Monitoring sea lice (*Lepeophtheirus salmonis*) sensitivity to emamectin benzoate in Atlantic salmon (*Salmo salar*)

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Abstract

The main objectives of thesis are: (1) to describe sea lice management on the east coast of Canada in the absence of standardized methods for monitoring sea lice abundance on Atlantic salmon production sites, (2) to develop and optimize field bioassays to monitor for changes in sea lice sensitivity toward Emamectin Benzoate (EMB), and (3) to optimize and use monooxygenase testing to describe enzyme activity in field collected sea lice populations with exposure to EMB.

'Sea lice' is a generic term used to refer to ectoparasitic caligid copepods which occur on wild and farmed marine fish world-wide. The requirement for continual monitoring and control of sea lice is a costly economic burden for Atlantic salmon *(Salmo salar)* producers throughout North America and northern Europe.

There are currently no regulations for the reporting of sea lice burdens on salmon farms in Atlantic Canada, nor are there officially standardized methods for conducting sea lice counts in the field, although sea lice do receive close attention and management for health and production purposes. A survey was conducted of the Atlantic salmon farming industry to describe the different methods of sea lice sampling employed, the types of sea lice treatments being used, and the factors considered when deciding to treat for sea lice. Participants in the survey included 83 of the approximately 94 sites in operation in the Bay of Fundy at the time the survey was conducted. The survey indicated that the majority of fish farmers, in concert with their veterinarians, practice intensive monitoring and control of sea lice burdens. Ninety percent of sites surveyed are using emamectin benzoate (EMB; active chemical in SLICE®) to treat their smolt and premarket salmon for sea lice. Despite the current absence of clinical resistance of sea lice to EMB, continued reliance on it for sea lice control on Atlantic salmon farms in the Bay of Fundy raises concern regarding the potential for sea lice to develop resistance to the drug.

Reports from Europe of reduced sensitivity and resistance development of sea lice towards several chemotherapeutants necessitate the development of resistance management strategies, including methods to monitor changes in sensitivity to treatments. A bioassay for sea lice sensitivity towards EMB was developed and validated for field use. Bioassay optimization included an evaluation of the inter-rater reliability of sea lice responsiveness to EMB and an evaluation of gender-related differences in susceptibility. The concordance correlation coefficient was used to compare the agreement between the proportion of lice categorized as moribund or dead by two independent raters prior to and following the adoption of a set of bioassay response criteria. The adoption of a set of bioassay response criteria improved the concordance between raters' assessments and it also improved the model estimation of the EC_{50} values (the "effective concentration" leading to a response of 50 % of the lice not prone to natural response) for subsequent bioassays. An evaluation of gender-related differences in EMB susceptibility for 19 bioassays indicated that preadult stage female sea lice from field populations exhibited a significantly higher sensitivity towards EMB compared to preadult males in 12 of 19 bioassays; 7 of 19 bioassays showed no significant difference in gender susceptibility, although all male EC_{50} values were higher than female values. Preadult stage females from one population of laboratory cultivated sea lice also exhibited a significantly higher EMB sensitivity compared to preadult males. The EMB

bioassay was implemented for field use and a total of 38 field bioassays were completed from 2002 to 2005 using preadult stage sea lice collected from Atlantic salmon farms in four regions in the Bay of Fundy salmon farming area. There was no significant overall effect of region or year on EC_{50} values, and although the range of sensitivities obtained in this three-year study did not appear sufficient to affect current clinical success in the control of sea lice, the results suggest that there is a seasonal or temperature associated variation in sensitivity of preadult sea lice to EMB with sensitivity decreasing in the fall and winter months.

As a potential means of EMB detoxification by sea lice, oxidase enzymes are of particular interest as an alternative to the use of bioassays for resistance detection. The results of this study indicated significant effects of region and a seasonal trend on median haem peroxidase values for 2001 to 2004 field sea lice samples. There were no significant effects in the 2005 field data. There was a positive, but not statistically significant, correlation between EMB sensitivity (bioassay EC_{50} values) and median haem peroxidase activity in field sea lice samples for the years 2001 to 2005.

An evaluation of potential factors influencing total oxidase activity indicated no significant time-dependent changes in haem peroxidase activity up to 72 h post mortem for sea lice stored at 3 to 4°C, suggesting that oxidase enzymes in sea lice are remarkably stable after post-mortem delays typically encountered between farm collection and arrival at the laboratory. There was no significant difference between haem peroxidase values for live and dead or frozen and non-frozen sea lice, suggesting that the method of freezing used in this study offers a convenient and economical alternative to use of liquid nitrogen for freezing of sea lice samples. There was a significant effect of storage at -80°C up to 30 days on haem peroxidase levels which requires further evaluation. Three sources of adult female sea lice were exposed to four EMB concentrations (0, 100, 200 and 300 ppb) for 24 and 48 h exposure periods. The effect of concentration and condition across the three sea lice sources was variable (e.g. sea lice exposed to 100 ppb had the highest haem peroxidase levels for source 1 but the lowest for source 2, dead sea lice had the highest haem peroxidase values for source 1 and the lowest for source 3).

This research has demonstrated the widespread use of EMB for sea lice control in the Bay of Fundy and has resulted in the development of an EMB bioassay which could be used to verify clinical resistance in the field. The developed bioassay does however lack rapidity and simplicity for use as a routine test for a field monitoring program. The measurement of total oxidase activity in individual sea lice is difficult to confirm as a diagnostic tool for resistance detection due to the absence of sea lice populations known to be resistant to EMB, and the inability of the assay to indicate the specific cytochrome P450 enzymes responsible for resistance development. However, the ability of the haem peroxidase assay to identify individuals displaying elevated general oxidative activity is encouraging, as field populations are likely to consist of a mixture of susceptible and less susceptible individuals. This study also highlights the importance of obtaining data on the parameters influencing oxidase levels in EMB-susceptible sea lice populations in order to standardize the haem peroxidase assay methodology before implementation in a resistance monitoring program. The haem peroxidase assay should be used in combination with bioassays or other tests (i.e. molecular) when resistance is suspected and the mechanism has been identified. The finding of a seasonal effect on the bioassay and oxidase values requires further investigation.

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List of Abbreviations

ACh	acetylcholine
AChE	acetylcholinesterase
ANOVA	analysis of variance
AVMs	avermectins
BC	British Columbia
BC MAFF	British Columbia Ministry of Agriculture, Food and Fisheries (now BC Ministry of Agriculture and Lands)
BSA	bovine serum albumin
CAIA	Canadian Aquaculture Industry Alliance
CCC	concordance correlation coefficient
CYP	cytochrome P450
CD	Campobello Island and Deer Island
d	day
EC_{50}	the effective concentration leading to a response (morbidity and death) of 50% of the test subjects
EMB	emamectin benzoate
FF	frontal filament
GABACl	gamma aminobutyric acid-gated chloride channels
GluCl	glutamate-gated chloride channels
GM	Grand Manan Island
GST	glutathione S-transferase
h	hours
IVM	ivermectin

ISA	infectious salmon anemia
LD_{50}	concentration leading to 50% mortality of test subjects
LD_{90}	concentration leading to 90% mortality of test subjects
LK	Lime Kiln Bay
min	minutes
nm	nanometers
PB	Passamaquoddy Bay
PBO	piperonyl butoxide
PCR	polymerase chain reaction
ppb	parts per billion
ppm	parts per million
ppt	parts per thousand
rpm	rotations per minute
TMB	3,3′,5,5′ tetramethylbenzidine
TMS	tricaine methanesulfonate
UK	United Kingdom

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Chapter 1 General Introduction

1.1 Sea lice

'Sea lice' is a generic term used to refer to ectoparasitic caligid copepods which occur on wild and farmed marine fish world-wide. They belong to the Phylum Crustacea (Margulis and Schwartz 1998), Class Copepoda (Margulis and Schwartz 1998), Suborder Siphonostomatoida and Family Caligidae (Kabata, 1984). *Lepeophtheirus* and *Caligus* are two genera of sea lice, containing approximately 100 and 200 known species, respectively (Kabata 1984). Several species of sea lice from these genera can pose an on-going management challenge because of their affect on the health and productivity of commercial salmonid culture world-wide. Two of these species include *Lepeophtheirus salmonis* and *Caligus elongatus.* Important differences between *L. salmonis* and *C. elongatus* include geographical distribution, host specificity, size and the nature of infestation.

L. salmonis has a circumpolar distribution (Kabata 1979) and is highly host specific to salmonids of the genera Salmo, Onchorhynchus and Salvelinus (Kabata 1979, Pike and Wadsworth 1999). It is the dominant sea lice species on cage-cultured salmonids on the east coast of Canada, northeastern USA, and Northern Europe (Pike and Wadsworth 1999, Johnson *et al.* 2004) and has also been reported on the west coast of Canada. Although *L. salmonis* has been reported on coho salmon (*Oncorhynchus kisutch*) and rainbow trout (*Oncorhynchus mykiss*) in Japan, infestation is not a serious economic problem and does not require treatment (Nagasawa 2004). Atlantic salmon, coho salmon, and rainbow trout are also farmed commercially in Chile, New Zealand, and Tasmania. Sea lice have not been reported to cause disease in New Zealand or

Tasmania. Outbreaks of *L. salmonis* at sea cage sites were first reported in Norway in the 1960's, followed by Scotland in the mid 1970's (Stuart 1990; Pike and Wadsworth 1999), Ireland in the late 1980's and the east coast of Canada in the 1990's (Hogans 1995). Initial outbreaks were likely attributed to an increasing trend in finfish production in these countries. In Chile, only *Caligus teres* and *Caligus rogercressyi* are considered economically important parasites of Atlantic salmon, coho salmon and rainbow trout (Johnson *et al.* 2004).

C. elongatus has a world-wide distribution with low host specificity, having been reported on over 80 species of fish including non-salmonid hosts (Kabata 1979). Although both species of sea lice are capable of inflicting damage upon their hosts, damage inflicted by *L. salmonis* is more severe than *C. elongatus*, owing to its larger size and the fact that *L. salmonis* infection is chronic and persistent, compared to that of *C. elongatus* which is acute and transient.

1.1.1 History of sea lice on the east coast of Canada

Despite early reports of the presence of *L. salmonis* on wild fish from the east coast of Canada (White 1940), and in varying degrees at several inspected commercial production sites (Hogans and Trudeau 1989), *C. elongatus* was initially the dominant species reported on farms in the lower Bay of Fundy (Hogans and Trudeau 1989). In the fall of 1994, *L. salmonis* became a clinically important issue in cage-cultured salmon in two areas of the Bay of Fundy (Hogans 1995), continuing to affect over 50 farms by the spring of 2005, and quickly establishing itself as the major cause of fish mortality and economic loss to the aquaculture industry in eastern Canada (O'Halloran and Hogans

1996, MacKinnon 1997). Sea lice were initially difficult to manage and control due to the lack of products registered to treat sea lice at that time (O'Halloran and Hogans 1996). Presently, *L. salmonis* remains an economically significant pathogen to the lower Bay of Fundy Atlantic salmon farming industry, although there are now two products available for use as in-feed treatments. *C. elongatus* is also prevalent (Hogans and Trudeau 1989, Hogans 1995) on cultured Atlantic salmon on the east coast of Canada, but its impact is minor compared to *L. salmonis* and infection intensities have generally remained insufficient to warrant intensive monitoring and control.

1.1.2 L. salmonis life cycle and differentiation

The life cycle of *L. salmonis* is direct with no intermediate hosts. Like most caligid copepods, the life cycle consists of 10 stages, comprising five distinct phases: nauplius (two stages), copepodid (one stage), chalimus (four stages), preadult (two stages), and adult (Kabata 1972) (Appendix A). Between each of the developmental stages, sea lice undergo a moult. Moulting ceases once sea lice have developed to the adult stage. The life cycle is temperature-dependent. Generation time has been estimated as 7.5 to 8 weeks at 10°C (Johnson and Albright 1991a) under laboratory conditions, and 6 weeks at 9-12 °C on farmed salmonids (Wootten *et al.* 1982).

The initial phase in the lifecycle, following eclosion (hatching from egg), represents the first planktonic and free-swimming larval phase. Both nauplii I and nauplii II are active and intermittent swimmers (Pike and Wadsworth 1999). They are similar in shape, with nauplii II being longer and more narrow; nauplius I and II have an approximate length and width of 0.5 and 0.2 mm, and 0.6 and 0.2 mm, respectively

(Johnson and Albright 1991b, Schram 1993). Both stages are photopositive (Johannessen 1978). A pair of eyespots is visible on the dorsal surface of each stage (Johnson and Albright 1991a). Both stages depend upon internal reserves for nutrients (Pike and Wadsworth 1999) and lack mouth and anus (Johannessen 1978). Yolk is visible within the almost translucent body of nauplii I (Johnson and Albright 1991b). Under laboratory conditions, the average duration of the first nauplius stage varied from 52 h at 5°C to 9.2 h at 15°C; that of nauplius II varied from 170 h at 5°C to 36 h at 15°C (Johnson and Albright 1991a, Pike and Wadsworth 1999).

The newly moulted copepodid stage is initially a free-swimming larval stage. However, the moult from nauplii II to copepodid marks the end of the planktonic form as it remains planktonic only until it finds a suitable host on which to attach. It attaches to the host by means of its hooked second antennae and legs at which point it becomes parasitic and capable of infecting the host fish (Wootten *et al.* 1982, Pike and Wadsworth 1999). Transmission of sea lice is dependent solely on this stage. The ability of the copepodid to locate and attach to the host fish is influenced by light (Hevrøy *et al.* 2003, Browman *et al.* 2004), salinity (Heuch 1995), and hydrodynamic factors (Costelloe *et al.* 1996a, Heuch and Karlsen 1997). Time and temperature will limit the length of time the copepodid will remain infective (Pike and Wadsworth 1999). The copepodid is longer and wider than the nauplius stages, having a length and width of 0.7 and 0.2 mm, respectively (Johnson and Albright 1991b). Its body now has two distinct regions: the cephalothorax (a fusion of the head and thorax) and a posterior region consisting of four segments (Johnson and Albright 1991a). Like the nauplius stages, the copepodid stage also exhibits positive phototaxis (Wootten *et al.* 1982). Although the copepodid stage

remains non-feeding, it now possesses a functional gut (Bron *et al.* 1993a) and external mouth parts (Pike and Wadsworth 1999). Its survival is dependent on energy reserves remaining within the yolk sac (Pike and Wadsworth 1999).

Sea lice moult from copepodid through four successive chalimus stages. This phase is marked by the development of a frontal filament (FF) which projects from the leading edge of the carapace and allows the chalimus to attach firmly to the host fish. Although it is not yet mobile on the host, it is able to cause localized damage to the host as it feeds in the area of attachment. Chalimus stages are most often found attached to the dorsal and pelvic fins or around the anus (Wootten *et al.* 1982). As early as the fourth chalimus stage, *C. elongatus* can be differentiated from *L. salmonis* by differences in the morphology and origin of the FF (Pike and Wadsworth 1999). Aside from function, there is very little similarity between the FF of *C. elongatus* and *L. salmonis*. The FF of *C. elongatus* is pre-formed inside the body of the copepodid where it adds a new basal portion following each successive moult, resulting in its long and slender appearance. It fixes directly to the scale of the host by first removing the epidermal layer prior to attachment (Pike *et al.* 1993). In contrast, the FF of *L. salmonis* is continuous with that of the chalimus body. It is replaced with each moult through the successive chalimus stages (Johnson and Albright 1992, Gonzalez-Alanis *et al.* 2001), resulting in a short and stumpy appearance. It attaches to the host fish by insertion through the epidermis covering the scale, followed by the production of an adhesive. The chalimus stages begin to resemble the preadult stages as the genital segments of each sex begin to enlarge and the carapace becomes wider and shield-shaped (Wootten *et al.* 1982).

The loss of the FF marks entry to the preadult phase of the life cycle. This allows the preadult I and II stages to move freely on and between hosts (Ritchie 1997). They are most commonly found on the dorsal surface, head, and on the posterior ventral surface. An increase in mobility results in an increased pathology due to these stages. Preadult stages are similar in body shape to the adults except for the size of the genital complex and urosome (abdomen) (Pike and Wadsworth 1999). Sexual differentiation is possible at the preadult I and II stages due to a difference in the appearance of the genital segments of males and females; the male genital segment is oval or barrel shaped, while that of the female is triangular (Wootten *et al.* 1982). Preadult I females average 3.6 and 1.9 mm in length and width, and males average 3.4 and 1.6 mm, respectively (Johnson and Albright 1991b, Schram 1993). Although *C. elongatus* lacks the preadult stage (Piasecki and MacKinnon 1995), there are a number of features that allow for differentiation between species. *C. elongatus* are smaller in size, possess paired lunules on the anterior edge of the carapace, and have paired eyespots. *L. salmonis* are larger in size, lack paired lunules (sucker-like organs) and have a single eyespot (Wootten *et al.* 1982). Mature adults of both sexes are mobile and can transfer between hosts. There is extreme sexual dimorphism (Revie *et al.* 2002), with females being almost twice the size of adult males; averaging 8 to 11 and 5 to 6 mm in length, respectively (Schram 1993).

Adult males will mate with preadult I, II, and adult females (Pike and Wadsworth 1999). The male will attach to the female, waiting for her to complete the final moult. Sperm, which is enclosed in a protective sac, the spermatophore, becomes attached to the female's genital orifices (Pike and Wadsworth 1999) and then passes through the ovogenital pores. Several sets of paired egg strings can result from a single mating (Pike

and Wadsworth 1999, Schering-Plough Animal Health 2000). Paired egg strings, which are extruded through gonopores (genital openings) in the genital complex, are a chain of fertilized embryos. The strings are attached to the genital complex by an apparatus of paired hooks through the proximal ends of the strings (Schram 2000). The number of eggs per egg string is related to length of the egg string. Egg strings collected from farmed Atlantic salmon at lower water temperatures are longer and have more eggs per string, although the eggs are smaller (Heuch *et al.* 2000, MacKinnon 1997). Reports on the estimation of number of eggs per string vary. Estimates range from between 180 to 300 eggs (Schram 1993) to above 900, depending on the host species of salmon (Johnson 1993), the time of year (Tully 1989), and whether or not the salmon was wild or farmed (Jackson and Minchin 1992). Newly extruded egg strings are transparent or light in color but they darken as they develop, owing to the appearance of pigment in the embryos; fully developed egg strings ready for eclosion (hatching) appear dark brown (Johannessen 1978, Pike and Wadsworth 1999). The expected number of *L. salmonis* generations per year could be upwards of six; this is based on previous estimations of three or four generations between May and October (Wootten *et al.* 1982), and the ability of *L. salmonis* to overwinter (Jacobsen and Gaard 1997) and produce egg strings at lower water temperatures (Boxaspen and Næss 2000, Heuch *et al.* 2000).

There are a number of parasitic crustaceans, in addition to *L. salmonis*, that may be found on Atlantic salmon in Canadian waters (i.e. *Argulus alosae*, *Ergasilus labracis*, *Salmincola corpulentus* and *Salmincola salmoneus*). These parasites can easily be differentiated from the species of sea lice used in this study based on their habitat (i.e. freshwater or marine), geographic distribution, and physical appearance. In addition to *L.*

salmonis and *C. elongatus*, *C. curtus* is another species of sea lice that can also be found on Atlantic salmon in New Brunswick, Canada. Both *C. elongatus* and *C. curtus* can easily be differentiated from *L. salmonis* based on the presence of lunules, as well as other differentiating physical characteristics (Margolis and Kabata 1984, Appendix B).

1.1.3 Pathological effects

Sea lice intensity and the pathogenic effects of infestation will depend upon a number of factors. Host susceptibility will determine the number of sea lice that are able to successfully establish themselves on the host, and may be influenced by host species (Johnson and Albright 1992, Johnson 1993), age, nutrition (Johnson 1993), stress level and immunocompetency (MacKinnon 1998). The sea lice species to which the host is exposed, as well as the developmental stages of the species present, will affect the host's response to infestation (Pike and Wadsworth 1999). The pathogenic effects of sea lice infestation will increase with size, mobility, and number of sea lice present, since the pathology associated with sea lice infestation is primarily due to the effects of feeding on the host fish. Sea lice feed on host mucus, skin, and blood which cause direct mechanical damage such as skin and fin erosion (Kabata 1974, Brandal *et al.* 1976, Wootten *et al.* 1982). The chalimus stages, which attach to the fish by means of the FF, cause only a local cellular response in the radial area of attachment (Pike and Wadsworth 1999). Black spots are a common indication of this epidermal reaction (Grimnes and Jakobsen 1996), which is insufficient to cause osmoregulatory problems (Dawson *et al.* 1999). An increase in the severity of mechanical damage due to feeding is an indication of the increase in pathogenicity of the mobile stages (Grimnes and Jakobsen 1996). Aggressive

feeding by mobile stages may result in the disruption of the overall integrity of the epithelia which can affect the ability of the host fish to osmoregulate (Wootten *et al.* 1982) and may leave the host susceptible to secondary pathogens (Nolan *et al.* 1999). Host mortality may occur in extreme cases of infestation (Grimnes and Jakobsen 1996). Heavy infestations may cause primary (e.g. high levels of cortisol), secondary (eg. osmoregulatory problems) and tertiary (e.g. increased disease incidence) stress responses in salmonids (Grimnes and Jakobsen 1996, Bowers *et al.* 2000).

1.2 Sea lice control and management

Sea lice control and management has improved since the first reports of industry outbreaks in the 1970's. This is largely due to the development of integrated sea lice management strategies employing both biological and chemical control methods. There are several bay and individual farm management practices which are useful methods for managing sea lice abundance on and between farms. These practices include fallowing, single year-class sites, the coordination and strategic timing of sea lice treatments, as well as general husbandry practices aimed at reducing sea lice abundance.

The objective of fallowing is to break the life cycle of existent sea lice populations (Grant and Treasurer 1993) by leaving a site or area devoid of fish for a specified period of time, disinfecting the hardware and restocking the site with healthy smolts (Rae 2002). Fallowing has been shown to lower numbers of *L. salmonis* on newly introduced fish for several months after stocking and substantially delays the need for chemical control due to a slower acquisition of sea lice (Bron *et al.* 1993b, Revie *et al.* 2003). The length of the fallow period should be based on the water temperature and the

relative generation time of the sea lice life cycle to ensure sufficient time has lapsed to break the cycle. Fallowing should be coordinated within bays or areas to eliminate the movement of sea lice from stocked to fallowed sites, although lengthy periods of fallowing may not be economically feasible for all farms.

The objective of single year-class sites is to reduce the transfer of sea lice infestation from existing year classes to newly introduced smolts in an effort to delay the acquisition of sea lice. This is accomplished through a physical and geographical separation of year classes. Although smolts may be generally more susceptible to sea lice infestation following transfer to salt water due to their small size and stressed state owing to transport from the hatchery and to physiological changes associated with smoltification, water temperature near smolt transfer times are not amenable to settlement of copepodids. Single year class sites represent an important barrier to transferring sea lice from one generation to the next, delaying the need for initial treatment and reducing the number of treatments required during the first year at sea (Bron *et al.* 1993, Revie *et al.* 2003). This 'all-in-all-out' strategy reduces the transfer of sea lice infestation from older year classes to smolts by having all fish enter the salt water phase of the production cycle after all fish have been harvested.

Coordinated and strategic timing of treatments within sites and within areas is an important means of sea lice management and control. As some of the chemotherapeutants used for sea lice control target specific, and not necessarily all, stages of the sea lice lifecycle, strategic timing of treatments will ensure that the correct treatment method is selected to target the sea lice stages present. It is beneficial for farmers to treat their fish in the late fall to target remaining gravid females in order to

prevent them from over-wintering on fish. This is necessary to lower spring lice loads by preventing a spring 'explosion' of sea lice when water temperatures rise and the sea lice life cycle becomes more rapid. Coordination of sea lice treatments within a bay or area is ideal; one farm neglecting to control sea lice puts all neighboring farms within that area at greater risk of infestation due to the fact that the infective copepodid stage can travel great distances by water currents and tides and sea lice can move between hosts.

Reducing stocking densities may also contribute to better sea lice management as higher stocking densities can increase the chance of copepodid transmission (Wootten *et al.* 1982) between susceptible hosts through an increase in the probability of host location by the infective copepodids. Severe crowding may also increase stress in fish, increasing their susceptibility to sea lice infestation.

Regular monitoring for sea lice is important to ensure that levels remain at acceptable levels and to allow for strategic timing of treatments. It will also help predict later sea lice developments and the need for future treatments, as well as providing an indication of the success of administered treatments.

The transmission of sea lice is dependent solely on the infective copepodid which has a limited amount of time to find a host on which to attach. Although it is impossible to prevent the presence and movement of copepodids it is beneficial for farmers to implement husbandry practices aimed at decreasing the probability that the copepodid will find a host on which to attach. Several general husbandry practices can aid in minimizing copepodid abundance, including regular net changes to minimize fouling. Net fouling has the potential to reduce water flow through cages, thereby increasing the retention of copepodids inside (Costelloe *et al.* 1996). Host location behaviors of

copepodids include positive phototaxis and a diel vertical migration involving daily migration to the surface during daylight and movement to deeper water at night. Caged salmon feeding at the water surface during the day may be at an increased risk of coming into contact with infective copepodids (Heuch 1995). Ideally, it would be beneficial for farmers to feed during the night when the copepodids have moved deeper within the water column or to employ feeding technologies that do not require feeding at the surface. Unfortunately, such ideas are not practical under commercial conditions. However, farmers are able to control the timing of treatments to reduce the reproductive output of sea lice when it is naturally high (i.e. during spring and summer), which will reduce the number of new infective copepodids in subsequent generations.

Revie *et al.* (2003) investigated environmental and management risk factors for sea lice burdens using data collected from 40 Scottish salmon farm sites during 1996 to 2000. Mean mobile sea lice abundance during six month periods within the salmon production cycle (i.e. the first and second halves of the first year of production, and the first half of the second year of production) were used to explain the variation in sea lice numbers across sites. Nine potential risk factors for mobile sea lice abundance were identified by an expert panel as being within management control. These included treatment level and type, the use of strategic treatments, stocking density and biomass levels in the first and second halves of the year, historical farm size, cage volume and the number of days the site was fallowed. Of these nine factors only the level of treatment and cage volume partially explained the abundance of mobile sea lice on salmon in the second half of the first year of production; the frequency of treatments increased as sea lice abundance increased and sea lice abundance decreased with increasing cage volume.

