice trends in the Bay of Fundy region would be an important next step. There has been limited formal epidemiological analysis of sea lice trends. While some factors can be extrapolated from other locations, there are likely local variables which can cause variations in sea lice abundance. Knowing the risk factors for increased sea lice abundance or any disease process in the aquatic environment is important when planning a preventative medicine program. In the Bay of Fundy, we found variations in treatment effectiveness by location. We have reason to believe that there are natural differences in sea lice trends based on pre-treatment levels and studies from other salmon farming regions. Examination of these trends through the use of historical sea lice count data would allow for tracking of these changes. This may be important in the future planning of salmon farm locations in that region. If some locations were found to be at high risk for severe sea lice infestations, relocation of those sites may be warranted. New Brunswick uses Aquaculture Bay Management Areas (ABMAs) for the grouping of farming sites. Currently, each ABMA practices single year class stocking, mandatory fallow periods (i.e. four months per site,

two months per ABMA), and a three year rotation system (Chang et al. 2011). Some ABMAs have a higher density of farms than others. This variable would be interesting to evaluate as off-farm infection pressure would likely vary depending on overall farm density within an ABMA. Findings from such a study may aid in the restructuring and relocation of farms to better control sea lice populations.

There needs to be more collaboration between laboratory studies and the analysis of field data to aid in the early detection of resistance emergence. Unfortunately, there is no single screening test which will reliably allow for the detection of resistance emergence in the early stages. Currently, the best plan for monitoring of resistance would involve a collaborative effort between the evaluation of field efficacy and laboratory monitoring of sea lice sensitivity. While monitoring through the use of field data should include as many treatment episodes as possible, laboratory bioassays might benefit from targeted sampling since these tests are costly and require considerable time and effort. Perhaps suspect treatment failures should be followed up with a review of field data, treatment information and environmental conditions and then these sites could be targeted for future monitoring through bioassays. In British Columbia, researchers have begun to monitor treatment effectiveness in the field in conjunction with instituting the examination of sea lice sensitivity to emamectin benzoate through the use of laboratory bioassays (Saksida et al. 2012).

There has been little epidemiologic research in the area of sea lice on farmed Atlantic salmon in the Bay of Fundy. The research presented in this thesis adds insights around the treatment of sea lice and the development of resistance. With further research (i.e. epidemiologic analysis of sea lice population trends), knowledge can be expanded

to provide a more solid foundation for decision making in regards to salmon farming and disease management in this area. With respect to treatment monitoring, this study demonstrated the importance of monitoring individual life stages together with total mobiles. Through a careful examination of sea lice epidemiology and treatment effectiveness, we believe that this research furthers the knowledge of the subject area on the east coast of Canada. With further understanding, the development of improved monitoring and management practices may delay the emergence of resistance in the future and allow for earlier detection of emergence. This work provides solid evidence of the development of emamectin benzoate resistance prior to the problem becoming evident to producers and the rest of the industry in 2009 and suggests the basis for monitoring of parasiticide resistance development in the future.

4.4 Literature cited

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Chapter 5 Appendix

Appendix A An alternative method for tracking resistance development to emamectin benzoate in separate sea lice (Lepeophtheirus salmonis) life stages

Chapter 3 evaluated two groups of life stages for changes in treatment effectiveness over time and location. An alternative method to the one presented in Chapter 3 was explored. In general, the abundance of 'other mobile' lice tended to be higher in comparison to adult female lice making comparisons between the two groups challenging. In an effort to determine if resistance developed in adult females prior to the other stages or if there were differences in the ratio of adult females to other mobiles which occurred over time, an approach which calculated the proportion of total mobiles which were adult females was considered. In theory, if resistance developed in one stage before another then there should be an increase in the proportion of that life stage as resistance emerged over time. The year 2004 was eliminated from the analysis as that year only had seven treatment episodes and all the episodes involved data collected from one company. Given that there were concerns around misclassification error, ensuring that data was from a variety of sources minimized these concerns. Weeks were grouped into pairs in the post-treatment period to ensure the results were robust.