In the first half of the second year, treatment level, treatment type and cage volume were found to have an influence on mobile sea lice abundance; sea lice abundance decreased as the level of treatment increases and as the type of sea lice treatment changed from hydrogen peroxide to organophosphates or synthetic pyrethroids. Factors investigated as outside farm management control, which are principally environmental factors, included current pattern, current speed, water temperature, loch flushing time, freshwater run-off/salinity, ambient light level and wind, and sea lice levels in the preceding 6 month period. Of these factors only current speed, loch flushing time and sea lice levels in the preceding 6 month period were key explanatory factors of variations in mobile sea lice abundance between farms.

Overstocking of cages will increase the probability of sea lice finding a host on which to attach and the stress of crowding from overstocking can increase fish susceptibility to sea lice infestation. The size and depth of nets, as well as their position on site, may influence infection rates; fish deeper in the water column are less likely to come into contact with copepodids higher in the water column, and outside or end cages tend to have heavier lice burdens than inside cages. Mixed year class sites will promote the transfer of sea lice from older to younger fish and heavily fouled nets may reduce water flow through nets, thereby trapping sea lice within and improving the potential for host location.

At the individual level, sea lice susceptibility has been shown to vary by salmon species (Johnson and Albright 1992) and diseased or stressed fish may be more susceptible to sea lice infection. Moribund fish require regular removal from cages as

they may function as sea lice attractants, harboring higher lice loads which may infect healthy fish (Kvenseth 1997).

1.2.1 Biological control

1.2.1.1 Wrasse (cleaner-fish)

The use of wrasse as cleaner-fish for salmon was developed in Norway in the late 1980's and they have been subsequently used in Scotland and Ireland (Kvenseth 1996, Rae 2002). Wrasse stocked from wild sources are typically placed in salmon cages at a ratio of one wrasse to 50 to 150 salmon (Costello 1993), where they swim amongst the salmon and pluck lice from their skin (Costello *et al.* 1996b). Wrasse are not used in Canada because there are no known native species capable of controlling sea lice. There are 5 species of wrasse available in northern European waters; the goldsinny (*Ctenolabrus rupestris*), rock cook (*Centrolabrus exoletus*), corkwing (*Crenilabrus melops*), ballan (*Labrus bergylta*) and cuckoo wrasse (*Labrus mixtus*). The goldsinny, corkwing and ballan wrasse seem to have the best delousing potential (Kvenseth and Kvenseth 2000). The main advantages of using wrasse are environmental and economical. The cost of buying wrasse for a whole generation of salmon is approximately the same as the cost of one in-feed or bath treatment (Kvenseth and Kvenseth 2000). They offer the advantage of continuous sea lice treatment as long as they survive in pens. However, there are problems with high mortality rates after introduction due to susceptibility of wrasse to both typical and atypical strains of *Aeromonas salmonicida* as well as *Vibrio anguillarum* (Gravingen *et al.* 1996). Survival of overwintering wrasse is also a problem, and escapees are common particularly with

increasing net mesh size. Wrasse need to be captured in the wild and screened before use. They are not captured in high numbers until May or June, and given that smolts are often transferred from March to June, there may be a period when wrasse are unavailable for lice control (Costello 1993). Other disadvantages to using wrasse include the fact that their feeding activity decreases with decreasing water temperature (Kvenseth and Kvenseth 2000) and they will eat net fouling so this must be minimized to achieve efficacy. They are also more effective at controlling sea lice when the lice reach mobile stages and are larger in size; chalimus stages are often left behind.

1.2.1.2 Vaccines

Immunologic methods offer a number of advantages over chemical and biological control. In the case of sea lice management, vaccine development would mean the prevention of sea lice infection through the development of long-term immunity, resulting in a decrease in the number of sea lice treatments needed during the production cycle. Although treatment failure can also occur with the use of vaccines, resistance does not develop, suggesting that the effectiveness of sea lice vaccines could potentially be indefinite. Vaccines also require little or no withdrawal times prior to marketing (Raynard *et al.* 2002). As well, they would be made to specifically target the vaccinated fish, thus having no adverse effects on non-target species or the environment. They would also be easy to administer and more cost-effective versus chemical treatments and could be administered in the hatchery prior to saltwater transfer so that fish would have sustained protection as soon as they entered the salt water phase of production.

There are currently relatively few metazoan vaccines in practical commercial usage. Strategies for sea louse vaccine development have involved the adoption of methods used for the immunization of agriculture hosts against various ectoparasites. The concealed antigen approach has been used to develop vaccines against various parasites including the cattle tick *Boophilus microplus* (Willadsen *et al.* 1989, Lee *et al.* 1991), larvae of the sheep blowfly *Lucilia cuprina* (East *et al.* 1993), the ixodid tick *Rhipicephalus appendiculatus* (Rechav *et al.* 1992) and the nematode *Haemonchus contortus* (Smith *et al.* 1993). Concealed antigens are components of the parasite which are capable of eliciting a protective immune response in the host, but do not come into contact with the host's immune system during an infection (Raynard *et al.* 2002). In the case of vaccine development for sea lice it would be based on targeting the sea louse gut such that sea lice ingestion of salmon blood containing antibodies would result in antibody binding to the sea louse gut, thereby interfering with normal gut function, and ultimately impairing louse physiology, nutrition and fecundity (Raynard *et al.* 2002). There are several challenges to using this approach in sea louse vaccine development. The degree of success with the concealed antigen method is dependent on the sea louse mode and rate of blood feeding. Sea lice ingestion of salmon blood would be required to ensure significant ingestion of antibody. Although blood may be an important component of the sea louse diet, the rate of blood consumption may vary between sea louse developmental stages (Brandal *et al.* 1976) and is low compared to other ectoparasites. The structure and biochemical characteristic of the sea louse gut may also effect the concealed antigen approach (Raynard *et al.* 2002).

Successful vaccines against sea lice have not yet been developed, although research is on-going (Roper *et al.* 1995, Nilsen 2004). Nilsen (2004) and Fast *et al.* (2003) have been working on targeting the proteins involved with digestive processes of the sea louse, particularly proteases. Nilsen (2004) has also been targeting proteins involved in reproduction, such as vitellogenins.

1.2.2 Chemical control

Chemicals can be applied in a number of ways. These include bath, dip, oral (in-feed), or injection (Costello 1993). Bath and in-feed treatments are the most common application methods employed by Atlantic salmon farmers for treating sea lice infections. Bath treatments have a number of inherent disadvantages. They are very labor-intensive due to the need for tarping or skirting of cages, which makes it difficult to simultaneously treat all cages on site. Doses and concentrations have to be carefully calculated based on water volume which is often difficult to do in a skirted or tarped cage and the crowding of fish is stressful. Appetite suppression may occur following treatment, resulting in lost growth. Proper training and experience in pesticide application is necessary for administrators of certain bath treatments as there may be concerns with regards to the dangerous effects of certain chemicals to the administrator. It can be difficult to administer bath treatments under adverse weather conditions and lengthy treatments result in the inability to simultaneously treat all cages on site which may allow for reinfestation of treated cages from untreated cages. There are environmental concerns with regards to the release of chemicals into the surrounding water following treatment (Davies *et al.* 2001, Ernst *et al.* 2001, Haya *et al.* 2001) and their effects on non-target

organisms (Burridge *et al.* 2000). There is a need for repeated bath treatments because most have no residual or prophylactic action, they may be affected by temperature, and it may be difficult to ensure a proper mixing of the chemical within the cage. Bath treatments are advantageous because all fish are treated equally despite the presence of hierarchies within cages.

There are a number of advantages and disadvantages associated with the use of in-feed treatments. For example, disadvantages include the withdrawal times that are often necessary prior to marketing as the drug is fed to the fish and can accumulate within the tissues. It is often difficult to control the dose that each fish receives; making it difficult to ensure that each fish receives the therapeutic concentrations. This may be due to the fact that diseased or stressed fish may not be feeding. There may be different rates in drug uptake under field conditions due to the natural size variations of fish that exist under cultured conditions. Advantages include the ability to simultaneously treat all cages on site and the fact that the method of application is a lot less labor intensive and stressful to fish as they don't have to be crowded or handled. In-feed treatments also have a minimal environmental effect and they allow for medication of fish during adverse weather conditions.

1.2.2.1 Hydrogen peroxide

Hydrogen peroxide was first used in Canada in 1994 following sea lice outbreaks in the Bay of Fundy salmon farming regions. Its use continued up until 1998 although it had not gained full regulatory approval and was available under special permit only (Burka *et al.* 1997). It had a full product license in the United Kingdom (UK) (Pike and Wadsworth 1999) and it was used consistently in Scotland up until 1999 (Revie *et al.*

2002). It was also used in Norway from 1993 to 1997 due to problems with resistance development of sea lice towards other chemotherapeutants used at that time (Grave *et al.* 2004). However, its use in Norway and Ireland was not widespread, owing to its narrow safety margin, moderate efficacy (Grave *et al.* 2004) and the availability of more effective chemotherapeutants (Pike and Wadsworth 1999). Hydrogen peroxide is a strong oxidizing agent that acts by liberating large amounts of oxygen within treated sea lice, which results in the development of oxygen emboli in the gut and haemolymph causing sea lice to lift off the host and float to the water surface (Thomassen 1993; Burka *et al.* 1997). Hydrogen peroxide is administered as a bath treatment at concentration of 1.5 g l^{-1} for an average of 20 min. It is not recommended for use above 14°C (Thomassen 1993) as gill irritation and mortality have been shown to occur at higher water temperatures (Kiemer and Black 1997). Concentration during treatment has to be carefully monitored as it has a very narrow safety margin for Atlantic salmon. Treatment with hydrogen peroxide elicits a short-term stress effect in Atlantic salmon but physiological parameters return to normal within 24 h (Bowers *et al.* 2002). It is most effective against preadult and adult stages but it will affect some chalimus. Removal of 85 to 100% of mobile sea lice is possible at a concentration of 1.5 g l^{-1} for 20 min at 8 to 12°C (Thomassen 1993). There is evidence that the treated stages recover, but there is no evidence of them resettling on fish treated in sea cages (Treasurer and Grant 1997). Under laboratory conditions mature egg strings hatched following treatment, although in significantly reduced numbers; immature ones failed to hatch. Viable copepodids were produced from treated egg strings, although in significantly reduced numbers compared to those from sea lice on control fish (McAndrew *et al.* 1998). Hydrogen peroxide breaks

down to water and oxygen leaving a minimal environmental impact. Due to its narrow margin of safety for salmon at higher water temperatures, it could be used effectively during winter months when water temperatures and sea lice abundance are lower and the safety margin is wider (Kiemer and Black 1997). However, it is relatively expensive to use and treatment requires large volumes of the chemical (Burka *et al.* 1997) which can cause logistical problems on site. Toxicity to salmon and gill irritation increases with increasing concentration, exposure time and water temperature (Thomassen 1993, Kiemer and Black 1997), a trend which is seen in other salmonid species (Rach *et al.* 1997). It is not effective against all stages of sea lice and it is difficult to simultaneously treat all cages on site.

1.2.2.2 Organophosphates

Trichlorfon (Neguvon®), introduced in Norway in 1974, was the first organophosphorus compound developed for sea lice control (Treves-Brown 2000). It was first administered as an in-feed treatment (Brandal and Egidius 1977) and later as a bath treatment at 300 ppm for 15 to 60 min, depending on water temperature (Brandal and Egidius 1979). It is now seldom used for sea lice control (MacKinnon 1997) as it was superseded by the development of dichlorvos. Dichlorvos (Nuvan®, Aquagard®), a breakdown product of trichlorfon, that is much more toxic to fish and much less expensive was introduced in Scotland in the mid-seventies (Roth *et al.* 1993) and in Norway in 1986 (Grave *et al.* 2004). It is administered as a bath treatment at a concentration of 1 ppm for 60 min at 5 to 16°C and is successful at removing mobile stages only (Wootten *et al.* 1982), thus necessitating repeated treatments. Neither

trichlorfon nor dichlorvos is licensed for aquaculture use in Canada or the USA (Burka *et al.* 1997) although dichlorvos is registered for use in Norway and Scotland (MacKinnon 1997). Azamethiphos (Salmosan®) is the newest of the organophosphorus compounds used in aquaculture. Preliminary trials by Roth and Richards (1992) found a 1 h bath at a dose rate of 0.01 mg l^{-1} effective at removing adult and preadult stages of *L. salmonis.* It was used in the early to late nineties in Norway (Grave *et al.* 2004) and Scotland (Roth *et al.* 1996) and first used in North America in 1995 (O'Halloran and Hogans 1996). In Canada it was registered for use up until 2003 (Burridge *et al.* 2005) when a decline in its use, due to the availability and widespread use of more effective chemotherapeutants, led to the lapse of its registration. Although organophosphates were the drugs of choice for sea lice treatment in the eighties in Norway, their use declined in the late nineties due to resistance development and the availability of additional chemotherapeutants (Grave *et al.* 2004). The mode of action of organophosphates is through acetylcholinesterase (AChE) inhibition, the enzyme which catalyses the hydrolysis of acetylcholine (ACh), the neuromuscular transmitter in both vertebrates and arthropods (Treves-Brown 2000). Lowered levels of AChE result in an accumulation of ACh which ultimately leads to death of the sea louse due to overstimulation of the nervous system which is manifested by spastic paralysis (Taylor 2001). Targeted stages include mobile sea lice only. Azamethiphos is administered as a bath treatment at a dose of 0.1 ppm for 30 to 60 min. Humans and fish also have AChE so its use poses a risk to both the fish and the administrator. Oxygenation of the bath during treatment helps salmon maintain AChE levels. The efficacy of these bath treatments does not extend beyond the treatment period

because they have no residual or prophylactic action, necessitating repeated treatments (Treves-Brown 2000).

1.2.2.3 Pyrethrin and synthetic pyrethroids

Pyrethrum is an extract of naturally occurring pyrethrins obtained from flowers of chrysanthemums and African daisies that have been used administered in an oil layer on the surface of an enclosed pen (Roth *et al.* 1993). Administration involved fish having to jump through this layer. This impractical method was replaced by synthetic pyrethroids administered as bath treatments. Pyrethroids, introduced as treatments for sea lice in the early 1990's, are synthetic analogues of pyrethrins, with similar pharmacological properties (Burka *et al.* 1997), although they are more toxic and less degradable in the environment (Fallang 2005). Pyrethroids are registered for use in Norway and the UK, however, they are not registered for sea lice control in North America. The mode of action is through interference with nerve transmission by irreversibly opening sodium channels in nerve cells, inducing spastic paralysis (Arena 1995). Cypermethrin (Excis®, Betamax®) and deltamethrin (Alphamax®) are administered as bath treatments; cypermethrin at a dose rate of 0.005 mg L^{-1} for 1 h (Burridge *et al.* 2000, Treves-Brown 2000) and deltamethrin at 0.003 ml L^{-1} for 30 min (EMEA 2001). They are effective against all stages of sea lice and are very safe to use for arthropod parasites of mammals since the insect sodium channel appears to be approximately 1000 times more sensitive to these drugs (Burka *et al.* 1997). The safety margin for fish is considerably reduced and fish toxicity has been noted with the use of pyrethroids (Roth *et al.* 1993). Reduced

sensitivity of sea lice towards pyrethroids has been reported (Sevatdal and Horsberg 2003).

1.2.2.4 Chitin synthesis inhibitors

Diflubenzuron (Lepsidon®) and Teflubenzuron (Ektobann®, Calicide®) were the first in-feed treatments developed for sea lice treatment. Their mode of action is through interference with the synthesis of chitin, thereby interfering with cuticular formation (Roth *et al.* 1993). In sea lice, the demand for chitin is greatest at the moult between growth stages, making these chemicals effective against molting stages only. However, because adults have a reduced need for chitin they are not affected (Branson *et al.* 2000). Teflubenzuron is administered in feed at a dose of 10 mg kg^{-1} body weight d^{-1} up to about 7 d (Branson *et al.* 2000). Diflubenzuron is administered at a dose of 2.26 to 4.54 mg kg^{-1} for 14 d (Pike and Wadsworth 1999). Teflubenzuron has been in Norway since 1996, in the UK since 2000 (Branson *et al.* 2000), and is currently registered for use in Canada . Reported post-treatment efficacy ranged from 7 d at 12 to 15°C (Branson *et al.* 2000) to 26 d at 5.4°C (Ritchie *et al.* 2002). Diflubenzuron can bind to marine sediments and remain in the environment for a prolonged period of time (Burka *et al.* 1997; Selvik *et al.* 2002) and can be extremely toxic to marine crustaceans with effects ranging from direct mortality to indirect behavioral responses (Roth *et al.* 1993). However, the chitin-synthesis inhibitors are relatively non-toxic to mammals, resulting in short withdrawal periods prior to marketing (Branson *et al.* 2000).

1.2.2.5 Avermectins

Both ivermectin (IVM) and emamectin benzoate (EMB, the active chemical in SLICE®) are administered orally (in-feed) and are effective against all stages of sea lice (Grant 2002). Their mode of action is through irreversible binding to glutamate-gated chloride channels (GluCl), increasing neurone permeability to chloride ions at invertebrate synapses, resulting in paralysis and death (Arena *et al.* 1995). Both IVM and EMB have selective toxicity for nematodes and arthropods because they target the chloride channels in the peripheral nervous system of arthropods and helminths and the drug does not readily pass the blood-brain barrier in vertebrates (Burka *et al.* 1997).

IVM is widely used in human and veterinary medicine as an anthelmintic and ectoparasiticide (Lasota and Dybas 1991, Burka *et al.* 1997). Its use in aquaculture is limited due to its narrow margin of safety for fish, apparently due to their poorly developed blood-brain barrier (Høy *et al.* 1992, Burka *et al.* 1997). Although not registered for aquaculture use in any country, it was commonly used as an off-label prescription drug (MacKinnon 1997). It was most commonly used in smolts at a dose of 0.2 mg kg^{-1} body weight, divided into four treatments over a 2 week period (Burka *et al.* 1997). IVM was first used in the Bay of Fundy following sea lice outbreaks in 1994 but its use was superseded by the availability of EMB around 1999 through emergency drug release. It has a number of drawbacks when compared to EMB. It has a slow clearance rate from fish musculature (Høy *et al.* 1992, MacKinnon 1997), it persists unchanged in faecal material under sea cages (MacKinnon 1997) and, as noted above, has a poor safety margin in fish. Davies and Rodger (2000), have reported a high percentage of the administered dose being excreted in the feces of treated fish.

EMB is a semi-synthetic avermectin insecticide which was originally developed for pest control in edible plant crops (Lasota and Dybas 1991). It is incorporated in fish feed as a 0.2% premix (SLICE® Aquaculture Premix, Schering-Plough Animal Health) and is authorized for use in Scotland, Norway, Ireland, Iceland, the Faroes and Chile. It is currently available in Canada by emergency drug release only through veterinary prescription (Grant 2002). It is the most common drug used by Canadian farmers for sea lice control. It is approved as a pesticide in the United States and is made available for use in fish under a program similar to Canada's emergency drug release program (Health Canada 2005). It has a wide safety margin, being well tolerated by fish at 3.4 times the therapeutic dose (Stone *et al.* 1999). It is administered at an optimum therapeutic dose of 50 $\mu g\ kg^{-1}$ body weight d^{-1} for a period of 7 d (Stone *et al.* 1999) and has an extended efficacy of up to 10 weeks (70 d) post-treatment (McHenery and Johnson 2000), requiring fewer applications for effective sea lice control (Schering-Plough Animal Health 2000). The efficacy of EMB is unaffected by temperature, making it effective under a wide range of environmental conditions (water temperatures of 5 to 15°C and salinity from 23 to 35 ppt) (Schering-Plough Animal Health 2000). One drawback is the 68 day withdrawal period required (Health Canada 2005), which restrictss its use in fish ready for market. EMB is prone to overuse in Canada, owing to its advantages over currently available treatment methods and as a consequence of the limited therapies available for sea lice control.

1.2.3 Management

It is important to recognize that sea lice management practices, severity of lice infections, and strategies for control may differ amongst salmon producing countries (Heuch *et al.* 2003). Biological and chemical means of sea lice control are often used in combination to manage sea lice levels on Atlantic salmon farms, although the availability of chemicals used for sea lice control also varies by country. According to Costello (1993), there are a number of factors to be considered when deciding on a method of sea lice control. The efficacy of the treatment is important; choosing chemotherapeutants with an extended efficacy will reduce the number of treatments per year that will be required to control sea lice. It is in the best interest of the farmer to minimize stressful events during the Atlantic salmon production cycle so that growth is not impeded. This often means choosing treatment methods that are least stressful for the fish. The need for crowding of fish associated with bath treatments can be stressful. Keeping production costs low is also important to the farmer. Certain treatments are more expensive than others and the safety to the administrator and ease of application are also important considerations when deciding on a treatment. Some sea lice chemotherapeutants are neurotoxins which can be dangerous and require training and experience. The ease of application may also be important to consider; some treatments are much more labour intensive than others, limiting the ability to simultaneously treat all cages on site. Environmental effects associated with the choice of treatment are an important consideration. Discharging chemicals into the environment and the persistence of chemicals in sediment under cages, as well as effects on non-target species all have to be considered, particularly in the vicinity of other commercial aquaculture operations. The

availability of chemotherapeutants varies by salmon-producing country as not all chemotherapeutants are registered for use in each country. The effects of withdrawal time or residue limits on marketing fish and the timing of treatments is critical with market sized fish because certain treatments have mandatory withdrawal periods to ensure safety to the consumer. Certain chemotherapeutants may only target certain life stages and environmental parameters such as water temperature, water flow, salinity, pH, etc, may influence chemotherapeutic action.

1.2.4 Sea lice monitoring in Canada and Europe

The development of efficacious chemotherapeutants for treating sea lice and the adoption of Bay Management strategies have allowed for the effective year-round management and control of sea lice burdens on Atlantic salmon farms. As a result, *L. salmonis* outbreaks on cultured Atlantic salmon sea cage sites are rarely reported at present. However, sea lice will likely always be present at some level on cultured Atlantic salmon in marine grow-out sites and, as a result, they require constant year-round monitoring and control for fish health and production purposes. British Columbia (BC), Norway, Ireland and Scotland have established control programs and/or area management agreements to ensure the mandatory year-round recording and reporting on the incidence of sea lice. This is done in an effort to minimize sea lice abundance on Atlantic salmon sea cage sites and to allow for the strategic timing of treatments (Johnson *et al.* 2004, British Columbia MAFF 2005) on and between salmon farms. Sea lice monitoring and sampling methodologies vary between countries.

1.2.4.1 Canadian monitoring

There are currently no regulations for monitoring or reporting of sea lice on Atlantic salmon farms on the east coast of Canada. Due to a lack of standardized protocols for sea lice counting, monitoring methods vary between sites. Many farmers depend on their site veterinarian to conduct sea lice counts while other sites have trained personnel to conduct their own counts. Johnson *et al.* (2004) suggested that, depending on water temperature and season, treatments are often initiated when sea lice numbers exceed >5 preadults per fish and/or 1 gravid female per fish.

In BC, as a condition of license, provincial regulation stipulates the requirement for all Atlantic salmon farms to have a comprehensive Fish Health Management Plan which includes mandatory sea lice monitoring. All producers are required to adhere to BC Ministry of Agriculture, Food and Fisheries (MAFF) requirements. The sea lice monitoring program has two components: on-farm sampling and reporting by farmers based on internationally accepted protocols for sea lice monitoring, and MAFF audits to ensure validity of industry data. Monthly sampling is to be conducted on every site within each MAFF zone/sub-zone. These reports are given to the British Columbia Salmon Farmers Association for a confidential database which provides monthly reports to MAFF by specific fish health zones or areas. The action level is 3 mobile stages of lice per fish any time throughout the year, and the sampling strategy employed involves conducting lice counts on one standard and two random cages per visit. A total of 20 fish per pen are lice counted, and sea lice are categorized as *L. salmonis* adult females (with and without egg strings), mobiles (preadult and adult male/female), total chalimus and *C. elongatus*. Twice monthly sampling is required at any time throughout the year if the

number of mobile lice per fish exceeds the maximum allowable level of 3 per fish. Furthermore, action (treatment or harvest) should be taken during juvenile wild salmon out-migration (March to July) if lice levels on farms exceed 3 motile lice per fish. If broodstock are present on farms during the out-migration of wild salmon, they will also require sea lice monitoring.

Pacific salmon are also cultured on the west coast of Canada, but commercial operations are not required to follow the sea lice monitoring program as outlined for Atlantic salmon producers due to their lower susceptibility to sea lice. However, Pacific salmon producers are expected to monitor for sea lice at times when lice are observed or when they have been historically documented. If requested, such information must be available to MAFF for audit review.

1.2.4.2 Irish monitoring

The monitoring of sea lice on Irish salmon farms began in April of 1991. Lice monitoring is under the control of government scientists dictating that all farms submit to mandatory inspections 14 times per year (McMahon 2000, Copley *et al.* 2001). Inspections typically occur twice monthly from March to May. Additional inspections occur monthly for the remainder of the year, with the exception of December and January where only one inspection occurs (Copley *et al.* 2001, Jackson *et al.* 1997). The Irish methodology for sea lice sampling at each inspection involves one standard cage to be sampled at subsequent inspections, and one random cage which is chosen on the day of inspection, for each year class on site. A total of 30 fish per cage are lice counted and the mean numbers of lice per fish are calculated (Copley *et al.* 2001). These counts include

differentiation of sea lice by species and stage. *L. salmonis* and *C. elongatus* are counted and categorized according to developmental stage to include the number of mobile and gravid (egg-bearing) lice present. Treatment triggers vary from an average of 0.3 to 0.5 gravid females per fish during the critical spring period, to two gravid females per fish outside of the spring period (McMahon 2000).

1.2.4.3 Norwegian monitoring

The National Action Plan Against Salmon Lice on Salmonids, a national program for control of sea lice, was first implemented in 1997 by the Norwegian Animal Health Authorities (AHA), in co-operation with Directorate of Fisheries, Directorate of Nature Management, fish farmer's organization and private fish health services (Eithun 2000). The purpose of the program is to establish minimum measures to reduce the incidence of sea lice on farmed and wild fish. The objectives of the National Action Plan include the establishment of regional working groups to plan and coordinate sea lice control efforts, the compulsory reporting of lice numbers and lice treatments from all farms to the AHA, and the monitoring of sea lice on sea trout and running Atlantic salmon smolts (Heuch *et al.* 2005). In 2000, the "Regulation of the fight against sea lice" was established. The purpose of this regulation was to establish minimum measures of sea lice in an effort to reduce the damage to wild and farmed Atlantic salmon. These guidelines are continually under review and subject to change. Currently, under this regulation, farmers are required to conduct biweekly sea lice sampling at sea temperatures ≥ 4 °C. Mandatory delousing must occur between 1 November to 1 July if adult female numbers exceed 0.5 per fish, or 5 or more adult female and mobile stages per fish. Mandatory delousing must

also occur between 1 July to 1 November if adult female lice numbers exceed 2 per fish, or 10 or more adult females or mobile stages (McKinley *et al.* 2002). Administrative fines may be incurred if regulations are violated. At each sampling, one random cage and one standard cage is sampled and a minimum of 20 fish from each cage is counted. Sea lice are categorized as adult female lice with and without eggs, preadult stages and adult male lice and chalimus stages. Farmers are required to record and submit a monthly count of gravid and non-gravid adult female sea lice numbers to the District Veterinary Officer, as well as the number of treatments given on each site, and information on the use of wrasse.

1.2.4.4 Scottish monitoring

In 1998 the Scottish Salmon Growers Association published its Code of Practice on a National Treatment Strategy for the Control of Sea Lice on Scottish Salmon Farms (Anon 1998). Although there were no official action levels set, management agreements are in place to encourage the monitoring of sea lice at agreed intervals using standard protocols, and to coordinate the strategic timing of treatments (Rae 2000, Rae 2002). As a result, most farms in Scotland have implemented routine methodologies for the assessment of sea lice abundance (Revie *et al.* 2002). A common strategy employed by Scottish researchers involves conducting lice counts weekly from April to October and biweekly throughout the remainder of the year (Revie *et al.* 2002, Revie *et al.* 2003). Their sampling strategy for number of cages and fish per cage to be sampled is based on the descriptions by Treasurer and Pope (2000). This involves sampling five fish per cage from two to six cages, depending on the production capacity of the site. *L. salmonis*

counts are categorized by developmental stage to include chalimus, preadult, adult male/female and gravid female and total adult *C. elongatus* counts are also included.

1.2.4.5 Monitoring in other countries

There is currently no published information on sea lice monitoring in other Atlantic salmon producing countries such as Chile and Japan.

1.3 What is resistance?

Resistance evolves through a process of natural selection (Devine *et al.* 2000). It is defined as 'an increase in the quantity or dose rate of a chemotherapeutant required to elicit a given response due to a change in gene frequency in a population of the gene(s) that control susceptibility' (Schering-Plough Animal Health 2000). Resistance of sea lice to chemotherapeutants used for their control is likely to develop when chemotherapies are used exclusively without rotation of alternative control methods with differing modes of action. Intensive use of a chemotherapeutant will select for those individual sea lice in the population that are capable of surviving treatment. Survivors are genetically and physiologically resistant to the treatment and will reproduce and pass on the genes conferring resistance to their offspring (Prichard 1994). Further treatments of subsequent sea lice generations containing the genes for resistance will progressively select for resistance in the sea lice population.

It is possible that resistance may go undetected until it has reached a high level because these processes can continually occur to some extent with each successive sea lice treatment, although the chemotherapeutant may appear to maintain its effectiveness (Prichard 1994). Early indications of changes in the sensitivity of a sea lice population

towards a chemotherapeutant may be an increase in the number of treatments per production cycle or the shortening of intervals between treatments, as well as the requirement for higher doses of a chemotherapeutant to control sea lice at the appropriate threshold (Schering-Plough Animal Health 2000). When resistance has reached a high enough level to be detected, the effectiveness of the chemotherapeutant may already be compromised.

The inability of a chemotherapeutant to significantly suppress sea lice infections on farmed fish may lead to problems with production, especially in areas where limited chemotherapeutic options are available. Therefore, it is imperative to prolong the useful life of the chemotherapeutants available for sea lice control. There are a number of simple practices that can be followed. Avoiding the unnecessary use of sea lice treatments will reduce the risk of resistance development, as frequent treatments will naturally intensify selection pressures. For chemotherapies administered as a bath or orally (in-feed), it is important that the farmers administer the correct dosage for the prescribed medication period (without interruption) to avoid under- dosing and exposing fish and sea lice to non-therapeutic drug concentrations. Specifically for in-feed treatments, careful feeding practices to monitor feed consumption will improve the chances of fish receiving the therapeutic dose. This can be challenging in sea cage sites as large fish may eat more than small fish so it may be necessary to alter feeding practices to ensure smaller fish receive the required dose. In-feed products should only be used when fish are feeding actively. They should not be administered when fish are suffering from disease, if they are stressed, or under any other circumstance which affects their ability to feed normally.

The strategic rotation of chemotherapeutants with different modes of action will aid in prolonging resistance development by avoiding continuous selection for the same potential resistance mechanism. Simultaneous treatment of all fish on site will decrease the movement of sea lice from treated to untreated cages and the coordination of treatments between all farms in a bay system will be beneficial to prevent sea lice from spreading to untreated farms.

1.3.1 Treatment failures

There are a number of factors that can contribute to treatment failures in the field, therefore a treatment failure should not automatically be equated with resistance development without ruling out extenuating circumstances. Exposure of fish to sub-lethal or non-therapeutic concentrations of a drug administered in-feed may result in a treatment failure. For example , intentional under-dosing to save money, poor quality of feed preparation, failure to consider host health, natural hierarchies within a cage, and too short a treatment period could result in fish being exposed to non-therapeutic or sub-lethal concentrations of a drug administered in-feed. Inaccurate calculations of cage biomass could lead to inaccurate dose calculations. Cage biomass is an important factor in determining the amount of the drug to be fed to a cage, as dosages are based on a per kg body weight per day amount. Errors in cage biomass may result in over or under dosing fish. Failure to consider host health may lead to a treatment failure such that stressed or diseased fish may be off feed, making it difficult to ensure that each fish receives therapeutic concentrations of the drug. Natural hierarchies within a cage may result in variable feed intake between fish within a cage; larger, more aggressive fish may

receive an overdose of drug while small, subordinate fish may be under-dosed due to an inadequate consumption of feed. In the case of bath treatments, treatment failures may occur if the exposure time is too short, the water volume of the netpen is incorrectly calculated, the chemotherapeutant is insufficiently mixed in the netpen, there is insufficient oxygenation during treatment, there is an insufficient reduction of pen volume during treatment or there is an improper use of tarpaulins or skirts resulting in a leaking of the product (SEARCH 2004).

1.3.2 Resistance development in sea lice

When the availability of chemotherapeutants for sea lice control is limited, the development of resistance by sea lice to those chemotherapies is a concern. The success of several groups of chemotherapeutants used for sea lice control have been compromised due to changes in sea lice sensitivity and reports of resistance development. Variations in sea lice sensitivity to the organophosphates dichlorvos and azamethiphos on Scottish (Jones *et al.* 1992, Roth *et al.* 1996) and Irish (Tully and McFadden 2000) Atlantic salmon farms have been reported. Reduced sensitivity to the pyrethroids deltamethrin (AlphaMax®) and cypermethrin (Betamax®, Excis®) has been demonstrated in sea lice from Norway, Scotland and Ireland but it does not currently pose a problem with regards to the ability of these compounds to maintain sea lice control (Sevatdal *et al.* 2005).

The mechanisms most likely responsible for sea lice resistance to organophosphates and pyrethroids, which have been identified in other arthropods, are target site insensitivity resulting from structural changes to the target site (i.e. the presence of an altered AChE in cholinergic synapses of organophosphorous resistant

arthropods and knock down resistance against pyrethroids caused by structural changes in the voltage-gated sodium channel proteins of nerve membranes), metabolic detoxification due to the enhancement of detoxification systems (i.e. esterases, glutathione S-transferase, or cytochrome P450 (CYP)-dependent oxidation), and to a lesser extent reduced cuticular penetration (Scott 1990, Sevatdal 2005, Fallang *et al.* 2005). Sea lice resistance to hydrogen peroxide has been demonstrated from trials in Scotland (Treasurer *et al.* 2000). Suggested mechanisms of sea lice resistance to hydrogen peroxide include the genetic selection of individuals with cuticles that provide a barrier to hydrogen peroxide penetration and the presence of detoxifying enzymes (Treasurer *et al.* 2000). There are no documented reports of sea lice resistance to the chitin synthesis inhibitors, teflubenzuron or diflubenzuron, or to the avermectin, EMB. The underlying resistance mechanisms towards these chemotherapeutants are less well established but are most likely based on some form of increased detoxification or target site modification (SEARCH 2004). Reports of reduced sensitivity and resistance of sea lice towards these chemotherapeutants necessitates the development of resistance management strategies.

1.3.3 Anthelmintic and insecticide resistance

Nematode and arthropod infections are a medical and veterinary health problem in humans, domestic and livestock animals, and in some instances, in commercially cultured fish species. The AVMs are of major importance for antiparasitic and antipest control against nematode (worms), acarine (ticks and mites) and insect targets (Shoop 1995). The AVMs represent one of the three main groups of currently available anthelmintics used to control parasitic nematodes of cattle and sheep (Taylor *et al.* 2002).

In humans, IVM has played a key role in control of filarial nematodes, such as *Onchocerca volvulus*, which causes onchocerciasis (river blindness); and *Wuchereria bancrofti*, which causes lymphatic filariasis or elephantiasis (Prichard 2005). IVM is also important in the control of canine heartworm, *Dirofilaria immitis*. The AVM, EMB, which is used in commercially cultured anadromous fish species to treat sea lice infestations (Stone *et al.* 2000, Ramstad *et al.* 2002), was originally developed for use as an insecticide for controlling field crop pests (Ishaaya *et al.* 2002).

Antiparasitic resistance mechanisms vary among invertebrates and may include, but are not limited to: (i) target site insensitivity; (ii) a change in metabolism that inactivates or removes the drug, or that prevents its activation (eg. penetration, excretion, metabolism, detoxification); (iii) a change in the distribution of the drug in the target organism that prevents it from reaching the target site; or (iv) amplification of target genes to overcome drug action (Wolstenholme *et al.* 2004).

1.3.3.1 Anthelmintic resistance detection

Anthelmintic resistance is widespread in nematode parasites of sheep, goats and horses and is developing in nematode parasites of cattle and pigs (Prichard 1994). As previously stated, the AVMs affect nematodes through their action on ligand-gated channels, including GluCl and GABACl chloride channels. These are a family of receptors that regulate feeding, locomotion and reproduction in nematodes. Mechanisms of anthelmintic resistance vary between nematode species (Wolstenholme *et al.* 2004) and involve several mechanisms which include changes to the drug's target site (Kwa *et al.* 1995), increased enzymatic detoxification, and increased drug efflux by membrane

transport proteins such as P-glycoprotein (Blackhall *et al.* 1998, Xu *et al.* 1998, Kerboeuf *et al.* 2003).

Conventional methods of resistance detection in nematode populations involve a variety of *in vivo* and *in vitro* tests aimed at measuring the effects of anthelmintics on physiological processes such as development, growth and movement (Várady and Čorba 1999); each method has drawbacks in terms of either cost, applicability, reliability, reproducibility, sensitivity and ease of interpretation (Várady and Čorba 1999, Taylor *et al.* 2002). The faecal egg count reduction test (Taylor *et al.* 2002) is the most common *in vivo* method, while egg hatch assays and larval paralysis assays (Taylor *et al.* 2002), motility tests (Bennett and Pax 1986, Várady and Čorba 1999, Taylor *et al.* 2002), migration assays (Petersen *et al.* 2000), and larval and adult development tests (Stringfellow 1986, Várady and Čorba 1999) are common *in vitro* methods. There are also a number of biochemical and molecular tests available for resistance detection in nematodes. The development of molecular techniques for the diagnosis of resistance offers a number of advantages over conventional means. Molecular methods are highly specific and sensitive even with small quantities of DNA (Sangster *et al.* 2002); they are also rapid, highly reproducible and inexpensive (Álvarez-Sánchez *et al.* 2005). Most molecular methods of resistance detection are based on the polymerase chain reaction (PCR) (Elard *et al.* 1999, Silvestre and Humbert 2000). This technique is advantageous in that it allows for the simultaneous processing of large numbers of samples and the detection of emerging resistant alleles; conventional methods cannot detect resistance until it is well established in a population (Silvestre and Humbert 2000). PCR assays are currently only available for the detection of resistance to benzimidazoles (Elard *et al.*

1999, Silvestre and Humbert 2000, Coles 2005, Álvarez-Sánchez *et al.* 2005) as the molecular basis of resistance to other anthelmintic drugs such as IVM remains largely unknown.

1.3.3.2 Arthropod resistance

Resistance has developed to every chemical class of insecticide, including antimicrobial drugs and insect growth regulators (Brogdon and McAllister 1998). In arthropods, resistance mechanisms vary by species. Two major forms of biochemical resistance include target site resistance and detoxification enzyme-based systems (Denholm and Rowland 1992, Clark *et al.* 1994, Ahammad-Sahib *et al.* 1994, Brogdon and McAllister 1998, Liu and Yue 2001) (i.e. CYP-dependent monooxygenases, hydrolases, or GST). Additional mechanisms include delayed or decreased cuticular penetration (Denholm and Rowland 1992) and drug efflux by membrane transport proteins (i.e. P-glycoprotein) (Srinivas *et al.* 2004).

A number of established bioassays and biochemical assays have been used to detect resistance development in arthropods. No one technique is likely to be adequate for all chemicals used against any given species of arthropod. The currently available methods include *in vivo* assays on intact test subjects involving varying degrees of exposure to the test compound, and *in vitro* biochemical tests that typically assess enzyme activity or the activity, quantity or nature of DNA coding for specific resistance genes (*f*french-Constant and Roush 1990).

In vitro bioassay techniques have historically been used as an initial means of resistance detection, and are advantageous in that knowledge of the resistance mechanism

is not required. However, they are insensitive to small changes in resistance frequency. They typically involve exposure of large numbers of test subjects to a fixed range of test compound concentrations and exposure times in an effort to determine LD_{50}, LD_{90} or EC_{50} values. Exposure of the test subjects to the drug or pesticide being tested is usually via immersion, residue or surface contact, or topical application (*f*french-Constant and Roush 1990). Comparing the slopes of dose-response curves between field and laboratory populations may provide an indication of changes in sensitivity of a field population towards a compound used for their control.

Biochemical assays allow for the detection of resistance at much lower frequencies through the monitoring of resistant genotypes (*f*french-Constant and Roush 1990) and enzyme activity (Brogdon *et al.* 1997, Baxter *et al.* 1999) within the host and host population. Biochemical assays often use model substrates to detect enzyme activity in pooled and individual insect homogenates, while molecular techniques, such as PCR, are used to detect point mutations that cause target-site resistance or changes in detoxification enzyme specificity (Brogdon and McAllister 1998).

1.3.4 Resistance detection in sea lice

A conventional means of detecting insecticide resistance has been by bioassay (Brogdon 1989). A bioassay is an experiment in which a living organism is used as a test subject to quantify the response or responses of the subject to an agent or stimulus (Hubert 1980, Robertson and Preisler 1992). Bioassays have the advantage of being able to detect changes in the sensitivity of a population where the biochemical basis of resistance is not clearly understood. This makes them useful tools for monitoring

changes in sensitivity of sea lice towards newly introduced chemotherapeutants, such as emamectin benzoate, whose underlying resistance mechanisms are not well established, as resistance has not yet been reported. Previously established bioassays have been successful at detecting changes in sea lice sensitivity towards pyrethroids (Sevatdal and Horsberg 2003, Sevatdal *et al.* 2005).

Biochemical assays are advantageous due to their ability to diagnose qualitative or quantitative differences in enzymes between individual susceptible and resistant insects. These methods offer a faster and more sensitive method of resistance monitoring (Denholm 1990). Biochemical assays were used by Fallang *et al.* (2004) to provide evidence for the occurrence of an organophosphate-resistant type of AChE in strains of L. *salmonis*. Sevatdal *et al.* (2005) used a combination of bioassays and biochemical assays to provide evidence of monooxygenase-mediated pyrethroid detoxification in *L. salmonis*.

Molecular techniques for resistance detection in sea lice have recently been used to identify a point mutation in the sodium channel gene of pyrethroid-resistant *L. salmonis* (Fallang *et al.* 2005). Such techniques offer the potential for the development of diagnostic tools to identify mutations in gene sequences responsible for target site resistance in individual sea lice. This would allow for the quick and early detection of resistance in different sea lice populations.

Although resistance of sea lice to EMB has not yet been reported in the field, its widespread use for sea lice control in the aquaculture industry of Canada (Chapter 2), coupled with the development of nematode and, particularly, arthropod resistance to

AVMs in domestic and livestock animals, raises concerns that sea lice may develop resistance to EMB.

1.4 Current investigation

1.4.1 The problem

Sea lice resistance to chemicals used for their control, such as the organophosphates and pyrethroids, has previously been documented, although changes in sea lice sensitivity to pyrethroids do not currently pose a threat to chemical control. Changes in sea lice sensitivity and resistance development are likely due to the over-use of the limited number of therapeutic options available for use in each country. Prolonging the useful life of the limited number of control methods that are currently available is imperative for the future control of sea lice. Proper monitoring of all stages of sea lice is necessary to ensure the strategic timing of treatments and to reduce unnecessary treatments in during the Atlantic salmon production cycle. Regular monitoring for sea lice abundance on farmed fish before and after treatment represents an important component of sea lice management as it can provide an early indication of problems with chemical control, should they arise.

Standardized methods and mandatory counting and treatment now comprise an effective component of the multitactic sea lice management strategies employed by Atlantic salmon producing countries such as Norway, Ireland and Scotland. British Columbia has recently developed a mandatory monitoring strategy, but the east coast of Canada lacks such a plan. Although there have been no reports of sea lice resistance development to emamectin benzoate on Atlantic salmon farms, its widespread use in

countries such as Canada necessitates the development of standardized tools and protocols to monitor for changes in sea lice susceptibility towards the chemotherapeutant. Bioassays have been developed to monitor sea lice sensitivity towards pyrethroids, which are used extensively in Norway, and changes in sensitivity have been detected by these methods. For this reason, the development of bioassays for monitoring sea lice sensitivity to EMB is a promising research tool, as no such methods currently exist.

Up-regulation of metabolic oxidative pathways has been identified as one method by which arthropod resistance to xenobiotics has developed. Among the most important detoxifying enzymes identified are the mixed-function, or CYP, oxidases (also termed oxygenases or monooxygenases). Haem-peroxidase (or non-specific monooxygenase) testing was originally developed for the agriculture industry to detect the differences in oxidase levels between individual susceptible, resistance, or induced mosquitoes. This method has been used in combination with bioassays recently in Norway to identify monooxygenase mediated pyrethroid detoxification in sea lice (Sevatdal *et al.* 2005). Monooxygenase testing of individual sea lice may provide a method by which individual sea lice with elevated oxidase levels may be detected. Elevated monooxygenase levels may indicate a decrease in sea lice sensitivity towards treatment if oxidative metabolism is a mechanism involved. This non-specific method could then be used in combination with established bioassays to detect changes in sea lice sensitivity towards chemotherapeutants prior to resistance development.

1.4.2 Specific objectives

1) To describe sea lice management on the east coast of Canada in the absence of standardized methods for monitoring sea lice abundance on Atlantic salmon production sites.
2) To develop and optimize field bioassays to monitor for changes in sea lice sensitivity toward EMB. Previously established bioassay methods were used to develop a bioassay protocol for sea lice and EMB. The established protocol was used to determine if sensitivity to EMB from field samples of sea lice varied by year, region, or season. Bioassay optimization included an evaluation of inter-rater agreement and gender related differences in EMB susceptibility.
3) To optimize and use monooxygenase testing to describe enzyme activity in field collected sea lice populations with exposure to EMB. This method may have the potential to be used in combination with bioassays to monitor for changes in sea lice sensitivity towards EMB in field samples of sea lice. The use of this test for sensitivity monitoring is based on the hypothesis that sea lice have the ability to oxidatively metabolize EMB. Sea lice were individually assayed to determine overall monooxygenase levels in an effort to establish base-line data of enzyme activity in the absence of EMB resistance development. Assay optimization included an evaluation of the effect of the following on haem peroxidase levels: (i) freezing, (ii) storage, (iii) condition and exposure of sea lice to varying concentrations of EMB, and (iv) post-mortem enzyme stability.

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Chapter 2 Sea lice treatments, management practices and sea lice sampling methods on Atlantic salmon farms in the Bay of Fundy, New Brunswick, Canada

2.1 Abstract

Proper monitoring of all stages of sea lice is imperative to ensuring the strategic timing of treatments. Although sea lice receive close attention and management for production purposes, there are no regulations for the reporting of lice burdens on salmon farms in Atlantic Canada, nor are there officially standardized protocols for conducting sea lice counts in the field. The purpose of this study was to describe methods for sea lice sampling, types of treatments administered for control of sea lice, and management practices and decisions regarding the control of sea lice through a survey of Atlantic salmon farms in the Bay of Fundy, New Brunswick, Canada. A total of 83 surveys were completed from July to December 2002. Surveys were completed on 30 sites from the Lime Kiln Bay and surrounding areas, 23 sites from Grand Manan Island, 18 sites from Deer Island, 6 sites from Campobello Island and 6 sites from Passamaquoddy Bay. Fish farmers, in concert with their veterinarians, generally perform intensive monitoring and control practices. However, there is a continued reliance on emamectin benzoate (EMB; active ingredient in SLICE®) for sea lice control on Atlantic salmon farms in the Bay of Fundy, which raises concerns regarding the potential for sea lice to develop resistance to the drug.

2.2 Introduction

In 2001, Atlantic salmon production in New Brunswick was estimated at 33,900 tonnes, or 94% of total aquaculture (finfish and shellfish) production for the province

(CAIA 2003). Currently, there are approximately 94 marine production sites located in the Bay of Fundy, New Brunswick, and the majority of farms are owned and/or operated by companies that have one or more sites in the area (CAIA 2003).

Sea lice are ectoparasitic crustaceans that pose an on-going management issue for the health and productivity of commercially farmed Atlantic salmon *Salmo salar* throughout eastern North America and northern Europe (Costello 1993, Treasurer and Pope 2000). Two major species, *Lepeophtheirus salmonis* and *Caligus elongatus*, infest Atlantic salmon in the Bay of Fundy. *L. salmonis* is larger and more aggressive than *C. elongatus*, and can cause pathological damage to the host. High economic costs are incurred by the salmon industry in Atlantic Canada due to the continual need for monitoring and control of sea lice.

Although there are no documented or confirmed cases of treatment failures with emamectin benzoate (EMB, SLICE®; Schering-Plough Animal Health, Pointe Claire, QC) or of sea lice resistance to the chemotherapeutant, there is a concern that reduced sensitivity will develop due to the widespread use of the chemotherapeutant in the industry. Proper monitoring of all developmental stages of sea lice, particularly for *L. salmonis*, is imperative to ensuring the strategic timing of treatments. In Atlantic Canada, there are no regulations for the reporting of sea lice on Atlantic salmon farms, nor are there officially standardized protocols for conducting sea lice counts in the field. This survey was conducted in an effort to describe the different methods of sea lice sampling employed on Atlantic salmon farms in the Bay of Fundy, and to describe the types of treatments being used and the factors that are taken into consideration when deciding to treat for sea lice. To the authors' knowledge, this is the first survey of its kind conducted

on Atlantic salmon farms in the Bay of Fundy that focuses on sea lice sampling methods and treatments.

2.3 Materials and Methods

2.3.1 Subjects

Farm owners of Atlantic salmon sea cage sites that had stocked fish in 2000 and 2001 in the Bay of Fundy were asked to participate. Respondents representing eighty-three of the approximately 94 sites in operation in the Bay of Fundy at the time were surveyed. Individuals were initially contacted by telephone and interviews were arranged at the convenience of the interviewee.

2.3.2 Survey design

Each survey was 3 pages in length, and included a total of 25 questions. This survey was inserted into a larger survey which was designed to evaluate environmental and management risk factors for infectious salmon anaemia (ISA) virus outbreaks in farmed Atlantic salmon (McClure *et al.* 2005; Appendix C). The survey was pre-tested with two farm managers prior to the interviews, and the necessary amendments were made. It was deemed most efficient to incorporate both surveys into one and thereby minimize the time commitments of the industry participants. Once the survey was completed, the data for the sea lice components were extracted and analyzed separately. All results reported in this paper concern the sea lice components of the survey.

All questions included in the survey were in closed-end format, either requiring selection from a list of possible choices or a yes/no/uncertain and/or not applicable

response. Space was also available for additional comments. The following areas of concern were addressed in the survey: (1) site characteristics; (2) information on the site veterinarian and the frequency of veterinary visits; (3) methods of sea lice sampling used; (4) methods used for sea lice counting and classification; (5) management practices and policies regarding decisions to treat for sea lice; and (6) types of sea lice treatments administered over the past two years.

2.3.3 Survey implementation and data management

A total of 83 surveys were prepared. Six individuals were trained to administer the survey. Interviews began in July 2002, and all interviews were completed by December 2002. As this questionnaire was a component of a larger ISA risk factor questionnaire, to avoid over-burdens of time commitments by site managers, the first responses were used as an assessment of the survey questions and questions were then modified or added in the remaining surveys to clarify responses. This resulted in lack of responses to certain questions that were categorized as 'not applicable' as opposed to 'uncertain' to distinguish the fact that the questionnaire did not include those particular questions at the time of the interview. Follow-up questions were asked to address the deficient responses. However, participants had lower response rates on follow-up questions due to other time commitments. Interviews were done in person, when possible, or by telephone. Twelve of the individuals surveyed owned and/or operated more than one site and completed a survey for each site at the time of the interview.

All questions were coded and entered into Excel by one individual. Statistical analysis was completed with Intercooled STATA 7.0 software (Stata Corporation, College Station, TX, USA) to generate descriptive statistics.

2.4 Results

Survey information was collected from July to December 2002 on a total of 83 Atlantic salmon sea cage sites. The respondents included 44 site managers, 3 site owners, 4 production managers, 8 individuals who were both site owner and manager, and one site veterinarian. Surveys were completed on 30 sites from Lime Kiln Bay and surrounding areas, 23 sites from Grand Manan Island, 18 sites from Deer Island, 6 sites from Campobello Island, and 6 sites from Passamaquoddy Bay.