An increase in the percentage of adult females over time was observed. The pre-treatment proportion of adult female lice in 2005 was 12.8% (Table A.1). In 2006, a larger proportion of total mobiles were adult females at pre-treatment at 22.3% and 2007 had a similar value at 23.8%. In 2008, the pre-treatment percentage adult female lice was 15.2%. During the post-treatment period, in 2006, the percentage of adult females varied from 12.1% to 17.1%. A marked increase was noted in 2007, although the lowest proportion of adult females was 19.2% but this may not have been an accurate indicator

(during the last time period where data tends to be sparser and treatment effect was starting to wane). The next lowest proportion was 25.4%, which may have been a more accurate indicator of the true value. It should be noted that in 2007 there was not much change in the percentage of adult females present from the pre-treatment evaluation to the post-treatment assessments. In essence, the proportion of adult females present on fish during the post-treatment period did not decrease much from the pre-treatment value of 23.8%. In 2008, a similar finding was noted with an increased proportion of adult females being found.

A ratio of the mean proportion of adult female L. salmonis during the post-treatment period to the proportion pre-treatment abundance of adult female lice by year was calculated. This ratio increased with each successive year (Table A.1). This indicator shows that there was an increase in the presence of adult females which coincided with a reduction in the effectiveness of emamectin benzoate. In fact, in 2007 the ratio was 1.1 which was one year before a significant reduction in treatment effectiveness was noted (Chapter 2). The use of such an indicator warrants further investigation through the examination of similar data sets to determine if such a pattern is routinely present in the development of resistance in sea lice to treatment products.

Table A.1 Proportion of total mobile Lepeophtheirus salmonis that are adult females (AF) (+/- standard error) for emamectin benzoate treatment episodes by year in New Brunswick, Canada. [a]

Sample Week	2005	2006	2007	2008
Pre-Tx	12.8 (3.1)	22.3 (4.4)	23.8 (4.3)	15.2 (2.7)
3 & 4	8.9 (3.1)	16.6 (6.7)	26.7 (3.7)	23.3 (3.6)
5 & 6	1.6 (1.1)	13.4 (6.0)	29.3 (4.4)	30.6 (5.0)
7 & 8	8.8 (7.0)	14.2 (4.3)	25.4 (5.0)	27.2 (4.9)
9 & 10	5.5 (3.4)	17.1 (5.1)	25.5 (5.2)	16.1 (3.5)
11 & 12	3.9 (2.2)	12.1 (5.5)	19.2 (5.1)	14.3 (5.6)
Mean proportion of AF during post-treatment period	5.7	14.7	25.2	22.3
Ratio of mean proportion of AF post-treatment to proportion of AF pre-treatment [b]	0.4	0.7	1.1	1.5

[a] Proportion total mobile L. salmonis that are adult females = (mean abundance adult females/mean abundance total mobiles)*100%

[b] Ratio of mean proportion of adult females post-treatment to proportion of adult females pre-treatment = (mean proportion of adult females during post-treatment period/ proportion of adult females at pre-treatment)

One of the possible downfalls to using the proportion of adult female lice to track resistance emergence is that changes in the ratios of life stages may be occurring naturally in the environment. In other words, there may be some variation in the levels of one life stage over another. This has been noted during the winter months when adult females tend to be the life stage that persist on fish during these colder months. There are likely regional variations to this finding as well, with some areas having overall higher abundance than others. Another possibility could be variation between counters of sea lice, although the easiest stage to differentiate is probably adult females given that they are significantly larger than the other mobile stages. Lastly, there is variation in the pre-treatment levels of the percentage of total mobiles which are adult females. There were increases found in 2006 and 2007 and then a decrease for 2008. It could not be determined if this was influenced by the emergence of emamectin benzoate resistance or due to natural variations in sea lice populations. One could speculate that if resistance were evolving, then the life stages more prone to resistance development would be present in higher proportions. The cause of this variation cannot be determined in this study but it is interesting that an increase in the proportion of adult females does appear to coincide with a decrease in the effectiveness of emamectin benzoate over time.