2.4.1 Site veterinary visits

The large majority of respondents (90.4%; n=75) reported regular visits by the site veterinarian, and 9.6% (n=8) reported reason-specific visits. Most sites (77.1%; n= 64) have veterinary visits at least once per month (Table 2.1). 13.3% (n=11) reported visits every six to nine weeks; 8.4% (n=7) were visited only when called. 1.2% (n=1) of respondents reported some other frequency of veterinarian visits. The 'other' category included bringing in samples to the site veterinarian as needed.

2.4.2 Sea lice sampling

Most sites conduct lice counts either on a weekly (26.5%; n=22) or monthly (26.5%; n=22) basis (Table 2.1). 10.8% (n=9) of respondents reported conducting

biweekly counts and 36.1% (n=30) conducted lice counts at some other frequency. This 'other' category included responses such as lice counts weekly in the summer and biweekly in the winter, lice counts biweekly in the summer and once a month in the winter, or lice counts weekly in the summer and once a month in the winter. Other responses included lice counts every six weeks, every three months, 2-3 times per year when needed, only when lice were seen on the fish, and counts are done as needed for a SLICE® prescription. During summer months when lice burdens are of greatest concern, 48.19% (n=40 sites) of sites conducted at least biweekly counts.

Lice counts require that cages be selected to represent the overall lice burdens at the site (Table 2.2). This often involved sampling all the odd numbered cages on a site during one visit and all the even numbered cages on the next visit. Respondents for 60.2% (n=50 sites) of sites reported that purposefully selected cages where sampled and 34.9% (n=29) that every cage was counted, leaving 4.8 % (n=4) of sites using some other strategy. The 'other' category included monitoring every cage when fish are small and every 3 cages when fish are large, 75% of cages on site and 5-28 cages per visit. Of the 34.9% (n=29) of respondents reporting monitoring of every cage on site, one reported counting all cages when conducting lice counts but did not report how many cages were on site. Of the remaining 28 respondents which reported counting all cages on site, 10.7% (3 of 28) had an average total of less than 5 cages on site, 25.0% (7 of 28) had 6-10 cages on site, 39.3% (11 of 28) had 11-15 cages on site, 14.3% (4 of 18) had 16-20 cages on site, and 10.7% (3 of 28) of sites who count all of their cages when doing lice counts had 21+ cages on site and a minimum of 5-10 fish per cage were sampled when conducting lice counts (Table 2.3). In total, 50.6% (n=42 sites) of sites sampled 5-10 fish

per cage, whereas others sampled over 50 fish per cage. Respondents for 4.8% (n=4 sites) of the sites surveyed were uncertain as to the number of fish sampled per cage. This may be due, in part, to the fact that the site veterinarian was the individual who sampled fish for sea lice.

The majority of sites (57.8%; n=48 sites) anaesthetized their fish when conducting lice counts, whereas 25.3% (n=21 sites) of respondents reported that they did not. Meanwhile, 15.7% (n=13 sites) of respondents reported that the decision to anaesthetize fish when conducting lice counts depended on the size of the fish. Typically, smaller fish were anaesthetized when conducting lice counts and larger fish were not. One of the respondents surveyed was uncertain as to whether or not fish were anaesthetized when conducting lice counts because the site veterinarian was the individual who conducted the counts. Although there were 48 respondents that reported anaesthetizing fish when conducting lice counts, there were 54 that reported using tricaine methanesulfonate (TMS, Syndel Laboratories) (44 of these reported using TMS on all fish and 10 respondents were from the group that reported that the decision to anaesthetize fish when conducting lice counts depended on the size of the fish and they reported using TMS only on small fish). Thus, TMS was used at most sites (65.1%; n=54) (Table 2.4). Respondents for 4.8% (n=4) of sites reported the use of clove oil, while 2.4% (n=2 sites) of respondents reported being uncertain as to what type of anesthetic was used to anaesthetize fish when conducting lice counts.

2.4.3 Sea lice counting and categorization

Many sites (51.8%; n=43) had a staff person on site who was trained to identify the various life stages of lice (Table 2.5), whereas 24.1% (n=20) of respondents reported that they did not. On sites not having trained personnel, either the site veterinarian or their assistant conducted lice counts, or the sea lice burdens at those sites were historically low enough that sea lice counts were not formally conducted. In such a case, trained personnel were not required because a general uncategorized lice count was performed in order to obtain general lice numbers only. Only one respondent reported that they were uncertain as to whether or not there was a person on site who was trained to identify lice stages and 22.9% (n=19) of sites were in the 'not applicable' category because this particular question was not in the survey at the time of their interview and they did not answer it on follow-up interviews.

A large proportion of sites (94.0%; n=78) categorized lice by life stage when conducting lice counts, 4.8% (n=4) did not, and one respondent was uncertain. The proportion of respondents reporting the differentiation of *L. salmonis* copepodid, chalimus, mobile (preadult and adult) and gravid female stages and *C. elongatus* are presented in Table 2.6. 7.2% (n=6) of respondents reported that they counted and recorded copepodid numbers when conducting lice counts and 89.2% (n=74) did not. Nearly all sites counted and recorded chalimus (90.4%; n=75) numbers and only 6.0% (n=5) did not. 91.6% (n=76) of sites counted and recorded mobile numbers when conducting lice counts but 4.8% (n=4) did not. Most sites (89.2%; n=74 sites) recorded gravid female numbers, and a large proportion of sites (74.7%; n=62) counted and

recorded *C. elongatus* numbers when conducting lice counts. *C. elongatus* counts were not differentiated by stage.

2.4.4 Sea lice treatments

The majority of respondents indicated that sites based their decision to treat for sea lice upon a combination of factors that included lice counts, general fish appearance, recommendation by the site veterinarian and an expected problem (Table 2.7). An expected problem meant that site managers anticipated a lice problem based on a combination of environmental factors (ie. water temperature) and present or historical lice burdens for their site at a particular time of year. However, 48.2% (n=40) of sites based their decision to treat for sea lice upon lice counts alone; 15.7% (n=13) upon a recommendation by their site veterinarian; 8.4% (n=7) upon the general fish appearance; and 6.0% (n=5) upon an expected problem. The remainder used a combination of criteria.

The site owner was most frequently (34.9%; n=29) the person who made the final decision to treat for sea lice (Table 2.8). Other decision makers included the site veterinarian (31.3%; n=26) and site manager (14.5%; n=12). Some other person, including the general manager, aquaculture manager, site contractor or the technical manager made the final decision to treat for sea lice on 10.8% (n=9) of the sites. 8.4% (n=7) of respondents reported that the final decision to treat for sea lice was made by some combination of site manager, site owner and site veterinarian.

Respondents indicated that 91.6% (n=76) of sites had treated for sea lice at least once in the past two years, 6.0% (n=5) that they did not, while 2.4% (n=2) were uncertain

as to whether or not their site was treated for sea lice over the past two years. The 2 uncertain respondents were individuals who did not work on the site two years previous to the interview and as a result, could not accurately comment on this question.

Respondents were also asked what types of sea lice treatments had been administered on their farms in the past two years. The results for this question total more than 100% because several respondents reported using more than one type of sea lice treatment on their site in the past two years. A large majority, 90.4% (n=75), reported using an in-feed treatment to combat sea lice; 13.3% (n=11 sites) a bath treatment, and 4.8% (n=4) that no sea lice treatments had been administered on their site in the past two years.

EMB was the most frequent agent used to treat smolts (71.1%; n=59 sites) (Fig. 2.1). 8.4% (n=7) of respondents reported using ivermectin (22, 23-Dihydroavermectin B_1); 4.8% (n=4) reported using some other means of lice control; 3.6% (n=3) reported being uncertain as to the method of sea lice control used to treat smolt, and 12.1% (n=10) fell into the 'not applicable' category because this question was not answered. EMB was also used most frequently to treat premarket salmon (78.3%; n=65) (Fig. 2.1), whereas 3.6% (n=3) of respondents reported using azamethiphos (Salmosan®; Novartis, Mississauga, ON), 3.6% (n=3) were uncertain as to the method of sea lice control used, and 12.1% (n=10) fell into the 'not applicable' category because this question was not answered or was not in the survey at the time of the interview.

Respondents were asked about the average time to first treatment for sea lice after their current year class of salmon had been transferred to saltwater. A large percentage of people interviewed did not have access to the appropriate records to permit an accurate

answer to this question (Fig. 2.2), so 30.1% (n=25) of respondents were uncertain as to the time to first treatment for sea lice after saltwater transfer. Of the respondents who were able to answer this question, 26.5% (n=22) reported treating salmon for sea lice on average 5-8 weeks after saltwater transfer, 8.4% (n=7) of sites treated earlier, i.e.1-4 weeks after saltwater transfer, and the remainder treated anywhere from 9 weeks to greater than 6 months post-transfer. Another 7.2% (n=6) fell into the 'not applicable' category because the question was not in the survey at the time of the interview.

2.5 Discussion

2.5.1 Sea lice monitoring

Across salmon producing countries such as Canada, Norway, Scotland and Ireland, there are many differences in management practices, severity of lice infections, strategies and chemotherapeutants registered for sea lice control, and perceptions of environmental damage caused by lice (Heuch *et al.* 2003). This may result in a range of strategies and management practices regarding sea lice monitoring and control.

In Atlantic Canada, there are no regulations for the reporting of sea lice on Atlantic salmon farms, nor are there officially standardized protocols for conducting sea lice counts in the field. The results of this survey indicate that the methods of sea lice sampling being followed by the majority of New Brunswick fish farmers, in concert with their veterinarians, coincide with those of Europe (Copley *et al.* 2001, Treasurer and Pope 2002). In summary, most New Brunswick salmon farms conducted lice counts weekly during periods of high lice burdens; cages selected for lice counts were strategically chosen to provide usable information for control decisions; and a sample size of 5 to 10

fish per cage was the most common practice. Furthermore, the majority of fish farms anaesthetized their fish with TMS when conducting lice counts and most sites recorded the frequency of *L. salmonis* chalimus, mobiles and gravid female stages and *C. elongatus*.

In British Columbia, as a condition of license, provincial regulation stipulates the requirement for all Atlantic salmon farms to have a comprehensive Fish Health Management Plan which includes mandatory sea lice monitoring. All producers are required to adhere to BC Ministry of Agriculture, Food and Fisheries (BCMAFF) requirements of monthly sampling on every site within each MAFF zone/sub-zone. The action level is 3 mobile stages per fish any time throughout the year, and the sampling strategy employed involves conducting lice counts on one standard and two random cages per visit. A total of 20 fish per pen are lice counted and sea lice are categorized as *L. salmonis* adult females (with and without egg strings), mobiles (preadult and adult male or female), total chalimus and *C. elongatus*. Twice monthly sampling is required at any time throughout the year if the number of motile lice per fish exceeds the maximum allowable level of 3 per fish. Furthermore, action (treatment or harvest) should be taken during juvenile wild salmon out-migration (March to July) if lice levels on farms exceed 3 motile lice per fish.

In Ireland, lice monitoring appears to be much more formalized and controlled by government scientists dictating that all fish farms undergo lice inspections 14 times per year (Copley *et al.* 2001). These inspections typically occur each month on sites where fish are present, with two inspections occurring each month from March to May and one inspection occurring for December and January. The sea lice sampling protocol for

Ireland involves sampling one standard and one random cage of salmon for each year class on site at each inspection. A total of 30 fish per cage are anaesthetized in a container that is sieved at the end of sampling to account for any detached lice. Each fish is individually examined for mobile lice and the mean lice numbers per fish are calculated (Copley *et al.* 2001). A recent report by Revie *et al.* (2002) suggested that Scottish salmon farms follow a sampling similar to that described by Treasurer and Pope (2000). Weekly lice counts are conducted from April to October and biweekly counts are conducted throughout the rest of the year. At each sampling, between four to six cages are sampled, and stages (including gender) of *L. salmonis* are recorded, while only mobile stages of *C. elongatus* are recorded.

In Norway, a national program for control of sea lice was been established by the Norwegian Animal Health Authorities, in co-operation with the Directorate of Fisheries, Directorate of Nature Management, fish farmers' organization and private fish health services. In 2000, the "Regulation of the fight against sea lice" was established. The purpose of this regulation was to establish minimum measures of sea lice in an effort to reduce the damage to wild and farmed Atlantic salmon. These guidelines are continually under review and subject to change. Currently, under this regulation, farmers are required to conduct biweekly sea lice sampling at sea temperatures ≥ 4 °C. Mandatory delousing must occur between 1 November to 1 July if adult female numbers exceed 0.5 per fish, or 5 or more adult female and mobile stages per fish. Mandatory delousing must also occur between 1 July to 1 November if adult female lice numbers exceed 2 per fish, or 10 or more adult females or mobile stages (McKinley *et al.* 2002). At each sampling, one random cage and one standard cage are sampled. A minimum of 20 fish from each

cage is counted and sea lice are categorized as adult female lice with and without eggs, preadult stages and adult male lice and chalimus stages.

New Brunswick salmon farms appear to be more variable in their approach, with fewer fish and different cages being targeted, and at intervals that can fluctuate by perceived need for control measures. It should be noted, however, that many New Brunswick farms have sea lice monitoring that surpasses the Irish regulations for number of fish sampled, cages monitored, and frequency of sampling, despite the lack of regulated monitoring or reporting. Also, many of the veterinarians collect the information about lice burdens directly, through offering a lice counting service, or indirectly, by having the sites report lice count information to the practice on a regular basis to enable timely and appropriate prescriptions. Although not part of disease regulations, lice monitoring is practiced at a rigorous level at sites in New Brunswick.

2.5.2 Sea lice treatments

The results of this survey indicate that the majority of sites in the Bay of Fundy are using EMB to treat their smolt and premarket salmon for sea lice. It is not surprising that there is such a widespread use of EMB in Atlantic Canada. The agents registered for use in Canada at the time of the survey included teflubenzuron (Calicide®; Skretting, Bayside, NB) and azamethiphos (Salmosan®). Teflubenzuron is rarely used to control sea lice, and the use of azamethiphos, although successful at controlling mobile stages of lice, has declined with the widespread use of emamectin benzoate. Due to the lack of use, registration for Salmosan® has been allowed to lapse since the survey was conducted, resulting in the product eventually being unavailable (i.e. when current

supplies are exhausted). Currently progressing through the registration process in Canada, EMB is only available by Emergency Drug Release (EDR) (Health Canada 2003). All of the sea lice chemotherapies (bath or oral) are only available through a veterinary prescription in Canada. Thus, salmon farmers in New Brunswick depend heavily on their relationship with their veterinarian to monitor and implement timely and appropriate lice control measures.

The effectiveness of emamectin benzoate has quickly made it the drug of choice for sea lice control in New Brunswick. It is advantageous over other chemotherapeutants for a number of reasons. It combines highly effective control of all parasitic stages of sea lice and is safe for fish, easy to administer (orally), and exhibits a sustained duration of efficacy extending far beyond the 7-day medication period (Stone *et al.* 2000, SPAH 2001). Historically, such reliance on therapies with a single mode of action has proven to be a potent enhancement factor in the selection for resistance (Denholm *et al.* 2002). Alternating chemical treatments is important to prevent the development of resistant populations, especially for widespread problems such as sea lice (Costello 2001). However, Canada lacks multiple effective and approved control agents that could be used in a strategy of alternating chemotherapeutants with different modes of action, an accepted method of reducing the probability of developing resistance (Horsberg 2003).

2.6 Conclusions

This survey demonstrates that sea lice monitoring methods vary between sites within the Bay of Fundy salmon farming industry, but control strategies remain very similar due to a lack of effective treatment options. This may, in part, be due to the fact

that in Atlantic Canada, there are no regulations for the reporting of lice on Atlantic salmon farms, nor are there officially standardized protocols for conducting sea lice counts in the field. Furthermore, some sites depend on their site veterinarian to conduct sea lice counts on a regular basis, whereas other sites choose to do their own sea lice counts and consult with their veterinarian on control options. Comments by site workers suggest that there may be differences in sea lice pressures within different regions in the Bay of Fundy and so the need for sea lice counting and control is not uniform over the entire industry. The majority of fish farmers, in concert with their veterinarians, practice intensive monitoring and control of sea lice burdens. However, there is a continued reliance on EMB for sea lice control on Atlantic salmon farms in the Bay of Fundy, which raises concerns regarding the potential for sea lice to develop resistance to the drug.

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Table 2.1 Estimated frequency of veterinary visits and sea lice counts conducted on Atlantic salmon sea cage sites in the Bay of Fundy, New Brunswick, Canada (n=83 sites surveyed).

Frequency	Veterinary Visits		Lice Counts	
	n	%	n	%
Weekly	4	4.8	22	26.5
Biweekly	15	18.1	9	10.8
Monthly	45	54.2	22	26.5
Every 6-9 weeks	11	13.3	0	0
Only when called	7	8.4	0	0
Other[a b]	1	1.2	30	36.1
Total	83	100.0	83	100.0

[a] for veterinary visits the 'other' category included bringing in samples to the site veterinarian when necessary

[b] for lice counts the 'other' category included responses such as every 3 months, 2-3 times per year, when lice are seen on the fish and counts are done as needed for a SLICE® prescription, lice counts weekly in the summer and biweekly in the winter, lice counts biweekly in the summer and once a month in the winter, lice counts weekly in the summer and once a month in the winter

Table 2.2 Estimated proportion of cages sampled at a site when conducting sea lice counts on Atlantic salmon sea cage sites in the Bay of Fundy, New Brunswick, Canada (n=83 sites surveyed).

Proportion of cages	n	%
Every cage	29	34.9
Selected cages[a]	50	60.2
Other[b]	4	4.8
Total	83	100.0

[a] purposefully selected cages

[b] every cage when fish are small, and every 3 cages when fish are larger, 75% of cages on site, 5-28 cages per visit.

Table 2.3 Estimated number of fish sampled per cage and cages sampled per site when conducting sea lice counts on Atlantic salmon sea cage sites in the Bay of Fundy, New Brunswick, Canada (n=83 sites surveyed).

Number of fish sampled per cage when conducting sea lice counts	Every Cage		Purposefully Selected Cages	
	n	%	n	%
5-10	10	12.0	32	38.6
11-20	11	13.3	12	14.5
21-30	4	4.8	6	7.2
41-50	2	2.4	1	1.2
50+	1	1.2	0	0.0
Uncertain	1	1.2	3	3.6
Total	29	34.9	54	65.1

Table 2.4 Type of anaesthetic used to anaesthetize fish when conducting sea lice counts on Atlantic salmon farms in the Bay of Fundy (n=83 sites surveyed).

Type of anaesthetic	n	%
Tricaine methanesulfonate (TMS) [a]	54	65.1
Clove Oil	4	4.8
Uncertain[b]	2	2.4
Not applicable[c]	23	27.7
Total	83	100.0

[a] this group was comprised of 44 sites that reported using TMS to anaesthetize both small and large fish and 10 sites that reported using TMS to anaesthetize small fish only

[b] this group was comprised of one site that was uncertain as to the type of anaesthetic used and one site that reported anaesthetizing small fish and not large fish but was uncertain as to the type of anaesthetic used

[c] this group was comprised of 21 sites that reported not anaesthetizing fish when conducting lice counts, and two sites that reported large fish are not anaesthetized and did not comment as to whether or not small fish were anaesthetized and the type of anaesthetic used

Table 2.5 Person on site trained to identify stages of *Lepeophtheirus salmonis* on Atlantic salmon sea cage sites in the Bay of Fundy, New Brunswick, Canada (n=83 sites surveyed).

Variable	n	%
Yes	43	51.8
No	20	24.1
Uncertain	1	1.2
Not applicable[a]	19	22.9
Total	83	100.0

[a] this question was not added to the survey at the time of the interview.

Table 2.6 Classification of sea lice by species (*Lepeophtheirus salmonis* or *Caligus elongatus*) and life stage when conducting sea lice counts on Atlantic salmon sea cage sites in the Bay of Fundy, New Brunswick, Canada (n=83 sites).

Life Stage	Categorized		Not Categorized		Uncertain		Total
	n	%	n	%	n	%	n
Copepodid	6	7.2	74	89.2	3	3.6	83
Chalimus	75	90.4	5	6.0	3	3.6	83
Mobiles[a]	76	91.6	4	4.8	3	3.6	83
Gravid Female	74	89.2	6	7.2	3	3.6	83
Caligus elongatus	62	74.7	18	21.7	3	3.6	83

[a] this category includes preadult and adult sea lice

Table 2.7 Most important factors for sea lice treatment decisions on Atlantic salmon sea cage sites in the Bay of Fundy, New Brunswick, Canada (n=83 sites surveyed).

Variable	n	%
Sea lice counts	40	48.2
General fish appearance	7	8.4
Recommendation by site veterinarian	13	15.7
Anticipated problem	5	6.0
Combination of variables	18	21.7
Total	83	100.0

Table 2.8 Person who most influences the final decision to treat for sea lice on Atlantic salmon sea cage sites in the Bay of Fundy, New Brunswick, Canada (n=83 sites surveyed).

Position	n	%
Site manager	12	14.5
Site veterinarian	26	31.3
Site owner	29	34.9
Other[a]	9	10.8
Combination of positions	7	8.4
Total	83	100.0

[a] production manager, aquaculture manager, general manager, technical manager, site contractor

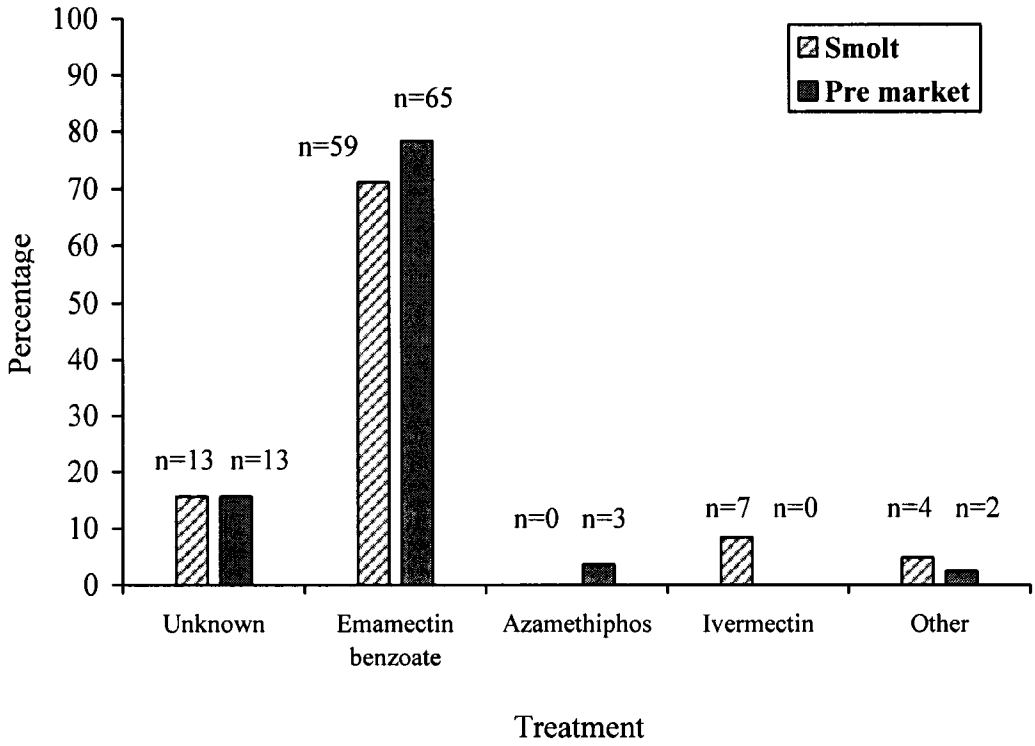

Figure 2.1 Chemotherapeutants used to treat smolt and pre market salmon on Atlantic salmon sea cage sites in the Bay of Fundy, New Brunswick, Canada as reported by site managers and owners (n=83 sites surveyed).

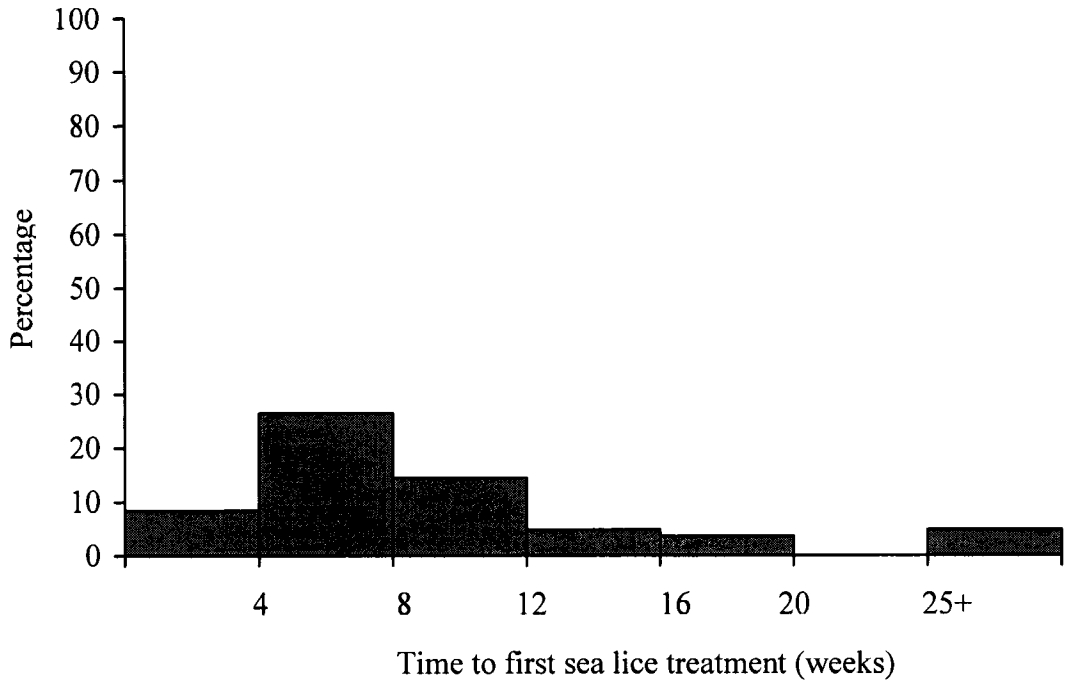

Figure 2.2 Estimated time to first treatment for sea lice after current year class of fish was transferred to salt water on Atlantic salmon sea cage sites in the Bay of Fundy, New Brunswick, Canada (n=52 sites surveyed).

Chapter 3 Emamectin benzoate bioassay optimization and gender-related differences in susceptibility of sea lice (*Lepeophtheirus salmonis)* from field samples

3.1 Abstract

A bioassay for sea lice sensitivity towards emamectin benzoate (EMB; active chemical in SLICE®) was validated for field use. Bioassay optimization included an evaluation of the inter-rater reliability of sea lice responsiveness to EMB and an evaluation of gender-related differences in susceptibility. The concordance correlation coefficient was used to compare the agreement between the proportion of lice categorized as moribund or dead by two independent raters prior to and following the adoption of a set of bioassay response criteria. The adoption of the bioassay response criteria improved the concordance between raters' assessments and it also improved the model estimation of the EC_{50} values (the "effective concentration" leading to a response of 50 % of the lice not prone to natural mortality) for subsequent bioassays. An evaluation of gender-related differences in EMB susceptibility for 19 bioassays indicated that preadult stage female sea lice exhibited a significantly larger sensitivity towards EMB in 12 of 19 bioassays compared to preadult males, and 7 of 19 bioassays showed no significant difference in gender susceptibility, although all male EC_{50} values were higher than female values. Preadult stage females from one population of laboratory cultivated sea lice also exhibited a significantly larger EMB sensitivity compared to preadult males.

3.2 Introduction

Sea lice (*Lepeophtheirus salmonis*) are ectoparasitic, copepodid crustaceans capable of inflicting serious physical damage upon their salmonid hosts if uncontrolled

(Ramstad *et al.* 2002). The requirement for continual monitoring and control of sea lice is a costly economic burden for Atlantic salmon (*Salmo salar)* producers throughout North America and northern Europe (Costello 1993, Treasurer and Pope 2000). Although there are a number of biological and chemical means of sea lice management and control, reduced sensitivity and resistance development of sea lice towards several chemotherapeutants have been reported (Jones *et al.* 1992, Treasurer *et al.* 2000, Tully and McFadden 2000, Sevatdal and Horsberg 2003).

The conventional and historical means of detecting insecticide resistance has been by bioassay (Brogdon 1989). A bioassay is an experiment which quantifies the response of a test subject to increasing concentrations or doses of an agent. The agent is typically a drug, and the subject response is a change in a particular characteristic, morbidity and/or death (Hubert 1980, Robertson and Preisler 1992). Bioassays are valuable tools in the detection of individuals or cases with a decreased sensitivity towards a chemotherapeutant, especially when the mechanism of resistance is unknown (Denholm *et al.* 2002). Bioassays represent the best method for the standardization of variables that may influence sea lice sensitivity to emamectin benzoate (EMB; active chemical in SLICE®) as non-responsive treatments in the field may be related to factors that cannot be detected through simple clinical observations. Using bioassays to aid in the early detection of changes in the sensitivity of a population of individuals before the onset of resistance development will allow for the adoption of alternative control measures and possible prevention or delay of resistance development. Bioassays for several chemicals administered as bath treatments to combat sea lice infections have been developed

(Sevatdal and Horsberg 2003, Sevatdal *et al.* 2005). However, the present study is the first report of a bioassay developed for a drug administered in-feed to farmed salmon.

The dependence of bioassays for sea lice on subjective assessments of sea lice responsiveness (i.e. vigour/mortality) to varying concentrations of a chemotherapeutant may influence the reproducibility of evaluations between two independent raters. The need to quantify agreement arises when 2 raters independently but simultaneously assess a response (King and Chinchilli 2001). The inter-rater reliability, or inter-observer agreement, is used to assess the consistency of results for the same outcome using the same subject/individual at the same time provided by independent raters. Lack of inter-rater reliability may arise from deviations between rater evaluations or instability over time of the attribute being measured.

Precise and frequent monitoring of changes in chemotherapeutant susceptibility of sea lice populations is essential for anticipating and contending with the development of resistance (Denholm 1990). Bioassays comprise an important component of resistance monitoring. Due to the current absence of standardized methods and techniques for detecting and monitoring resistance of sea lice to EMB, the objectives of this study were to: (1) develop and optimize a bioassay protocol for EMB using field-collected sea lice, (2) validate the bioassay protocol, (3) evaluate the inter-rater reliability of the subjective assessment of the bioassay protocol, and (4) evaluate gender-related differences in the susceptibility of sea lice to EMB.

3.3 Materials and Methods

3.3.1 Sea lice collections

Due to the fact that an element of subjectivity exists in this type of bioassay evaluation, specific criteria for lice condition was adopted (Table 3.1). An evaluation of inter-rater agreement prior to and following the adoption of the bioassay response criteria was conducted on sea lice collected in 2002 and 2003 from fish originating at 4 different Atlantic salmon *Salmo salar* sea cage sites located in the Bay of Fundy. Prior to the adoption of the bioassay response criteria, sea lice were collected on 4 separate days over a 1 week period from 1 site within the Bay of Fundy using market-sized fish (3 to 4 kg) that were part of a routine harvest (i.e. not anesthetized). Following the adoption of the refined bioassay response criteria, sea lice were collected from 3 different sites, on separate days, during routine sea lice counting on site. On those days, sea lice were collected from fish that were immobilized using tricaine methanesulfonate (TMS, Syndel Laboratories).

Sea lice used for the evaluation of gender-related differences in EMB susceptibility were collected during the years 2002 to 2005 from 16 Atlantic salmon marine farm sites in the Bay of Fundy. Sea lice were collected from smolts, pre-market fish, or broodstock, during routine sea lice counting or harvesting on site. Lice collected during routine sea lice counting were removed from fish anesthetized by TMS and those collected during harvest from fish that were lightly sedated with carbon dioxide or immobilized by percussive stunning.

In an effort to minimize potential variation in sensitivity that may be related to age or size of the test subjects, only live and healthy preadult (stage I or II) male and

female sea lice were collected for all bioassays. Sea lice were gently removed from Atlantic salmon using forceps and placed into a 2L container of seawater collected from the site. An additional 7 to 10 l of seawater was collected from each site for later use in bioassays. Battery operated air pumps were added to collection containers and ice packs were placed in a cooler with the collection containers to ensure that the sea lice were kept cool during transport to the laboratory.

3.3.2 Sea lice cultivation

Laboratory cultivated sea lice used in this study were the first generation progeny of *Lepeophtheirus salmonis* adult females collected in July 2005 during a cage harvest on an Atlantic salmon sea cage site in the Bay of Fundy. Gravid females were collected and transported to the laboratory as described above for preadult sea lice collections. In the laboratory, egg-strings were carefully removed from females and hatched and reared on Atlantic salmon in a re-circulation system containing 30 ppt synthetic saltwater (Instant Ocean®, Aquarium Systems, Mentor, OH). Following the development of sea lice to the preadult stages, fish were anaesthetized using TMS, and sea lice were removed and placed into Petri dishes containing synthetic saltwater. Two collection days were employed 2 weeks apart.

3.3.3 Bioassays

Bioassays involving laboratory cultivated sea lice were set up at the Atlantic Veterinary College (AVC), University of Prince Edward Island in Charlottetown, Prince Edward Island (PE), Canada, whereas all other bioassays involving field collected sea

lice were set up either at AVC or the New Brunswick Department of Agriculture, Fisheries and Aquaculture Diagnostic Laboratory in Black's Harbour, New Brunswick (NB).

All bioassays were initiated within a maximum of 6 h of collection. Seawater obtained from each sea lice collection site was used to prepare the bioassay dilutions. A stock solution was prepared for each bioassay by dissolving 5 mg EMB (Emamectin benzoate PESTANAL®, Sigma-Aldrich) in 50 ml of methanol. A working solution was prepared by diluting 10 ml of the stock solution with 990 ml of seawater. EMB concentrations were prepared by diluting the working solution with seawater in order to create 7 concentrations of EMB (0, 1, 3, 10, 30, 100, 300 ppb) (Table 3.2).

The number of sea lice used in each bioassay dish and the number of replicates at each treatment dilution was dependent upon the availability of sea lice on collection days. Due to the greater number of sea lice available from one site that was being harvested (prior to the adoption of the bioassay response criteria), an average of 15 sea lice (equal proportions of males and females were used, where possible) were carefully transferred from the collection containers into each of 28 or 35 (4 or 5 replicates of each of the 7 treatment dilutions) Petri dishes containing seawater from the collection site. In 2003 and 2004, sea lice collection resulted in fewer total sea lice due to low availability at sites in those years. Therefore, on these collections, an average of 10 to 15 live and healthy sea lice were transferred to each of 7 Petri dishes.

For laboratory cultivated sea lice, the proportion of males and females was approximately equal. Thus, subsequent bioassays contained 50 % males and 50 % females. Two bioassays were set up on each collection day; one with Instant Ocean® and

the other using Bay of Fundy seawater. Ten sea lice were transferred to each of 42 Petri dishes (3 replicates at each treatment dilution using natural seawater and 3 replicates at each treatment dilution using synthetic saltwater as the solvent).

For all bioassays, Petri dishes were maintained on a chilled surface and shaded from exposure to fluorescent lighting. Allocation of lice to Petri dishes, initially containing seawater (i.e. 0 ppb EMB), was blinded to the evaluator(s) to prevent bias during the bioassay outcome assessment. Following equal allocation of sea lice to each Petri dish, the seawater was replaced with approximately 50 ml volumes of the 7 different EMB dilutions assigned randomly to each Petri dish. Covers were placed on all dishes, which were incubated in a temperature controlled chamber at 10 to 12°C for an exposure period of 24 h. Sea lice were evaluated by individually categorizing each louse by dish as live, moribund, or dead according to an adopted set of bioassay response criteria similar to those established by Sevatdal & Horsberg (2003) (Table 3.1). The moribund and dead categories were combined for analysis to determine the EMB concentration immobilizing or inactivating 50 % of sea lice. All subjective bioassay evaluations used to assess rater agreement were conducted by 2 independent raters, except in the case of those used to evaluate gender differences in EMB susceptibility which were assessed by 1 individual.

3.4 Statistical analysis

3.4.1 Bioassay data

For each bioassay, the number of affected (moribund or dead) sea lice at different concentrations of EMB was analyzed by a probit regression model with natural

responsiveness (Finney 1971). Specifically, the modeling equation for the probability (p) of sea lice subjected to a certain dose of EMB being affected was $p = p_0 + (1 - p_0) *$ probit $(\alpha + \beta*\log(\text{dose}))$, where p_0 is probability of response in controls, log is the natural logarithm, and probit is the cumulative distribution function of a standard normal distribution. The parameter of primary interest was $EC_{50} = -\alpha/\beta$, the "effective concentration" leading to a response of 50 % of the lice not prone to natural response. For example, if the natural responsiveness was 40 %, the EC_{50} was the dose level corresponding to a 70 % (i.e. 40 % + 50 % of 60 %) mortality. Following Williams (1986), in order to improve identifiability of the EC_{50} parameter the equation was rewritten using $\alpha + \beta*\log(\text{dose}) = \beta*(\log(\text{dose}) - \log(EC_{50}))$. Parameter estimates were obtained by maximum likelihood estimation, and confidence intervals were computed by the profile likelihood method. The analyses were implemented in SAS 8.2 software (SAS Institute Inc., Cary, NC, US) using probit and nlmixed procedures (Stryhn & Christensen 2003).

3.4.2 Gender-related differences in EMB susceptibility

Gender-related differences in EMB susceptibility were evaluated by estimating separate-sex probit regression models with natural responsiveness. First, the previously described model was used separately for male and female sea lice. However, this approach required substantial lice counts of both sexes and was therefore limited to the subset of samples in which sufficient representation of both male and female sea lice occurred. Second, the model for data of both sexes was extended to allow for different EC_{50} values for males and females. To ensure identifiability, common values of the

natural responsiveness (p_0) and the dose-response regression coefficient (β) were retained. Fig. 3.1 shows results for the laboratory cultivated sea lice bioassay with the overlaid dose-response curves estimated from the model. In the latter model, a statistically significant difference in EC_{50} values between sexes was assessed by a likelihood-ratio test, a p-value < 0.05 being indicative of a statistically significant gender-related difference in sea lice susceptibility to EMB.

3.4.3 Rater agreement

The inter-rater agreement (2 raters) of the number of affected (moribund or dead) sea lice in the Petri dishes used for each bioassay was assessed prior to and following the adoption of the bioassay response criteria. Prior to the adoption of the bioassay response criteria, rater agreement was compared 3 times for each of the bioassays conducted on 6, 9, 10, and 11 September 2002. The first comparison involved rater agreement between the bioassays having replicates at each of the 7 bioassay concentrations (i.e. 1 to 4 or 1 to 5 dishes at each EMB concentration). The second and third comparisons involved an assessment of only the first and second dish read by each rater at each EMB concentration (i.e. dish 1 or 2 at each EMB concentration; Table 3.4). In each case, the rater's proportions of affected lice were compared using the concordance correlation coefficient (CCC) (Lin 1989). The CCC evaluates the agreement between 2 readings from the same sample by measuring the variation from the 45° line through the origin (line of perfect agreement) (Fig. 3.2). CCC values of 0 and 1 represent no and complete agreement, respectively. The analysis included approximate 95 % confidence intervals, based on a z-transformation, and was carried out using the concord command (Steichen

and Cox 2004) for Stata 8 software (Stata Corp., College Station, TX, USA, 2001). The agreement between raters was also assessed for EC_{50} values computed for each rater.

3.5 Results

3.5.1 Estimation of bioassay dose-response curves

Thirty-nine EC_{50} values were generated for the combined analysis (both genders) of the bioassay data (Table 3.3). One bioassay analysis was not included because it was not possible to calculate an EC_{50} value due to the absence of a dose-response relationship. The 95 % confidence intervals were computed for each of the 39 analyses; however, several intervals exhibited very wide ranges. EC_{50} values for bioassays involving field collected samples ranged from 25 to 118 ppb EMB. Natural mortality (at 0 ppb EMB) ranged from 0 to 60 %. The EC_{50} value for laboratory cultivated sea lice was 21 ppb EMB and natural mortality was 3.5 % among these lice.

3.5.2 Assessment of gender-related differences in EMB susceptibility

The number of preadult stage male and female sea lice collected for each bioassay was variable. There were a higher number of preadult stage males collected in the field than preadult stage females; on average, bioassays consisted of 15 % females and 85 % males. Preadult female sea lice were significantly more sensitive to EMB (i.e. lower EC_{50} values) compared to preadult stage males in 12 of the 19 bioassays assessed for gender-related differences in EMB susceptibility, with values ranging from 39 to 116 ppb EMB and 1 to 59 ppb EMB for males and females, respectively. 7 of the remaining 19 bioassays did not demonstrate significant gender-related differences in EMB

susceptibility, although all estimated EC_{50} values were higher for males than females (Table 3.3). Twenty of 39 bioassays were not included in the gender-difference analysis either because there were too few preadult stage female sea lice collected for the bioassay, or the information regarding the sex of susceptible sea lice in the bioassay was not recorded (early protocols did not include this assessment). Preadult female sea lice cultivated in the laboratory also exhibited the same increased EMB sensitivity as seen in field samples when compared to preadult males. The ratios of male to female EC_{50} values for significant and non- significant gender-specific analyses ranged from a minimum of 1.17 to a maximum of 39.00 with a median value of 3.04. This suggests that on average, the "effective concentration" leading to a response of 50 % of preadult female sea lice not prone to natural response was three times lower than that of preadult stage males.

3.5.3 Evaluation of inter-rater agreement

CCC's for bioassays with replicates ranged from 0.57 to 0.93, representing moderate to almost perfect agreement between raters. CCC's for bioassays in which the first plate read by each rater was evaluated for agreement ranged from 0.67 to 0.93, and CCC's for those in which the second plate read by each rater was evaluated for agreement ranged from 0.45 to 0.98. Following the adoption of the bioassay response criteria, due to the limited availability of sea lice, the 3 bioassays conducted did not have replicates. CCC's ranged from 0.95 to 1.00, representing almost perfect agreement between raters. The 95 % CI's for these coefficients were much narrower than earlier

assessments (Table 3.4). Also, the EC_{50} values were less variable between raters following adoption of the criteria.

3.6 Discussion

3.6.1 Gender-related differences in EMB susceptibility

An increasing number of human and animal studies show that males and females may differ in their biological response to drugs, and the safety and effectiveness of many drugs exhibit some degree of sex-dependence in both vertebrate and invertebrate species (Beierle *et al.* 1999, Miller *et al.* 2003, Pica-Mattoccia and Cioli 2004, Simon and Resnick 2004).

The reason for differences in male and female sea lice susceptibility towards EMB under bioassay conditions in this study are unknown. A gender difference in EMB efficacy following treatment was reported in a laboratory efficacy study involving sea lice grown on Atlantic salmon fed EMB in fresh water (Stone *et al.* 2002). The results of their study suggested that overall efficacy of EMB against preadult II sea lice following transfer to saltwater was higher in female sea lice than males on treated fish, although efficacy against adult males was lower compared to adult females. Bioassay studies conducted by Sevatdal *et al.* (2005) using pyrethroids suggested that adult females were five times less susceptible to pyrethroids than adult males. Although this study did not specifically look at gender-related differences in susceptibility, the results suggest that adult males and preadult I sea lice of both sexes displayed approximately the same sensitivity as mixed-sex populations of preadult II sea lice towards pyrethroids. It is evident that further studies will be required to quantify the effects of age or stage of sea lice on chemotherapeutant susceptibility under bioassay and field conditions.

The stages of sea lice that could be used in this bioassay were limited by the availability of sea lice in the field, the higher proportion of males collected in field samples, and the variation in size and weight between male and female sea lice at different stages of the life cycle. In an effort to minimize potential variation in sensitivity that may have been related to age and/or size of the test subjects, only preadult stage sea lice were used in the bioassays where possible, as males are similar in size to females at this stage (Schram 1993). However, there may have been adult males included in bioassays with preadult sea lice as it is difficult to distinguish between preadult stage I and II males and adult males without the assistance of a stereomicroscope. Immature males are distinguished from mature males by the surfaces of the second antennae and the presence of a rough surfaced pad (post-oral adhesions pad) located near the base of the first maxilla (Johnson and Albright 1991), distinctions which would require optical enhancement. Recent pyrethroid bioassay studies conducted by Sevatdal *et al.* (2005) suggested that adult males and preadult I and II sea lice displayed similar responses.

Further studies are required to understand the impacts of the effects of gender and sea lice stage-related sensitivity towards EMB for use in this bioassay.

3.6.2 Evaluation of rater agreement

Reproducibility is important to ensure uniformity among investigators testing different test subjects in different laboratories (Robertson and Preisler 1992). Reproducibility of a method can be determined by the test-retest of the assessments (intra-rater repeatability) or by measuring the reliability between independent raters (inter-rater reliability or inter-observer agreement). Discordance between the

assessments of independent raters is not uncommon when subjective measures are evaluated. Inconsistencies between the outcomes of diagnostic tests run by different labs on the same sample using the same test have been shown to be partly due to subjectivity of the diagnostic test evaluations experienced by independent raters (see for example McClure *et al.* 2005). Training and experience would likely improve inter-rater reliability (Seidman *et al.* 2003, Sevatdal 2005).

Although bioassays have been established to test sea lice sensitivity to several chemicals that are administered as bath treatments (Sevatdal *et al.* 2003, Sevatdal *et al.* 2005), those involving chemicals administered in-feed are still being developed. As a result, an appropriate gold standard or reference assay against which to compare the current EMB bioassay results is lacking. Furthermore, detailed descriptions of inter-rater agreement involving sea lice bioassays have not been published.

The inter-rater reliability of the current bioassay protocol was evaluated by comparing 2 independent rater's evaluations of sea lice responsiveness to EMB using the concordance correlation coefficient (CCC). Subjectivity of initial bioassay evaluations experienced by two independent raters led to the adoption of a set of bioassay response criteria. Although the CCC's prior to the adoption of the bioassay response criteria were reasonably high, our results suggest improvements in several aspects of rater agreement following adoption of the bioassay response criteria. These include a substantial improvement in rater agreement, as well as an improvement in EC_{50} estimation.

3.6.3 Susceptibility of laboratory cultivated and field collected sea lice

In this study, laboratory cultivated sea lice, with no direct exposure to EMB, had an EC_{50} value of 21 ppb, which was lower than any of the values recorded for field samples. The wide range of EC_{50} values for field samples of lice (e.g. 25 to 118 ppb) may reflect the potential influence of previous exposure to routine EMB treatments experienced by sea lice under field conditions, or the variability experienced in the collection.

3.6.4 Recommendations for optimizing bioassays

In this section, we review a number of factors that we considered or experienced to be of importance for optimization of the EMB bioassay protocol. Only live and apparently healthy sea lice were used in the bioassay to reduce pre-exposure factors affecting the reliability of the bioassay results (Sevatdal 2005). Careful removal of sea lice from fish using forceps reduced physical damage of the sea lice and minimized handling. As the distance between farms and the nearest laboratory caused variable transport times, battery-operated air pumps were used in collection containers to provide continual aeration during transport to the laboratory and maximize survival of the sea lice.

We preferred using glass Petri dishes instead of polystyrene plastic to minimize changes in EMB concentration. In a pilot assay using EMB in plastic containers, the concentration of EMB following an incubation of 24 h was 50 to 60 % of the initial concentration (observations made in our laboratory and by Sevatdal 2005), likely due to adherence of the EMB to the plastic dishes. We used sea lice of equal size to ensure

comparable adherence and absorption of EMB by all sea lice in each dish. Where possible, increasing the number of replicates within each bioassay is desirable as is the standardization of the number of sea lice used in each Petri dish. Although it may not be possible to collect equal numbers of males and females from field samples for use in the bioassay, it is important to ensure adequate samples sizes are used and the sex of each louse is recorded. The use of 2 independent evaluators for each bioassay was advantageous to ensure consistency of bioassay evaluations, and blinding of evaluators to test concentrations was essential to reduce any preconceived expectations of treatment effects during bioassay evaluations.

3.7 Conclusions

The high level of agreement achieved between the bioassay evaluations of 2 independent raters provides confidence that the adopted bioassay response criteria were clearly defined, the raters understood consistent definitions of live, moribund, and dead sea lice, and were able to consistently apply those definitions to their evaluations.

Although the bioassay protocol shows promise as a method to verify clinical resistance, it lacks rapidity and simplicity for use as a routine test. However, the time requirement for sea lice collection, bioassay set-up, and the 24 h incubation period, as well as the difficulty in consistently obtaining sufficient numbers of viable sea lice in the field make this bioassay impractical in many situations. As a result, the bioassay protocol is useful as a research tool at the descriptive level, but has limited used as a field resistance monitoring tool.

3.8 Acknowledgements

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Table 3.1 Bioassay response criteria (adapted from Sevatdal & Horsberg 2003).

Response	Criteria
Live	1) normal swimming behavior (ability to swim in a straight line) 2) securely adheres to Petri dish 3) normal movement of extremities
Moribund	1) disabled swimming but capable of weak uncoordinated movement (loop to loop swimming) 2) inability to firmly adhere to Petri dish (adherence to dish for a period before dropping off) 3) minimal movement of extremities
Dead	1) inability to swim 2) floating in Petri dish 3) no movement of extremities

Table 3.2 Emamectin benzoate (EMB) stock solution, working solution and dilution concentrations used in bioassays.

Stock Solution	**Working Solution**	**Bioassay Dilutions**		
5 mg EMB	10 ml stock		ml	ml
+	solution	conc.	working	sea-
50 ml methanol	+	(ppb)	solution	water
	990 ml	0	0	1000
	seawater	1	1	999
		3	3	997
		10	10	990
		30	30	970
		100	100	900
		300	300	700

Table 3.3 Combined (n=39) and gender-specific (n=19) EC_{50} (the effective concentration leading to a response of 50% of sea lice not prone to natural mortality) estimations used to determine gender-related differences in sea lice susceptibility to emamectin benzoate.

	Combined EC_{50} estimation		Gender-specific EC_{50} estimation		
Date	EC_{50} (95% CI)	natural mortality	male	female	p (gender)
Jul 2002	25 (17,35)	0.132	[a]	[a]	[a]
Jul 2002	41 (34,76)	0.156	[a]	[a]	[a]
Aug 2002	75 (42,115)	0.249	[a]	[a]	[a]
Aug 2002	73 (38,142)	0.228	[a]	[a]	[a]
Sep 2002	35 (26,48)	0.072	[a]	[a]	[a]
Sep 2002	35 (28,45)	0.290	[a]	[a]	[a]
Sep 2002	48 (35,78)	0.607	[a]	[a]	[a]
Sep 2002	[b]	[b]	[a]	[a]	[a]
Sep 2002	28 (23,33)	0.444	[a]	[a]	[a]
Sep 2002	44 (30,88)	0.262	[a]	[a]	[a]
Oct 2002	102 (80,134)	0.095	[a]	[a]	[a]
Oct 2002	103 (80,148)	0.056	114	34	<0.001
Oct 2002	48 (37,62)	0.029	95	20	<0.001
Oct 2002	107 (96,151)	0.162	116	31	<0.001
Oct 2002	103 (55,300)	0.038	110	18	<0.001
Oct 2002	98 (71,123)	0.093	104	59	<0.05
Oct 2002	39 (31,82)	0.057	[a]	[a]	[a]
Nov 2002	118 (106,292)	0.158	[c]	[c]	[c]
Nov 2002	107 (95,165)	0.078	[c]	[c]	[c]
Nov 2002	90 (54,104)	0.184	94	17	<0.05
Nov 2002	95 (55,116)	0.175	99	29	<0.05
Aug 2003	31 (23,43)	0.049	39	1	<0.001
Aug 2003	65 (41,104)	0.048	83	30	ns[d]
Sep 2003	87 (48,97)	0.135	86	31	ns
Nov 2003	60 (39,96)	0.068	85	30	ns
Nov 2003	104 (56,152)	0.037	115	61	ns
Nov 2003	40 (32,85)	0.077	55	17	<0.05
Nov 2003	37 (30,57)	0	35	30	ns
Oct 2004	26 (17,42)	0	55	5	<0.001
Oct 2004	38 (31,56)	0	55	28	0.01
Oct 2004	29 (18,48)	0	55	30	ns
Nov 2004	89 (53,103)	0.054	[c]	[c]	[c]
Dec 2004	42 (27,69)	0.138	50	20	ns
Dec 2004	79 (48,92)	0.048	[c]	[c]	[c]
Feb 2005	116 (86,163)	0.020	[c]	[c]	[c]
Feb 2005	100 (71,138)	0	[c]	[c]	[c]
Feb 2005	104 (79,152)	0	[c]	[c]	[c]
Feb 2005	113 (100,182)	0.020	[c]	[c]	[c]
Mar 2005	49 (31,80)	0	[c]	[c]	[c]
Lab cultivated[e]	21 (17,26)	0.035	37	12	<0.001

[a]gender data not recorded an these dates of the bioassay protocol
[b]no dose response
[c]too few females to obtain reliable gender-specific estimates
[d]actual p-value = 0.058
[e]sea lice from one laboratory cultivated population of sea lice (bioassay set up using Bay of Fundy seawater) in July 2005
ns: not significant (p>0.05)

Table 3.4 Agreement (Concordance Correlation Coefficient) of emamectin benzoate (EMB) bioassay evaluations of individual or multiple dishes of sea lice between two independent raters prior to and following the adoption of the bioassay response criteria.

Date	Dish(es) evaluated by each rater at each EMB concentration	Bioassay response criteria adopted (Y/N)	EC_{50} (ppb) EMB Rater 1	EC_{50} (ppb) EMB Rater 2	Concordance Correlation Coefficient (95 % CI)
6 Sept 2002	1-4	N	35	27	0.93 (0.86-0.97)
9 Sept 2002	1-4	N	51	36	0.85 (0.69-0.93)
10 Sept 2002	1-5	N	[a]	[a]	0.57 (0.31-0.75)
11 Sept 2002	1-5	N	28	32	0.78 (0.62-0.88)
6 Sept 2002	1	N	48	20	0.72 (0.08-0.94)
6 Sept 2002	2	N	[a]	[a]	0.98 (0.91-1.00)
9 Sept 2002	1	N	71	33	0.93 (0.64-0.99)
9 Sept 2002	2	N	71	[a]	0.47 (-0.07-0.80)
10 Sept 2002	1	N	[a]	[a]	0.67 (-0.06-0.93)
10 Sept 2002	2	N	[a]	[a]	0.45 (-0.33-0.86)
11 Sept 2002	1	N	21	[a]	0.90 (0.55-0.98)
11 Sept 2002	2	N	29	42	0.88 (0.48-0.98)
2 Oct 2002	1	Y	39	43	0.99 (0.96-1.00)
12 Aug 2003	1	Y	31	31	1.00 (0.98-1.00)
11 Sept 2003	1	Y	83	76	0.95 (0.74-1.00)

[a] EC_{50} estimation impossible because data did not exhibit a dose-response relationship

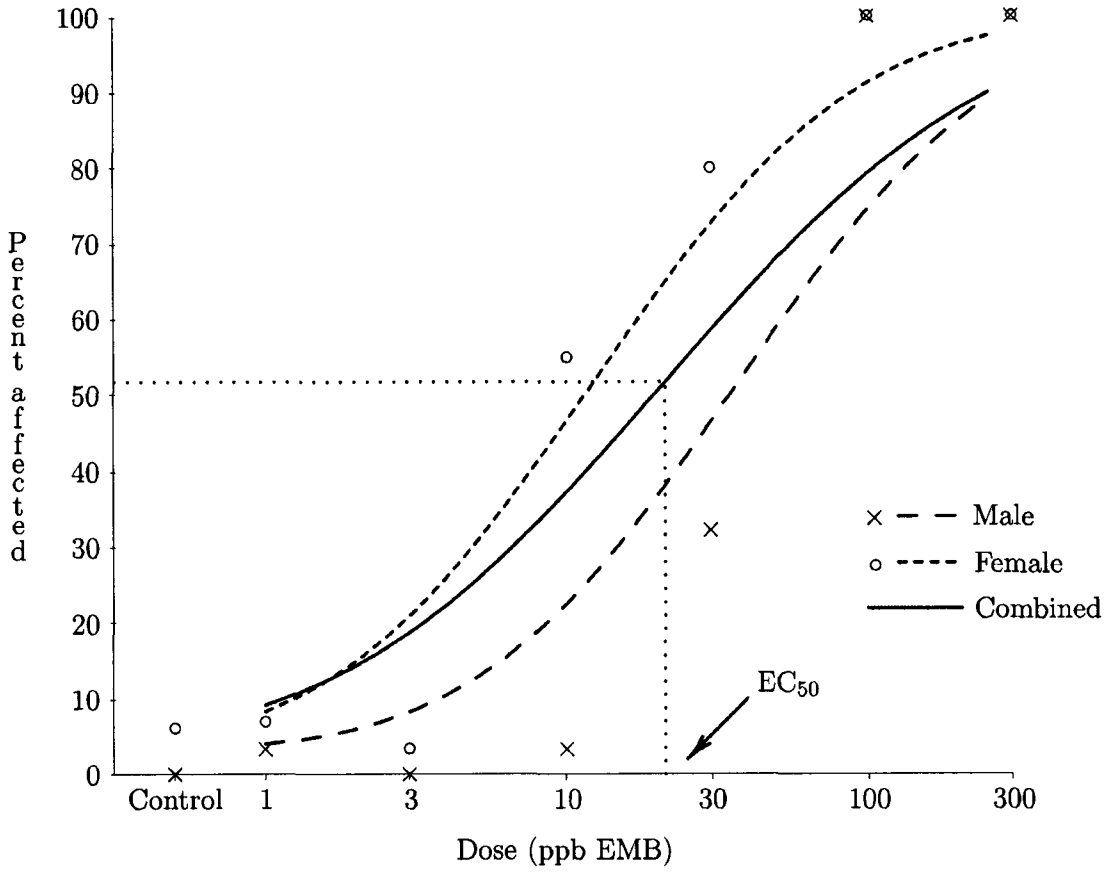

Figure 3.1 Dose-response curves for laboratory cultivated sea lice showing the combined and gender-specific curves and the determination of the combined EC_{50} (the effective concentration of emamectin benzoate (EMB) leading to a response of 50% of sea lice not prone to natural response) value.

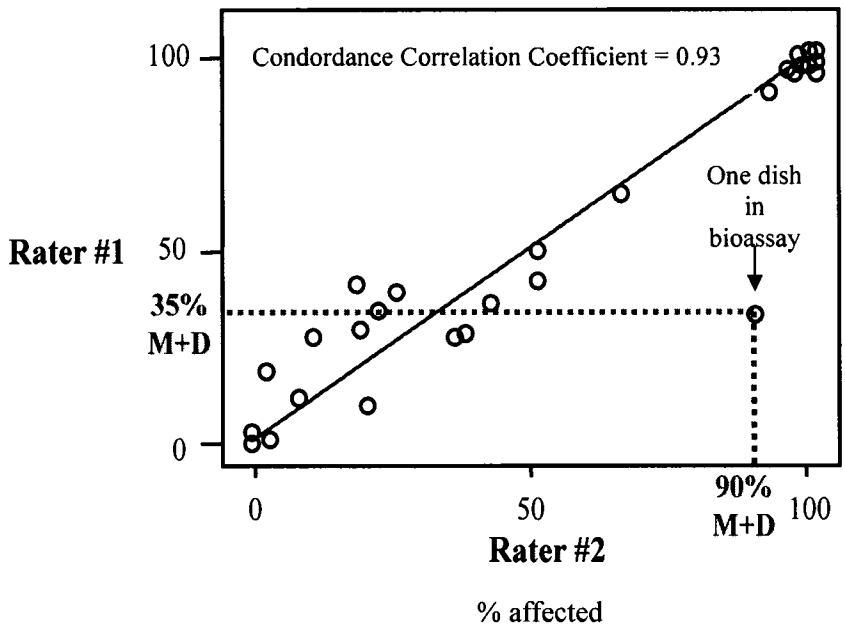

Figure 3.2 Agreement (Concordance Correlation Coefficient) of dish evaluations, the number of moribund and dead (M+D) sea lice, between two independent raters for the September 9, 2002 bioassay.

Chapter 4 Monitoring field sea lice (*Lepeophtheirus salmonis)* from the Bay of Fundy, Canada, for sensitivity to emamectin benzoate using a bioassay

4.1 Abstract

A bioassay for sea lice (*Lepeophtheirus salmonis)* sensitivity towards emamectin benzoate (EMB) was implemented for field use. The salmon farming area in the Bay of Fundy, New Brunswick, Canada, was divided into 4 distinct regions: Campobello Island and Deer Island (CD), Grand Manan Island (GM), Lime Kiln Bay (LK), and Passamaquoddy Bay (PB), based on industry health management practices and hydrographics. A total of 38 bioassays were completed from 2002 to 2005 using populations of preadult stage sea lice collected directly from 16 distinct Atlantic salmon farms within the 4 described regions. A probit regression model with natural responsiveness was used for the number of affected (moribund or dead) sea lice in bioassays involving different concentrations of EMB. There was no significant overall effect of region or year on EC_{50} values; however, analysis of variance indicated a significant effect of time of year on EC_{50} values in 2002 and 2004 to 2005. Although the range of EC_{50} values obtained in this three-year study did not appear sufficient to affect current clinical success in the control of sea lice, the results suggest that there is a seasonal or temperature associated variation in sensitivity to EMB.

4.2 Introduction

Resistance development is a continued challenge to pest control in both the agriculture and aquaculture industries. Over 500 species of arthropods, most of which are pests of crops, livestock, and humans, are reported to have developed resistance to at

least one class of chemical compounds (Denholm *et al.* 2002). Many of the therapies used by the aquaculture industry world-wide for sea lice control belong to the same classes of compounds as those used to control agricultural pests. Reports of reduced sensitivity and resistance of sea lice towards several chemotherapeutants (Jones *et al.* 1992, Treasurer *et al.* 2000, Tully and McFadden 2000, Sevatdal and Horsberg 2003) necessitate the development of resistance management strategies, including methods to monitor changes in sensitivity to treatments.

A successful sea lice resistance management strategy would require taking full advantage of biological and chemical means of control (i.e. fallowing, single year class sites, cleaner-fish, and rotation of chemicals with differing modes of action). Fallowing and single year class sites now comprise important components of area health management strategies in the Bay of Fundy and this affects biological control of sea lice in that area. An important principle of preventing or minimizing the development of resistance is the avoidance of reliance on single products, or on those treatments most likely to select for the same mechanism of resistance (Denholm *et al.* 2002). This is often difficult when a limited range of chemotherapies is available. In the Bay of Fundy, the only drugs currently available for sea lice control are the chitin-synthesis inhibitor teflubenzuron (Calicide®) and the avermectin, emamectin benzoate (EMB; active chemical in SLICE®), both of which are administered in-feed. A recent survey indicated that over 90% of sites in the Bay of Fundy are using EMB for sea lice control (Chapter 2). With such a limited range of medicines registered for use, and widespread reliance on a single chemotherapeutant, the potential for resistance development is an ongoing concern. Although there have been no known documented cases of treatment failures

with EMB or of sea lice resistance to the drug, early detection of changes in the sensitivity of sea lice towards EMB should be a key component of a successful resistance management strategy.

The conventional means of detecting insecticide resistance has been by bioassay. A bioassay is an experiment in which a living organism is used as a test subject to quantify the response or responses of the subject to an agent or stimulus (Hubert 1980, Robertson and Preisler 1992). A number of standard insecticide bioassay techniques have been developed to establish baseline levels of susceptibility for a wide range of insects (Roush and Tabashnik 1990). Bioassay methods used to determine the sensitivity of sea lice towards pyrethroids have recently been described (Sevatdal and Horsberg 2003, Sevatdal *et al.* 2005a). Bioassays are a valuable tool in the detection of cases with a decreased sensitivity towards a chemotherapeutant, especially when the mechanisms of resistance are unknown (Denholm *et al.* 2002). Resistance of pests to chemotherapeutants has typically been detected in the field due to an observed treatment failure or the need for repeated treatments. However, many factors unrelated to parasite susceptibility to the compound can be responsible for treatment failure occurrence in the field, including insufficient product or underestimated group biomass. The development of reliable and cost-effective bioassay protocols capable of detecting changes in sensitivity of sea lice to chemotherapeutants in the field will be necessary to improve our surveillance of resistance (Denholm *et al.* 2002). If monitored on a regular basis, an increase in resistance can be detected before control failures occur, which would allow for the adoption of more effective resistance management (Rousch and Tabashnik 1990).

Due to the current absence of standardized methods and techniques for detecting and monitoring of sea lice resistance to EMB, the objective of this study was to use a simple and suitable bioassay protocol with field-collected sea lice from Atlantic salmon farms in the Bay of Fundy region of New Brunswick, Canada, to test for differences in the susceptibility to EMB over a three-year period.

4.3 Materials and Methods

4.3.1 Sea lice collections

Based on hydrographics and industry health management policies, the Bay of Fundy salmon farming area, located on the east coast of Canada and bordering the United States, was divided into 4 distinct regions: Campobello Island and Deer Island (CD), Grand Manan Island (GM), Lime Kiln Bay (LK), and Passamaquoddy Bay (PB) (Fig. 4.1). Bioassays were conducted on sea lice collected during the years 2002 to 2005 from 16 Atlantic salmon *(Salmo salar)* marine farm sites located in these 4 regions; 4 sites in CD; 2 in GM; 9 in LK; and 1 site in PB. Only healthy preadult stages of both male and female *L. salmonis* were used in the bioassay to minimize potential variation in sensitivity that may be related to age or size of the test subjects (Sevatdal 2005). Sampling was dictated by the sporadic availability of sea lice and the cooperation and participation of sea cage site manager/owners in the Bay of Fundy. Sea lice were collected at sea cage sites from Atlantic salmon smolts, pre-market fish, or broodstock, during routine sea lice counting or harvest(s). Sea lice collected during routine sea lice counting were removed from fish anesthetized by tricaine methanesulfonate (TMS, Syndel Laboratories), and those collected during the harvest were collected from cage-side fish lightly sedated with carbon dioxide or those immobilized in ice water or by

percussive stunning. Sea lice were gently removed from Atlantic salmon using forceps and placed into a sealed container of seawater collected from the site. Except for initial collections, battery operated air pumps were added to collection containers. An additional container was filled with 7 to 10 l of seawater collected from the site for use in later bioassay EMB dilutions. Ice packs were placed in a cooler with the collection containers to ensure that the lice were kept cool during transport to the laboratory. Laboratory cultivated sea lice used in this study were the first generation progeny of *L. salmonis* hatched and reared in a re-circulation system containing 30 ppt synthetic saltwater (Instant Ocean®, Aquarium Systems, Mentor, OH).

4.3.2 Bioassays

The bioassays were set up as described in Chapter 3. Briefly, all bioassays were initiated within a maximum of 6 h of collection and subsequent bioassay evaluations were performed by the same individual to ensure consistency. An average of 10 to 15 sea lice were carefully transferred from the collection containers into each of 7 glass Petri dishes containing seawater from the collection site. Placement of sea lice in Petri dishes was conducted in such a manner as to ensure that dishes were filled simultaneously. Seawater collected from the Bay of Fundy was used in dishes for control sea lice and as the solvent when mixing EMB concentrations. Petri dishes were maintained on a chilled surface, and shaded from exposure to fluorescent lighting. Seven EMB concentrations (0, 1, 3, 10, 30, 100, 300 ppb) were prepared as described in Table 3.2 of Chapter 3. Allocation of Petri dishes to treatment dilutions was blinded to prevent bias during the bioassay outcome assessment. Seawater was replaced with approximately 50 ml volumes

of 7 different EMB dilutions allotted to 1 of 7 Petri dishes. Petri dishes were incubated in a temperature controlled chamber at 10 to 12°C for an exposure period of 24 h. The conditions of the sea lice were evaluated after 24 h according to an adopted set of bioassay response criteria (Sevatdal and Horsberg 2003, Chapter 3).

4.3.3 Bioassay data

The parameter of interest was EC_{50}, the "effective concentration" of EMB leading to morbidity or mortality of 50 % of the sea lice not prone to natural response. Therefore, the sea lice experiencing morbidity or mortality in the absence of EMB exposure (i.e. natural response) were not considered at risk. For each bioassay, the number of responding (moribund or dead) sea lice at different doses of EMB was analyzed by a probit regression model with natural responsiveness (Finney 1971), and parameter estimates were obtained by maximum likelihood estimation (for detailed description, see Chapter 3).

4.3.4 Analysis of EC_{50} values

An analysis of variance (ANOVA) for a linear model was used to determine whether EC_{50} values showed any significant differences between years, regions and time of year (modeled by a linear effect of the number of days elapsed within a year since 24 July, the earliest sampling time in the 3 years). All first order interactions were initially included and later removed if judged unimportant. The residuals were used to validate model assumptions and determine any influential observations. The significance level was set at $p < 0.05$.

4.3.5 Analysis of clustering in time and space

The temporal correlation derived from repeated measures on the same sites was assessed in a linear mixed model with the correlation, ρ, between two measurements on the same site being ρ^d, where d is the number of days between them. The presence of spatial correlation in the residuals of the linear model was assessed by computing Moran's *I*. This analysis was based on digitized site coordinates and was carried out using R 2.10 software (Ihaka and Gentleman 1996) with the spdep library.

4.4 Results

4.4.1 Bioassay data

A total of 38 field bioassays were completed during the years 2002 to 2005; the EC_{50} values are shown in Fig. 4.2 and were listed in Table 3.3. Sea lice collected from October 2004 to March 2005 were grouped as 2004 lice since the fish populations were the same and new sea lice infestations do not normally occur until after this point in time. Most bioassays were completed for the LK region due to a higher concentration and accessibility of sites within this area. Relatively few bioassays were completed in 2003 due to lower preadult lice burdens on the sampled sites during that year, making it difficult to consistently obtain the minimum 70 preadult sea lice required for each bioassay. The EC_{50} values reported for this study were the combined estimates for males and females. On average, bioassays using field collected sea lice consisted of 15 % females and 85 % males. However, bioassays using sea lice derived from a laboratory grown generation contained 50 % males and 50 % females. EC_{50} values ranged from 25

to 118 ppb EMB in 2002; 31 to 104 in 2003; 26 to 89 in 2004; and 49 to 116 in 2005. The EC_{50} value for laboratory cultivated sea lice was 21 ppb EMB.

4.4.2 Analysis of variance

Region, year, days and the interaction term between days and year were included in the final ANOVA model. The ANOVA indicated no overall significant effect of region ($p=0.68$) and year ($p=0.36$) on EC_{50} values. There was a significant effect of time ($p=0.007$) (expressed in days) on EC_{50} values, indicating a seasonal and/or temperature associated variation in efficacy of EMB. Although the interaction term between year and time was not significant ($p=0.10$), from Table 4.1 it is evident that there is a significantly positive slope ($\beta=0.62$, $p=0.002$) of the 2002 data indicating an increase in EC_{50} values towards late fall and early winter. The 2003 data showed no increase in EC_{50} values later in the sampling season ($\beta=0.03$, $p=0.9$). In 2004 to 2005, the positive slope of the data ($\beta=0.29$, $p=0.09$) reflects an increase in EC_{50} values towards fall and winter. The EC_{50} value for the 30 March 2005 bioassay was an influential point and, although not formally considered an outlier based on investigation of its leverage and Cook's distance, this point affects the slope of the 2004 to 2005 data and its significance ($\beta=0.59$, $p=0.03$, without 30 March 2005 value).

4.4.3 Analysis of spatial clustering

There was a marginally significant ($p=0.09$) and moderate correlation between measurements taken at the same site close in time ($\rho=0.50$, $SE=0.25$, for measurements taken 7 days apart). Only minor effects were seen in the results of the linear model when

accounting for the repeated measures. Spatial clustering of the bioassay data from the years 2002 to 2005 was determined to be of no statistical significance for this data set (Moran's I=-0.31, p=0.93).

4.5 Discussion

The results of this three-year study suggest that there is a seasonal or temperature associated variation in efficacy of EMB in field collected sea lice. Increased efficacy with increasing water temperature, as indicated by a lower EC_{50} values earlier in the sampling season, was found for sea lice collected and assayed in 2002 and 2004 to 2005. It is possible that variations in the field, transportation, and laboratory temperatures have affected the bioassay results (Schouest and Miller 1988). For example, lack of acclimation from the collection water temperature to the bioassay temperature may have altered the observed response.

Temperature is one of the most important factors affecting biological processes and the effects of temperature on pesticide toxicity have been well documented in mammals, birds, insects and other invertebrates (Scott 1995). Alterations in penetration, metabolism, and distribution within the animal and/or altered effectiveness at the target site have been suggested as possible factors contributing to temperature-toxicity effects (Scott 1995). Temperature has been shown to increase the toxicity of insecticides, possibly due to lower rates of physiological and metabolic processes of the test subject at lower temperatures (Johnson 1990). Although seasonal variations in the metabolism of other copepods have previously been documented (Siefken and Armitage 1968), the metabolic rate response of *L. salmonis* to acclimation periods or to small differences in

field and test temperatures are unknown (Tully *et al.* 2000) and there is a lack of information on temperature compensation in *L. salmonis*. However, previous arthropod research has shown that acclimation prior to insecticide treatment had no effect on toxicity in insecticide-susceptible and resistant German cockroaches (Valles *et al.* 1988). Thus, the effects of temperature on EMB toxicity of *L. salmonis* is evident but mechanisms of the effect require further investigation.

Although as many as 120 test subjects are required for a reliable full-scale dose-response experiment, as few as 60 test subjects can be used successfully as long as the doses are carefully selected (Robertson and Preisler 1992). In the current study, all bioassays were performed on a minimum of 70 healthy preadult sea lice exposed to 7 concentrations of EMB. However, a sufficient number of test subjects is often difficult to consistently obtain under field conditions (Brogdon 1989). Some areas in the Bay of Fundy have historically low lice burdens, while burdens in other areas may vary depending on environmental and management factors that contribute to reduced sea lice burdens. Although the number of sea lice collected in the field may have exceeded the minimum requirement for each bioassay, in some instances, upon arrival at the laboratory, the number of sea lice available for inclusion into bioassays was often limited due to the variability in the health of field-collected sea lice potentially affected by rougher handling when removing sea lice from fish and the effects of transport to the laboratory.

Day-to-day variations (i.e. water temperature, time of year, etc.) in bioassay results using field samples of sea lice are substantial, as was evident from the repeated measures analysis where only a marginally significant correlation between bioassay EC_{50}

values taken at the same site close in time was found. A feature of all biological assays, even under carefully controlled experimental conditions, is the variability in the reaction of test subjects and the difficulty in reproducing the same result in successive trials (Finney 1971). Variability between bioassay results from field samples may be attributed to a number of factors, including the total number of EMB treatments on a site throughout the year, and the proportion of sea lice from successive generations surviving EMB treatment. It is well established that the genes conferring resistance towards a chemotherapeutant are passed from one generation to the next as individuals within a population survive treatment. Resistance genes may increase in frequency within a sea lice population towards the fall of the year given the fact that as many as 5 or 6 successive generations of *L. salmonis* are possible in 1 year. Furthermore, the frequency of treatments on a site may be sporadic or regular depending on sea lice abundance in a given region or area. Thus, field populations of sea lice are likely comprised of a mixture of susceptible and less susceptible individuals. The apparent temperature-related decrease in EMB sensitivity experienced by sea lice through the fall of the year may be due to a decrease in EMB sensitivity through successive sea lice generations. An increase in the total number of EMB treatments in the field towards the fall of the year may also have resulted in previous and multiple exposures of successive generations of the same population of sea lice to EMB.

Sea lice from the same generation might be more alike in their response to a pesticide compared with sea lice in another generation. Unlike laboratory populations of sea lice where individuals of the same generation can be chosen for inclusion in a bioassay, under field conditions it is likely that sea lice collected for the bioassays are of

mixed genetic backgrounds. Thus, their susceptibility to EMB may vary naturally. The laboratory cultivated sea lice used in the current study, with no previous direct exposure to EMB, showed a lower EC_{50} value (21 ppb EMB) when compared to field samples (ranging from 25 to 118 ppb EMB). As a result of the inherent variability between bioassays, this protocol applied to field samples of sea lice should be used to describe population trends of EC_{50} values only. Individual bioassay values should not be interpreted in isolation as indicative of resistance development.

Bioassay development to date has focused on chemotherapeutants used as bath treatments. The development of bioassays using chemotherapeutants applied in-feed is a new application of the bioassay assessment. Under field conditions, sea lice become exposed to EMB by ingestion while feeding on the mucus, epidermis, and blood of treated fish. Following a week long medication period (50 $\mu g\, kg^{-1}$ feed d^{-1}), the average concentrations of EMB in Atlantic salmon were shown to be 128 ppb in plasma; 105 ppb in mucus; and 68 ppb in muscle. (Sevatdal *et al.* 2005b). The EMB concentrations chosen for inclusion in the bioassay cover the range of concentrations of EMB that sea lice would be naturally exposed to under field conditions while feeding on the mucus and blood of Atlantic salmon treated with SLICE®. The EMB bioassay relies on direct penetration and absorption of EMB through the cuticle of the sea louse so that it reaches its target site, the glutamate-gated chloride channels. The bioassay provides no information on the amount of EMB penetrating each sea louse. EMB may be inadvertently lost or its absorption limited due to adherence of EMB to the glass Petri dishes or non-target tissues in the sea louse, or the use of seawater as a solvent. Seawater collected from each site was used when setting up the bioassays and, although EMB

degradation is relatively slow in distilled water (less than 10 % over 30 days) (Roberts and Hutson 1999), its degradation in seawater is unknown. Seawater also contains organic particles that may bind EMB (Sevatdal 2005), limiting the amount of active EMB available to sea lice in the bioassay.

Accurate and regular monitoring for changes in susceptibility of sea lice populations, such as in the Bay of Fundy, will be essential for early detection of emerging resistance problems and the subsequent management (Denholm 1990). No information on resistance mechanisms is provided by the bioassay. Thus, it is important to recognize that even the occurrence of an apparent lack of response to an EMB treatment on a farm does not automatically indicate a change in sea lice sensitivity to EMB because there maybe other extenuating circumstances resulting in treatment success.

Future research should include the development of successive generations of a laboratory population of sea lice from the Bay of Fundy to use as a comparison to bioassays using field populations. Further investigation of resistance mechanisms would also be greatly facilitated by a known resistant sea lice population maintained in the controlled conditions of the laboratory. Information regarding the timing and frequency of field treatments using EMB should be investigated to determine a correlation between treatment frequency and increased EC_{50} values.

4.6 Conclusions

The results of this study suggest that sea lice collected during warmer field temperatures, when control is critical (i.e. in the spring and summer months), are susceptible to EMB. This seasonal and/or temperature associated variation in EMB

efficacy suggests that sea lice may be less sensitive to EMB at colder water temperatures or as they approach the colder seasons of fall and winter. Temperature may be an important variable when assessing sea lice sensitivity towards EMB in the field. It was not possible to fully validate the bioassay because there were no proven EMB resistant sea lice populations to use for comparison. Reduced treatment response should not automatically be equated with resistance development without eliminating possible extenuating factors more related to host or environmental factors.

4.6 Acknowledgements

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Table 4.1 Slopes and descriptive statistics of EC_{50} data for the years 2002 to 2004 (days, year[a] and region were included in the ANOVA model). CD: Campobello Island and Deer Island; GM: Grand Manan Island; LK: Lime Kiln Bay; PB: Passamaquoddy Bay.

Predictor	Predictor Level	Coefficient	SE	p-value
Region				0.682
	CD	21.20	18.53	
	GM	40.88	21.61	
	LK	27.79	14.57	
	PB	18.18	15.02	
Year				0.360
	2002	0		
	2003	35.58	24.79	
	2004	-1.71	31.66	
Days 2002		0.62	0.18	0.002
Days 2003		0.03	0.23	0.907
Days 2004		0.29	0.17	0.090

[a]2004 and 2005 data were grouped together as 2004 for ANOVA

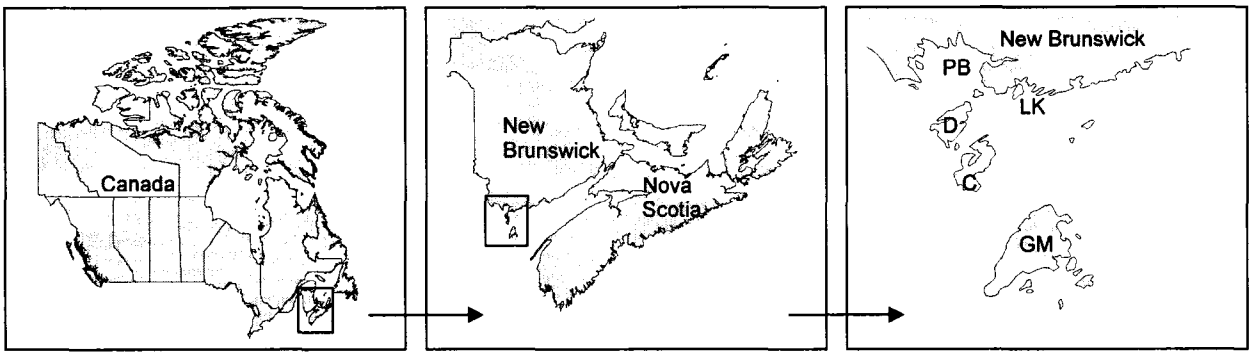

Figure 4.1 Bay of Fundy, New Brunswick, Canada, sea lice sampling regions: Campobello Island (C), Deer Island (D), Lime Kiln Bay (LK), Grand Manan Island (GM), and Passamaquoddy Bay (PB).

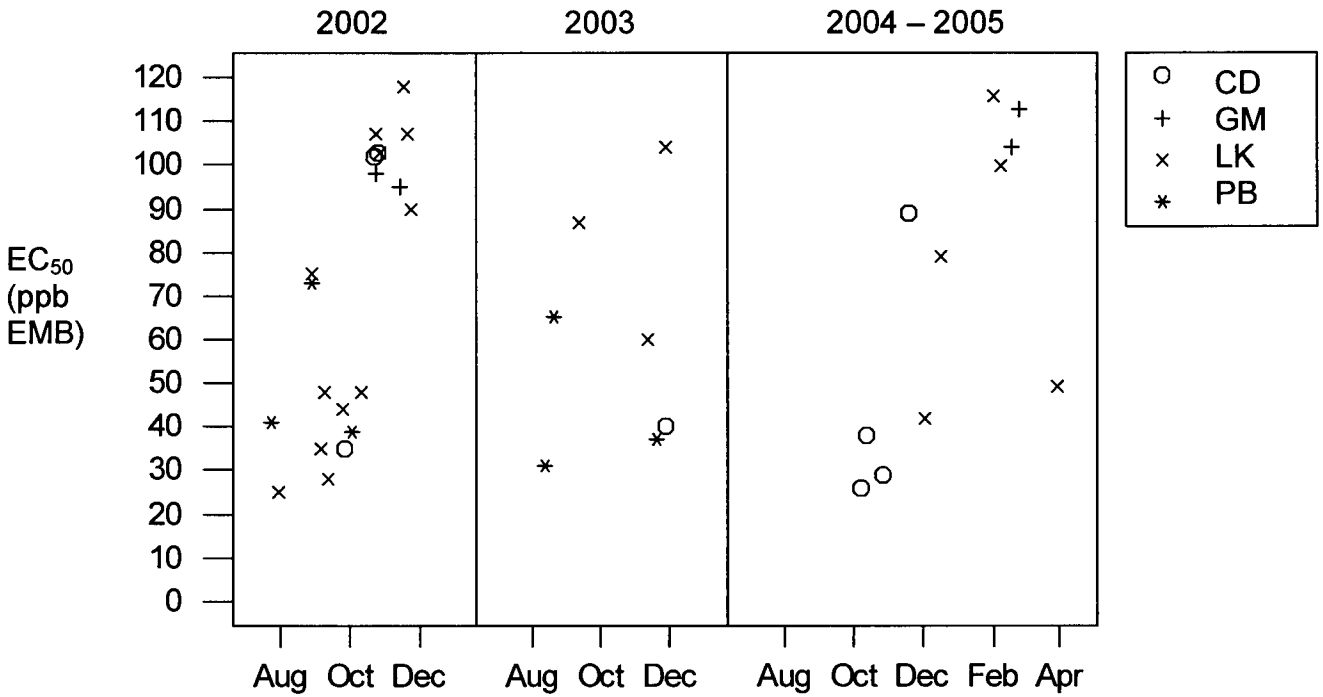

Figure 4.2 Emamectin benzoate bioassay EC_{50} values (ppb Emamectin Benzoate) for sea lice collected from Campobello Island and Deer Island (CD), Grand Manan Island (GM), Lime Kiln Bay (LK), and Passamaquoddy Bay (PB) for the years 2002 to 2005.

Chapter 5 Haem peroxidase activity measured in individual sea lice (*Lepeophtheirus salmonis*) from field and laboratory sources

5.1 Abstract

Up-regulation of metabolic oxidative enzyme-based systems, such as the cytochrome P450 (CYP) monooxygenases, is a major form of arthropod resistance. The haem peroxidase assay is a method used for measuring levels of haem-containing enzymes, such as the CYP monooxygenases, in individual test subjects. As a possible mechanism of sea lice resistance to EMB, oxidase enzymes are of particular interest. The haem peroxidase assay was evaluated for potential future utility in detecting changes in the sensitivity of field samples of sea lice (*Lepeophtheirus salmonis*) to EMB. The Bay of Fundy salmon farming area was divided into 4 distinct regions: Campobello Island and Deer Island (CD), Grand Manan Island (GM), Lime Kiln Bay (LK), and Passamaquoddy Bay (PB), based on hydrographics and industry health management practices. Field samples of adult female *L. salmonis* were collected during the years 2001 to 2005 from Atlantic salmon (*Salmo salar*) marine farm sites in the four described regions. Additionally, a single-generation laboratory population of sea lice was cultivated in 2005. The haem peroxidase assay was used to measure total non-specific oxidase activity (expressed as mg cytochrome C-equivalents g^{-1} protein) in a total of 1162 individual adult female *L. salmonis* collected from field sources and 82 laboratory sea lice samples. Median haem peroxidase values for field and laboratory samples were 3.7 and 13.0, respectively. There were significant effects of region ($p=0.003$), and a seasonal trend ($p=0.03$) on median haem peroxidase values for 2001 to 2004 field sea lice samples, but no significant effects in the 2005 field data. There was a positive, but not statistically

significant, correlation between emamectin benzoate sensitivity (bioassay EC_{50} values) and median haem peroxidase activity in field sea lice samples for the years 2001 to 2005 ($r=0.23$; $p=0.28$). The current utility of a test measuring total oxidase activity within individual adult female sea lice is limited as a stand-alone diagnostic tool for resistance detection

5.2 Introduction

Sea lice (*Lepeophtheirus salmonis*) are ectoparasitic crustaceans afflicting Atlantic salmon (*Salmo salar*) throughout eastern North America and northern Europe (Costello 1993, Treasurer and Pope 2000). A combination of year-round biological and chemical methods of sea lice control is required to maintain the health and productivity of commercially farmed Atlantic salmon. Teflubenzuron (Calicide®, Trouw Aquaculture) and emamectin benzoate (EMB; SLICE®, Schering-Plough Animal Health) are the only in-feed chemotherapeutants currently available for sea lice control in Canada (SLICE® is available through Emergency Drug Release as it progresses through the drug approval system at Veterinary Drugs Directorate of Health Canada) . The use of EMB, the active compound in SLICE®, for sea lice control in the Bay of Fundy has become widespread due to the limited availability of effective control options, and its advantages over other currently available chemotherapeutants, such as extended field efficacy and effect on all sea lice stages. Although it has yet to be detected, there is a concern that reduced sensitivity of sea lice to EMB will develop. The limited number of chemotherapeutants being commercialized for sea lice control, and the widespread use of available chemotherapies, necessitates the development of methods to monitor for changes in sea

lice sensitivity towards EMB to provide early indication of changes in sea lice susceptibility.

Two major forms of biochemical resistance in arthropods include target site resistance and up-regulation of metabolic oxidative enzyme-based systems (Denholm and Rowland 1992, Clark *et al.* 1994, Ahammad-Sahib *et al.* 1994, Brogdon and McAllister 1998, Liu and Yue 2001). The most prominent group of drug metabolizing enzymes is a superfamily of cytochrome P450s (also called mixed-function oxidases or P450 monooxygenases) (Rang *et al.* 2003). P450 monooxygenase-mediated metabolism is a common mechanism by which insects become resistant to insecticides (Ahammad-Sahib *et al.* 1994, Kasai *et al.* 1998, Scott 1999). The mechanism of resistance associated with P450 is either the overproduction of the P450 enzyme involved, or point mutations in the P450 gene increasing the enzyme's detoxification capacity (Fisher *et al.* 2003, Nikou *et al.* 2003).

Measuring the level or degree of resistance in a population requires comparisons between susceptible and resistant individuals within the population. Approaches to the measurement of oxidative resistance in insects include *in vivo* measurement of the ability of antioxidant synergists to increase insecticide toxicity in resistant populations and *in vitro* measurement of an increase in total mixed-function oxidase activity (Plapp 1975); such tests are often used in combination with each another. Total oxidase measurement in individual mosquitoes associated with insecticide metabolism has been established using a haem peroxidase assay that measures total non-specific oxidase activity in single mosquitoes as a means to identify individuals expressing elevated oxidases for insecticide resistance (Brogdon *et al.*, 1997). Research by Sevatdal *et al.* (2005) using piperonyl

butoxide (an oxygenase inhibitor) and total non-specific oxidase activity coupled with bioassay EC_{50} values for pyrethroids has provided evidence for monooxygenase mediated pyrethroid detoxification in sea lice.

As a potential means of EMB detoxification by sea lice, oxidase enzymes are of particular interest despite the fact that the mechanisms of sea lice resistance to EMB have yet to be identified in the absence of reported resistance. Effective resistance management strategies could include the development of biochemical tests with the ability to detect elevated enzyme levels associated with resistance development in individual sea lice. Such diagnostic tests of resistance development to EMB could be useful in the field as a means of early detection of changes in the frequency of resistant individuals where populations are likely to consist of varying proportions of susceptible and resistant individuals. However, inherent variability of oxidase levels within EMB-susceptible populations must be evaluated if the haem peroxidase assay is to be used to diagnose resistance development to EMB in sea lice populations. The monooxygenase system has been used as an indicator of stress and contamination in fish species and factors such as temperature, nutritional stress, season, and reproductive state have been shown to influence monooxygenase activity (Jimenez and Stegeman 1990).

The haem peroxidase assay was evaluated for potential utility in detecting changes in the sensitivity of field samples of sea lice (*Lepeophtheirus salmonis*) to EMB. The specific objectives of this study were: (1) to quantify total non-specific oxidase activity in individual adult female sea lice from two EMB-susceptible populations, a field population with potential variable exposure to EMB, and a laboratory population with no direct exposure; and (2) to correlate haem peroxidase values with EMB bioassay EC_{50}

values conducted on preadult stage male and female sea lice in the same sample populations.

5.3 Materials and Methods

5.3.1 Field collections

Based on ocean current patterns and industry health management policies, the Bay of Fundy salmon farming area was, for study comparison purposes, divided into 4 distinct regions: Campobello Island and Deer Island (CD), Grand Manan Island (GM), Lime Kiln Bay (LK), and Passamaquoddy Bay (PB) (Fig. 4.1). Sea lice were collected from areas where previous exposure to EMB for sea lice management purposes was possible, and where there were no documented reports of treatment failures with the chemotherapeutant. Enzyme assays were conducted on sea lice collected during the years 2001 to 2005 from Atlantic salmon (*Salmo salar*) marine farm sites in the four described regions. For sufficient sample volume to conduct the laboratory testing, only live adult female *L. salmonis* were used in the bioassay. Sampling was dictated by availability of sea lice, and the cooperation and participation of sea cage sites in the Bay of Fundy. Sea lice were collected from Atlantic salmon smolts, pre-market fish, and/or broodstock, during routine sea lice counting or harvesting on site. Lice were removed from fish anesthetized with tricaine methanesulfonate (TMS, Syndel Laboratories, Vancouver, BC) at a concentration of 50 to 100 mg l^{-1} for routine sea lice counting, or from fish immobilized for harvest (carbon dioxide, ice water, or percussive stunning). Sea lice were gently removed from Atlantic salmon using forceps and placed into a container of seawater collected from the site. Battery operated air pumps were added to

collection containers. Ice packs were placed in a cooler with the collection containers to ensure that the lice were kept cool during transport to the laboratory. The bodies of sea lice were blotted on paper towel to remove excess water and rapidly frozen by placing them on a thin metal plate previously stored at -80°C.

5.3.2 Sea lice cultivation

Laboratory cultivated sea lice used in this study were the first generation progeny of *L. salmonis* adult females collected from the Bay of Fundy during a cage harvest in July 2005 on an Atlantic salmon sea cage site. Gravid females were collected and transported to the laboratory as described above. Egg-strings were carefully removed from female sea lice and hatched and reared in a re-circulation system containing Atlantic salmon in synthetic saltwater (Instant Ocean®, Aquarium Systems, Mentor, OH). Sea lice were grown on Atlantic salmon held in the re-circulation system. On two separate collection days, two weeks apart, Atlantic salmon were anaesthetized using TMS (50 to 100 mg l^{-1}), and adult female sea lice were collected using forceps and placed into Petri dishes containing synthetic saltwater.

5.3.3 Sample preparation

Groups of ten individually frozen adult female sea lice were removed from storage in the -80°C freezer and individually homogenized on ice in 500 µl of chilled sodium acetate buffer (0.25 M, pH 6.0, adjusted with acetic acid) in separate 1.5 ml microcentrifuge tubes using separate motorized plastic pestles. Tubes were vortexed for 30 s and centrifuged at 7500 rpm for 4 min at 4°C. The supernatant was transferred to a

Spin-X tube, centrifugation was repeated, and the supernatant for individual samples from each of the Spin-X tubes was pooled. The resulting supernatant was stored on ice and used directly for the determination of haem peroxidase activity and a 100 µl aliquot of supernatant was added to 200 µl of sodium acetate buffer in a new 1.5 ml microcentrifuge tube and temporarily stored at -20°C for use in the total protein determination of individual samples (as a means of standardizing for enzymatic activity) (Sevatdal *et al*, 2005).

5.3.4 Haem peroxidase assay

This indirect method for determination of haem peroxidase activity in single adult female sea lice is an adaptation of the established method for detecting haem peroxidase activity in single mosquitoes (Brogdon *et al.*, 1997). It is based on the measurement of peroxidase activity in haem-containing enzymes using the 3,3′,5,5′- tetramethylbenzidine (TMB) liquid substrate system (Sigma T 8665) (Sevatdal *et al.*, 2005). A 200 µl aliquot of TMB was added to 100 µl aliquots of supernatant in duplicate on a flat-bottomed microtitre plate. The plate was shaken briefly (20 to 30 s) by hand, incubated for 3 min at room temperature, and read in kinetic mode at 630 nm every 20 s for 5 min at 25°C on a Bio-Tek Micro Titre Plate Reader. Serial dilutions of cytochrome C (equine) (Sigma C 8857) (ranging from 0.391- 12.5 µl cytochrome C ml^{-1}) were used to produce a standard curve against which the maximum slope of the kinetic curve was compared in order to determine enzyme activity of individual sea lice samples.

5.3.5 Protein assay

Measurement of total protein was determined per homogenate supernatant using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA), a commercially available protein assay based on the method of Bradford (1976), modified for microtitre plates (Brogdon 1983), using bovine serum albumin (BSA) as a standard. BioRad dye was diluted 1:5 with deionised water and filtered to remove particulates. The standard curve was prepared from BSA stock (0.5 mg ml^{-1}) stored at -20°C. BSA concentrations used to prepare the standard curve were 0, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 mg ml^{-1}. A 200 µl aliquot of diluted dye reagent was pipetted into each of seven wells containing 10 µl of BSA standard, in triplicate. Plates were incubated at room temperature for 5 min and absorbance measured at 595 nm on the Bio-Tek Micro Titre Plate Reader.

5.3.6 Statistical Analysis

5.3.6.1 Calculation of haem peroxidase activity

Haem peroxidase activity, expressed as mg cytochrome C-equivalents g^{-1}protein, was calculated using the formula (previously used in a study by Sevatdal *et al.* 2005):

$$\frac{\text{µg cytochrome C-equivalents}}{(\text{mg ml}^{-1}\text{protein} * \text{protein dilution} * 0.0001)}$$

Cytochrome C-equivalent values falling outside the quantification limit of the standard curve led to a censoring of the cytochrome value, and hence, also of the haem peroxidase values. Cytochrome values below the quantification limit were left censored at 0.001, a value substantially below any observed cytochrome value (i.e. the unobserved

value was considered below 0.001. The upper cytochrome quantification limit was 13.105, so unobserved values were considered larger than this value (i.e. right censored at 13.105).

Descriptive statistics were computed separately for individual field and laboratory samples, and their distributions were fit to normal, log-normal and gamma distributions (a flexible family of right-skewed probability distributions with constant coefficients of variation, McCullagh and Nelder, 1989).

5.3.6.2 Analysis of variance for sea lice sampling

The unit of analysis of interest was sampling day, and the analysis was based on median haem peroxidase values among lice sampled on the same day. Sea lice collections for 2001 to 2004 data occurred between July and December of each year; 2005 collections occurred between January and August, and due to the different sampling periods, data for 2005 was analyzed separately from the 2001 to 2004 data. Region, year, and season were included in an analysis of variance (ANOVA) model for 2001 to 2004 data, whereas the year variable was omitted in a similar model for the 2005 data. Seasonal effects were modeled by linear trends spanning the respective sampling periods, represented in the models by the number of days elapsed since a fixed date within each year. All first order interactions were initially included and later removed if judged insignificant; the significance level was set at 0.05. The residuals were used to validate model assumptions and detect any overly influential observations.

5.3.6.3 Correlation of haem peroxidase and bioassay data

The Spearman correlation coefficient was used to evaluate the relationship between field-sourced preadult sea lice EMB sensitivity (expressed as EC_{50} values in ppb EMB) obtained through bioassay analysis (described in Chapters 3 and 4) and the corresponding average adult female haem peroxidase values for 2001 to 2005 data.

5.4 Results

5.4.1 Haem peroxidase values for field and laboratory lice

A total of 1162 individual adult female samples from 2001 to 2005 field sources and 82 laboratory source samples were assayed. A total of 6.0% (70 of 1162) of the field data were left censored (i.e. to 0.001), and 1.9% (22 of 1162) were right censored (i.e. to 15), whereas 7.3% (6 of 82) of the laboratory data were left censored and 0% were right censored. Frequency distributions for individual haem peroxidase values in field and laboratory samples were strongly right-skewed (Fig. 5.1), and the best fits were obtained by gamma distributions. Haem peroxidase values for field sea lice samples ranged from 0.002 to 97.1 μg cytochrome C-equivalents g^{-1} protein with a median value of 3.7 and a coefficient of variation of 143% between sea lice samples; laboratory samples ranged from 0.005 to 69.8 μg cytochrome C-equivalents g^{-1} protein with a median value of 13.0 and a coefficient of variation of 97%.

5.4.2 Analysis of variance

The data included 38 sampling days for 2001 to 2004 and 13 sampling days in 2005; on average, each sampling day comprised, on average, 23 sea lice (range 4 - 69).

The median haem peroxidase activity ranged from 0.005 to 24.34 μg cytochrome C-equivalents g^{-1} protein (Fig. 5.2). The censoring was only of importance for the computed median haem peroxidase activity on two sampling days where more than half of the lice were left censored. The final ANOVA model for 2001 to 2004 sea lice data had significant effects of region (p=0.003) and a seasonal trend (estimate=0.04, SE=0.02, p=0.033). For the 2001 to 2004 data, pairwise comparisons between regions (adjusted by the Bonferroni method) showed PB was significantly different from CD and LK. The two 2005 laboratory sea lice populations sampled at approximately 6 and 8 weeks post-copepodid challenge had median haem peroxidase activity of 5.56 and 16.95 μg cytochrome C-equivalents g^{-1} protein, respectively (p=0.002 by two-sample Mann-Whitney test).

5.4.3 Correlation of oxidase and bioassay data

A positive but not statistically significant correlation was found between EMB sensitivity (expressed as EC_{50} values) and median haem peroxidase values (r=0.23; p=0.28). These values reflect evaluations of adult female sea lice only. Preadult sea lice were not tested for individual haem peroxidase values.

5.5 Discussion

The results of this study demonstrate a seasonal or temperature associated variation in adult female haem peroxidase values for 2001 to 2004 field samples. Although the cause of this effect in the current study is not known, the influence of season and temperature on enzyme levels has been shown in other invertebrate and

vertebrate species. One common example is that of the biotransformation enzyme cytochrome P4501A monooxygenase (CYP1A). CYP1A induction is commonly used as a biomarker in fish (Behrens and Segner 2005) for exposure to xenobiotics and environmental contaminants, and has been used as a biomarker in the zebra mussel (*Dreissena polymorpha*) (Ricciardi *et al.* 2005) and earthworm (*Aporrectodea tuberculata*) (Lukkari *et al.* 2004). Season and temperature have been implicated among the exogenous factors influencing CYP1A induction (Arinç *et al.* 2000), although CYP1A has not been confirmed in sea lice. Behrens and Segner (2005) showed that CYP1A levels varied according to season in liver samples from brown trout exposed to small streams of an urbanised area over a five-year period, in which levels were higher for October and November compared to May, July, and September. A field investigation by Gorbi and Regoli (2004) showed that CYP1A levels in liver samples of eels (*Anguilla anguilla*) collected from an unpolluted lagoon were significantly higher in August and October compared to January and April.

In the case of the current study, seasonal variation may be related to an increase in exposure of successive generations of sea lice to EMB treatments throughout the salmon production cycle, as sea lice burdens and the frequency of EMB treatments varied between the study regions. Sea lice in some areas may have little or no previous exposure to EMB, while in other areas, sea lice may be exposed to repeated treatments over the Atlantic salmon production cycle. Ocampo *et al.* (2000) detected increased enzyme activity in susceptible mosquito populations that had previous heavy exposure to agricultural pesticides. Collection of EMB treatment information was beyond the scope

of this study, making conclusions regarding the effects of previous EMB exposure on haem peroxidase levels in sea lice from field sources an unanswered question.

High inter-individual variability of enzyme levels is not uncommon for insect enzyme activity. In a horn fly population that had not been exposed to insecticides for 8 years, considerable variation in general esterase activity within individual samples of each sex, with females having demonstrably greater variability (Pruett *et al.* 2001). Similarly, the results of this study demonstrate a high degree of inter-individual variability with respect to haem peroxidase levels in two sea lice populations.

The absence of a known sea lice population exhibiting clinical signs of resistance to EMB makes it very difficult to interpret the inter-individual variability that may exist in such a population. However, appreciation of the inherent variability of oxidase levels within clinically susceptible field populations is required knowledge if this testing is to be used to detect the emergence of resistance in sea lice populations. The large inherent variation in susceptible sea lice populations determined by this study suggests that small mean differences in total oxidase activity between populations will be difficult to attribute to changes in clinical sensitivity to EMB.

Although variations in CYP levels do occur at different stages of insect development (Agosin 1985, Snyder *et al.* 1995), it is not known whether the age of test subjects may influence the variability in haem peroxidase levels between individual adult female sea lice. In the current study, a small group of laboratory cultivated adult female sea lice had significantly higher haem peroxidase levels than those assayed two weeks earlier.

Quantification of haem peroxidase activity has been used successfully to identify

individual mosquitoes expressing an elevated oxidase for insecticide resistance (Brogdon *et al.* 1997) and also as a biomarker in *Daphnia magna* for toxicity assessments of contaminated groundwater (Connon *et al.* 2003). However, the variability in enzyme levels detected in the current study and the seasonal effect of the field data suggests that the utility of the haem peroxidase assay is limited as a stand-alone test.

An alternative to determination of total oxidative activity in individual sea lice is the N-demethylase assay. Oxidative N-demethylation is the main route of EMB metabolism (Mushtaq *et al.* 1996). However, results from our laboratory have indicated that the utility of this assay for field monitoring may be limited due to the requirement for pooling of large numbers of sea lice and the inability of the test to detect sensitivity changes in individual test subjects (unpublished data).

Non-specific assays, such as the haem peroxidase assay described in this study, have a number of inherent advantages and disadvantages. Non-specific oxidase testing does not require knowledge of the precise mechanism responsible for changes in oxidase levels and resistance. However, the assay has the advantage of being able to test duplicate and triplicate samples from individual sea lice, thus eliminating the requirement for pooling of samples and providing greater access to individual measurements for a more powerful statistical analysis. Testing individuals also allows for the evaluation of inter-individual variability in enzyme levels.

Future research should involve an evaluation of the effect of temperature on enzyme activity in field samples of sea lice. If resistance to EMB should occur it may be possible to indicate the involvement of non-specific oxidases through *in vivo* measurement of the ability of oxygenase inhibitors, such as piperonyl butoxide (PBO), to

increase EMB susceptibility in sea lice populations. Detection of elevated levels of general oxidase activity may then provide a means for the rapid detection of this mechanism as an indication of decreased sensitivity towards a drug, particularly when combined with bioassays.

5.6 Conclusions

Results from this study provide basic information regarding the natural variability that exists in total non-specific oxidase activity of EMB-susceptible populations of field and laboratory samples of adult female sea lice. The current utility of a test measuring total oxidase activity within individual adult female sea lice is limited as a stand-alone diagnostic tool for resistance detection due to the absence of sea lice populations known to be resistant to EMB, the inherent variability of enzyme levels, and poor correlation of oxidase values with bioassay results. However, the ability of the haem peroxidase assay to identify individual sea lice displaying elevated oxidative activity is encouraging, as field populations are likely to consist of a mixture of susceptible and less susceptible individuals.

5.7 Literature cited

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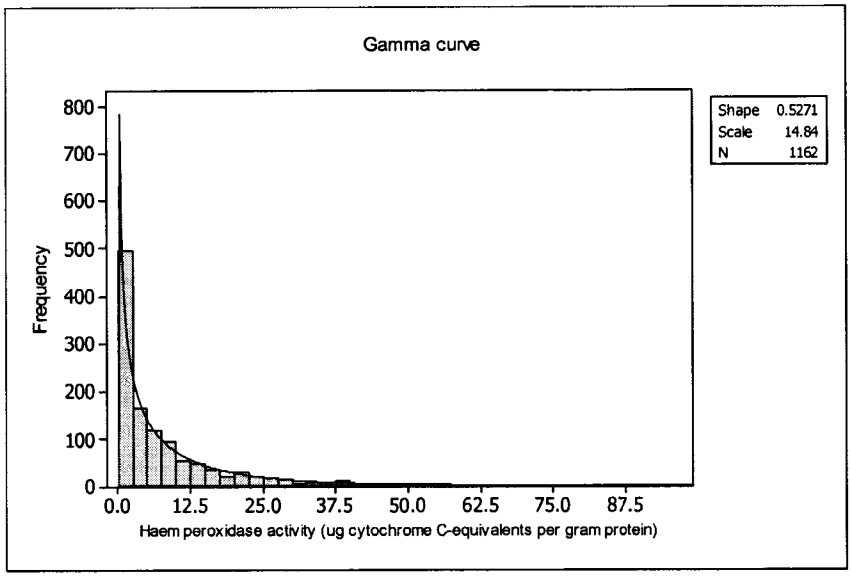
(a) Field samples from 2001 to 2005.



(b) Laboratory cultivated samples.

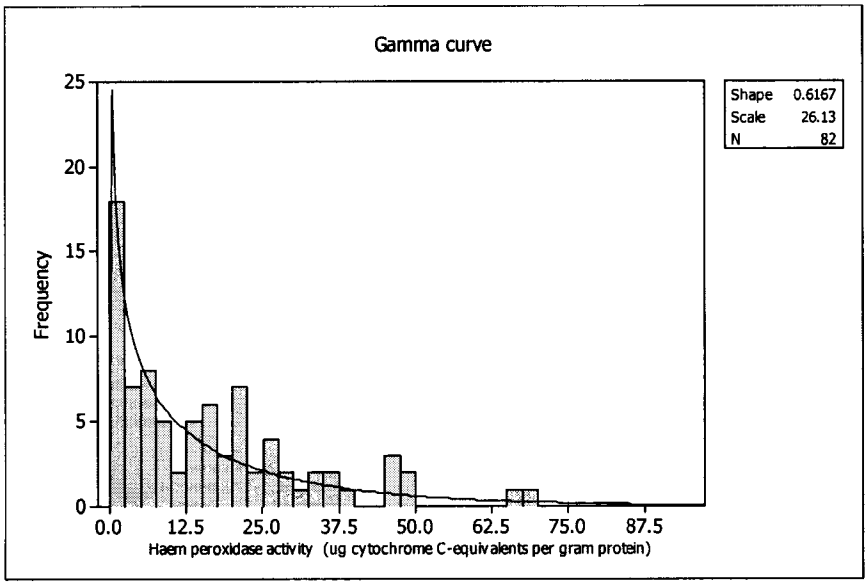


Figure 5.1 Frequency distribution of individual haem-peroxidase activity for field and laboratory samples of adult female sea lice (*Lepeophtheirus salmonis*), with overlaid best-fitting gamma distribution curves.

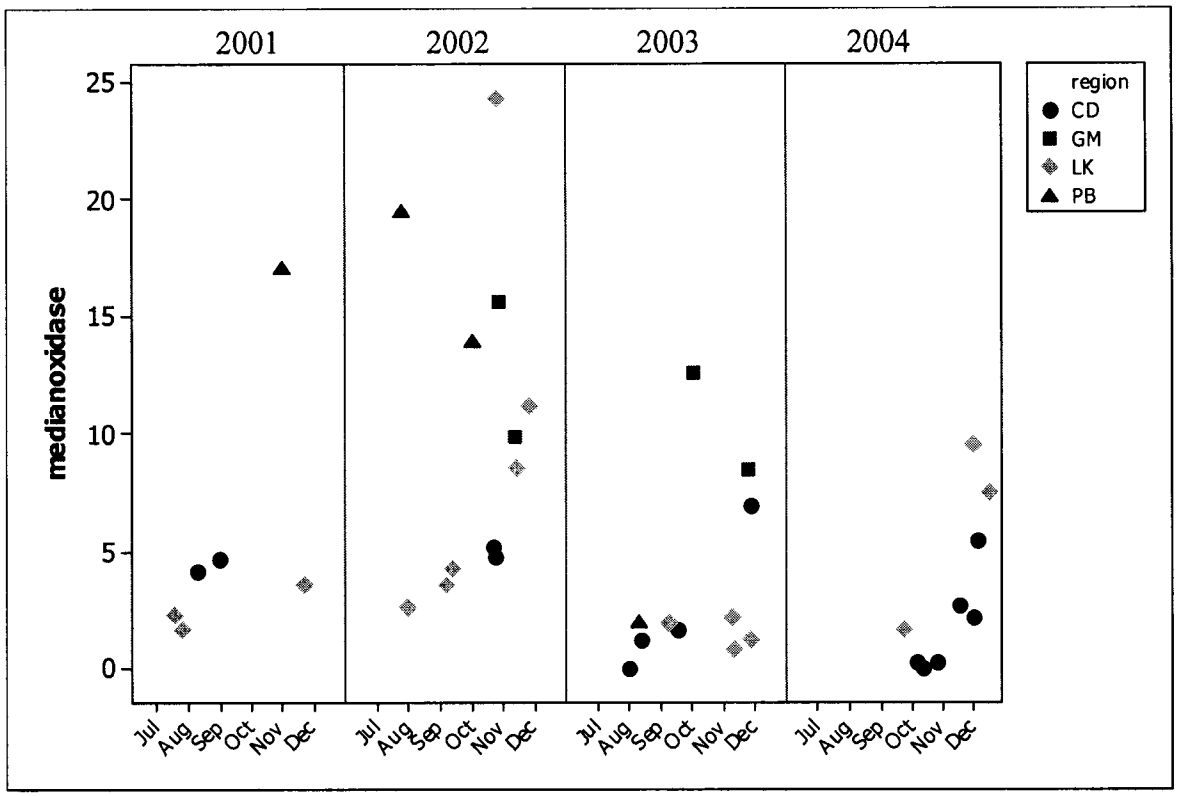


Figure 5.2 Median haem peroxidase activity (μg cytochrome C-equivalents g^{-1} protein) for adult female sea lice (*Lepeophtheirus salmonis*) sampled from four regions in the Bay of Fundy for the years 2001 to 2004. CD: Campobello Island and Deer Island; GM: Grand Manan Island; LK: Lime Kiln Bay; PB: Passamaquoddy Bay.

Chapter 6 Validation studies on total oxidase quantification for resistance of sea lice (*Lepeophtheirus salmonis*) to emamectin benzoate

6.1 Abstract

An increase in cytochrome P450 (CYP)-dependent monooxygenase detoxification activity is a common mechanism of drug resistance amongst arthropods. In an effort to evaluate the utility of total oxidase quantification in resistance monitoring of sea lice (*Lepeophtheirus salmonis*) to emamectin benzoate (EMB), studies were carried out to examine several potential factors influencing oxidase activity in EMB-susceptible field collected *L. salmonis*. Total oxidase activity was quantified in individual sea lice using an established haem peroxidase assay which measures levels of haem-containing enzymes, such as CYP monooxygenases. There were no significant time-dependent changes in haem peroxidase activity up to 72 h post mortem for sea lice stored at 3 to 4°C. There was no significant difference in haem peroxidase values for live and dead or recently frozen and non-frozen sea lice. Storage at -80°C for up to 30 days resulted in significantly lower haem peroxidase activity. Haem peroxidase values were higher following a 24 h versus 48 h EMB exposure period. Effects of condition and concentration were variable in the three sea lice sources. Dead sea lice had the highest haem peroxidase values in source 1 but the lowest in source 3. Sea lice exposed to 100 ppb EMB had the highest haem peroxidase values in source 1 and the lowest in source 2. Haem peroxidase levels cannot be used as stand-alone predictors of resistance. They should be used in combination with bioassays or other tests (i.e. molecular) when resistance occurs and the mechanism has been properly identified.

6.2 Introduction

Sea lice (*Lepeophtheirus salmonis*) are ectoparasitic copepodid crustaceans which occur on wild and farmed marine salmonids throughout eastern North America and northern Europe, posing an on-going management issue for the health and productivity of commercial culture (Costello 1993). Sea lice management and control is dependent upon a combination of biological and chemical methods. Several groups of chemotherapeutants used for sea lice control have been compromised due to changes in sea lice sensitivity and reports of resistance development (Treasurer *et al.* 2000, Tully and McFadden 2000, Sevatdal and Horsberg 2003).

Early indication of changes in the sensitivity of field population of *L. salmonis* towards a chemotherapeutant is imperative to detecting and managing resistance, especially when the number of chemotherapeutants being commercialized is limited. Monitoring sea lice abundance on farmed fish through regular sea lice counting after EMB treatment is one common approach that may provide farmers with an early indication of changes in sea lice sensitivity to EMB. Other signs of changes in sensitivity in the field may include an increase in the number of treatments per salmonid production cycle or the shortening of intervals between treatments, as well as the requirement for higher doses of a chemotherapeutant to control sea lice at the appropriate threshold (Schering-Plough Animal Health 2000).

There are many factors unrelated to resistance development that can contribute to a treatment failure in the field; therefore a more sensitive diagnosis of a decrease in sensitivity or of resistance development is required. Methods currently available for diagnosing sea lice resistance to chemotherapeutants are limited. Established bioassays

have been used to detect decreases in sea lice sensitivity and resistance development towards pyrethroids used for sea lice control in Europe (Sevatdal and Horsberg 2003). However, bioassays have a number of inherent disadvantages, including the requirement for numbers of test subjects in excess of that routinely available during field collections. Furthermore, bioassays are unable to detect sensitivity changes in individual sea lice and they do not provide information on the mechanism of resistance involved. These disadvantages necessitate the development of alternative tests for rapid and sensitive resistance detection. An alternative to the use of bioassays for resistance detection is the use of biochemical tests. Biochemical methods of resistance detection offer a number of advantages over bioassay methods which include the ability to detect sensitivity changes in individual test subjects and confirm resistance with the use of only a small number of subjects (Brogdon and McAllister, 1998).

Increased enzymatic detoxification due to the enhancement of enzyme systems (i.e. esterases, GST, or CYP-dependent oxidation) is a common resistance mechanism amongst arthropods (Brogdon and McAllister 1998). A number of biochemical assays have been established for quantifying the activity of esterases (Van Asperen 1962, Brogdon and Dickinson 1983, Grafton-Cardwell *et al.* 2004), monooxygenases (Brogdon *et al.* 1997) and GST (Brogdon and Barber 1990) associated with arthropod resistance. Measurement of esterase and monooxygenase activity in sea lice by Sevatdal *et al.* (2005) indicated that the overproduction of nonspecific esterases in adult female sea lice is not a significant resistance mechanism towards pyrethroids. However, they provided evidence of monooxygenase mediated pyrethroid resistance in sea lice.

The limited number of chemotherapeutants commercially available for sea lice control and the widespread use of the in-feed treatment EMB for sea lice control on fish farms in the Bay of Fundy area is cause for concern regarding the potential for sea lice to develop resistance (Chapter 2). Even in the absence of EMB resistance, the development of rapid and sensitive methods for the detection of enzymes associated with resistance may be beneficial so that early indication of changes in the sensitivity of field populations of sea lice towards a chemotherapeutant can be detected, and the resistance mechanisms responsible can be identified. If sea lice resistance to EMB is identified in the future and oxidative metabolism is indicated as one potential resistance mechanism, either through biochemical or molecular diagnostic tests, there may be utility of total oxidase quantification in individual sea lice. However, it will be necessary to understand the inherent variability of oxidative enzyme levels within EMB-susceptible populations of sea lice if such a test is to be used to diagnose resistance development (via the mechanism of oxidative metabolism) in sea lice field populations.

The objective of the current study was to provide basic information on the potential factors influencing total oxidase activity in EMB-susceptible field sea lice populations. The study included an evaluation of: (1) the effect of sample storage at -80°C on oxidative enzyme stability; (2) the effect of sea lice condition and freezing on oxidative activity; (3) post-mortem enzyme stability; and (4) a comparison of oxidative activity in live, moribund and dead sea lice following 24 and 48 h exposure periods to four EMB concentrations.

6.3 Materials and Methods

6.3.1 Sea lice collection

Healthy adult and gravid female sea lice (*L. salmonis*) were collected from Atlantic salmon (*Salmo salar*) marine farms sites in the Bay of Fundy, on the east coast of Canada, during routine harvesting on site. Fish were immobilized using carbon dioxide, ice water, or percussive stunning, and sea lice were gently removed using forceps and placed into a container of seawater collected near the farm site. Battery operated air pumps were added to collection containers and ice packs were placed in a cooler with the collection containers to ensure sea lice were kept cool and aerated during transport to the laboratory. In the laboratory, egg strings were carefully removed from gravid females using fine scissors. Prior to storage of individuals in separate 1.5 ml microcentrifuge tubes at -80°C, whole lice bodies were blotted on paper towel to remove excess water and quick-frozen by placement on a thin metal plate previously stored at -80°C.

6.3.2 Sample preparation

Detailed descriptions of the methods used for sample preparation, haem peroxidase and total protein determination are available in the chapter 5. Briefly, groups of ten individually frozen adult female sea lice were individually homogenized on ice in 500 µl of chilled sodium acetate buffer (0.25 M, pH 6.0) in separate 1.5 ml microcentrifuge tubes using separate motorized plastic pestles. Tubes were vortexed for 30 s, centrifuged at 7500 rpm for 4 min at 4°C and the resulting supernatant was transferred to a Spin-X tube. Centrifugation was repeated and the supernatant for

individual samples from each of the Spin-X tubes was pooled and stored on ice for use in the determination of haem peroxidase activity. An additional 100 µl of each supernatant was added to 200 µl of sodium acetate buffer in new 1.5 ml microcentrifuge tubes and temporarily stored at -20°C for subsequent protein determination.

6.3.3 Haem peroxidase assay

100 µl aliquots of supernatant from the haem peroxidase assay were added to 200 µl of sodium acetate buffer in new 1.5 ml microcentrifuge tubes. 200 µl of 3,3′,5,5′ tetramethylbenzidine (TMB) was added to 100 µl aliquots of supernatant in duplicate on a flat-bottomed microtiterplate. The plate was briefly shaken by hand, incubated for 3 min at room temperature, and read in kinetic mode at 630 nm every 20 s for 5 min at 25°C on a Bio-Tek Microtiter Plate Reader. Individual enzyme activity was determined by comparing the maximum slope of the kinetic curve to a cytochrome C (Sigma C9957) standard curve.

6.3.4 Protein determination

BioRad dye was diluted 1:5 with deionised water and filtered to remove particulates. The standard curve was prepared from bovine serum albumin (BSA) stock (0.5 mg ml^{-1}) stored at -20°C. A 200 µl aliquot of diluted dye reagent was added to each of seven wells containing 10 µl of BSA standard in triplicate. Plates were incubated at room temperature for 5 min and absorbances measured at 595 nm on the Bio-Tek Microtiter Plate Reader.

6.3.5 Freezing experiment

Adult female sea lice were collected as described previously. Upon arrival at the laboratory, 60 sea lice were identified as live (based on a normal swimming behavior and the ability to cling to the side of the collection container) and 50 as dead (based on lack of movement or response following stimulation with forceps); time to death was estimated as within 4 to 6 hours of collection. Live sea lice were further subdivided into two groups; 30 lice were immediately assayed (labeled L) and another 30 were quick-frozen at -80°C (labeled LF) and assayed at a later date. Dead sea lice were further subdivided into two groups; 30 lice were immediately assayed (labeled D) as described for live sea lice and 20 were quick-frozen at -80°C (labeled DF) and assayed at a later date. All individual haem peroxidase values were determined using the standardized protocol.

6.3.6 The effect of sample storage on enzyme stability

Sea lice were collected and frozen at -80°C as described previously. Ten sea lice were immediately removed from storage at -80°C and assayed. Subsequent groups of 10 sea lice were removed from -80°C and assayed at 6, 12, 24 and 48 h, one and two weeks, and one month post storage. All individual haem peroxidase values were determined using the standardized protocol.

6.3.7 Post-mortem enzyme stability

Field samples of sea lice were collected as described previously. Live adult female sea lice (with egg strings removed) (approximately 10 per dish) were maintained at 3 to 4°C in plastic Petri dishes containing seawater from the collection site. Eleven

live sea lice were immediately removed (t = 0) and quick-frozen at -80°C. Sea lice were monitored at 2 h intervals to identify dead sea lice (based on lack of movement or response following stimulation with forceps). Those sea lice identified as dead were moved to Petri dishes containing seawater and labeled with the approximate time of death. Dead sea lice were then removed from the Petri dishes and quick-frozen at -80°C at 1, 6, 12, 24, 48 and 72 h post-mortem. All sea lice were later assayed for individual haem peroxidase levels using the standardized protocol.

6.3.8 Total oxidase activity in live, moribund and dead sea lice following EMB exposure

Sea lice were collected as described previously, with the exception that live sea lice were used in this experiment. Ten adult females (with egg strings removed) were placed into each of 16 glass Petri dishes containing seawater collected near the collection site in the Bay of Fundy. Groups of four Petri dishes were allocated to each of four EMB concentrations; 0, 100, 200 and 300 ppb. Two of the four dishes at each concentration were allocated to a 24 h exposure period and two to 48 h. Seawater in each dish was replaced with the allocated EMB concentration and all dishes (with lids in place) were stored at 3 to 4°C. Following 24 h and 48 h exposure periods, the sea lice from each dish were individually categorized as live, moribund or dead according to the established bioassay response criteria. Sea lice were then quick-frozen on a thin metal plate stored at -80°C and placed into individually labelled plastic 1.5 ml microcentrifuge tubes with corresponding records of EMB concentration, exposure period, sea lice condition and dish number for storage. Lice were assayed at a later date for individual haem peroxidase values using the standardized protocol. These evaluations were repeated using sea lice

collected on three separate collection days from Atlantic salmon (*Salmo salar*) marine farms sites in the Bay of Fundy.

6.3.9 Statistical methods

6.3.9.1 Calculation of haem peroxidase activity

Haem peroxidase activity, expressed as mg cytochrome C-equivalents g^{-1} protein, was calculated using the formula (previously used by Sevatdal *et al.* 2005):

$$\frac{\mu g \text{ cytochrome C-equivalents}}{(\text{mg ml}^{-1}\text{protein} * \text{protein dilution} * 0.0001)}$$

Cytochrome C-equivalent values falling outside the quantification limit of the standard curve led to a censoring of the cytochrome value and hence also of the haem peroxidase values. Cytochrome values below the quantification limit were recorded as zero but subsequently left censored at 0.001 (a value substantially below any observed cytochrome value) (i.e. the unobserved value was considered below 0.001). The upper cytochrome quantification limit was 13.105; unobserved values were considered larger than this value (i.e. right censored at 13.105).

6.3.9.2 Analysis of variance (ANOVA)

Data from each of the studies were analyzed separately by ANOVA methods. Due to a strong right-skewness in the haem peroxidase values, these were log-transformed prior to the ANOVA; however, in the freezing experiment, a square-root transformation was used to normalize the data. The model assumptions were evaluated by the standardized residuals. Significant factors with more than two categories were further

explored by Bonferroni-corrected multiple comparisons. First-order interactions were assessed in all multifactorial models. The significance level was set at $p < 0.05$.

6.3.9.3 Tobit regression

In order to ensure that the left and right censoring would not invalidate the results of the ANOVA, supplementary analyses were carried out by Tobit regression (Amemiya 1984), which allows for such censoring. Maximum likelihood estimation and testing were based on a normal distribution on the chosen, transformed scale. The intreg command in Stata version 9 software was used for these analyses. All reported p-values and estimates are from the Tobit regression analyses.

6.4 Results

6.4.1 Freezing, storage and post-mortem experiments

Descriptive statistics for all experiments are shown in Table 6.1. The 2*2 factorial of the freezing experiment had non-significant effects of condition (live/dead, p=0.43) and freezing (p=0.68). ANOVA of the storage experiment showed a significant effect of length of storage (p=0.002). Haem peroxidase levels for the 30 day storage period were significantly different from all other storage times except 48 h and one week. ANOVA showed no time dependent changes in haem peroxidase activity up to 72 h post-mortem (p=0.23).

6.4.2 EMB exposure experiment

Separate 4*2*3 factorial analyses for the three source populations (of sea lice) showed varying effects across sea lice sources. In the combined analysis, source was therefore included with first-order interactions with the experimental factors. The combined ANOVA had a significant effect of exposure period ($p=0.01$), as well as significant sea lice source interactions with both EMB concentration ($p=0.02$) and sea lice condition ($p=0.01$). Table 6.2 presents estimated medians for all factorial levels at the three sources. For all sources, haem peroxidase values were higher following a 24 h than 48 h exposure period. Dead sea lice from source 1 showed the highest haem peroxidase values and the lowest from source 3. Across the three sources, the effect of EMB concentration was variable (e.g. 100 ppb had the highest in source 1 but the lowest in source 2).

6.5 Discussion

It is necessary to identify potential factors contributing to the inherent variability of oxidase enzyme levels within susceptible field populations of sea lice if the haem peroxidase assay is to be used to diagnose resistance development to chemotherapeutants used in the field. Previous studies involving the determination of haem peroxidase activity in mosquitoes and adult female sea lice involved the freezing of samples and/or storage of samples at -80°C (Brogdon *et al.* 1997, Sevatdal *et al.* 2005). Such knowledge is important if this assay is to be incorporated into a field resistance monitoring program as it will determine whether or not sea lice can be frozen and stored at -80°C prior to assay procedures. The effects of freezing and storage on enzyme activity in sea lice

samples is not well documented, and information regarding the effects of sample storage on enzyme stability in mammal tissues is variable. Yamazaki *et al.* (1997) found no significant decrease in catalytic activities of CYP enzymes in human liver by storage at -80°C for 5 years. In contrast, Hale *et al.* (1997) concluded that liver temperature, storage temperature (25, 4, -20, and -70°C) and post-mortem interval (time from death until sample collection and storage) affected enzyme activity in rat liver. Up to 40% of enzyme activity was lost in the first 24 h following storage at 25°C, and similar results were found by Gallenkamp *et al.* (1981). However, loss of enzyme activity was delayed in liver samples stored at 4°C and prevented in samples stored at -20 or -70°C for 96 h. Furthermore, Marshall *et al.* (2002) investigated the effect of freezing at -80°C on feline antioxidant activity determined from blood samples. They found that enzyme activity was significantly reduced after sample storage for 14 d at -80°C. However, they detected no significant differences between enzyme activity of fresh (non-frozen) samples and the other storage points measured up to one month, leaving them to deduce that the significant time point at 14 d was probably erroneous and due to assay variation. They concluded that sample storage up to one month at -80°C was suitable for subsequent determination of antioxidant activity from feline blood samples.

There is a lack of information regarding the post-mortem stability of oxidase enzymes in sea lice or any invertebrate. In the current study, there was no detectable time-dependent decrease in haem peroxidase activity of sea lice stored at 3 to 4°C up to 72 h post-mortem. These results suggest that oxidase enzymes investigated in this study are remarkably stable after post-mortem delays in excess of those typically encountered in field collection and transport to the laboratory. Knowledge of enzyme stability is

important if haem peroxidase testing were to be used in field monitoring of sea lice where transport times to the laboratory may be variable, possibly resulting in death during transit to the laboratory and, thus, limiting the number of viable sea lice samples available for assays. Information regarding the post-mortem effects of freezing on enzyme activity is variable and seems to be dependent on the species, biological material or tissue sampled and the type of enzymes present. Ritchie *et al.* (1986) showed only slight losses of enzyme activity in post-mortem rat brain kept at 4 °C for up to 72 h. Yamazaki and Wakasugi (1994) investigated post-mortem changes in the drug-metabolizing enzymes in rat liver microsomes from livers stored in situ at 25°C for 0, 6, 12, 18, 24, 36 and 48 h. Post-mortem time-dependent changes in specific activities of CYP-linked monooxygenase, such as aminopyrine *N*-demethylase activity, aniline *p*-hydroxylase activity and p-nitroanisole *O*-demethylase activity showed decreased activity by 48 h post-mortem to 87%, 98% and 75% of relative activities, respectively.

The results of the current study indicated no significant differences in haem peroxidase values between live or dead sea lice. This is likely due to the fact that haem peroxidase enzymes exhibited stability up to 72 h post-mortem and the dead sea lice used in the freezing experiment were dead within only 4 to 6 h of collection making detection of oxidase activity likely. Also, there were no significant differences in haem peroxidase values between frozen and non-frozen sea lice. This suggests that the method used in this experiment offers a convenient and economical alternative to the use of liquid nitrogen for flash-freezing of biological samples to be used for haem peroxidase assays.

There were inconclusive results regarding oxidase activity in live, moribund

and dead sea lice following 24 and 48 h exposure periods to four EMB concentrations. Given that oxidase levels are detectable in dead sea lice up to 72 h post-mortem, it is not surprising that in the current study haem peroxidase levels were similar in live, moribund and dead sea lice. Inter-individual variability may have prevented detection of small differences in mean levels. It is possible that the chosen EMB concentrations were inadequately separated to detect significant differences in haem peroxidase activity.

6.6 Conclusions

The current study highlights the importance of obtaining data on the variables influencing oxidase levels in EMB-susceptible sea lice populations, in order to standardize the haem peroxidase assay methodology before implementation within a resistance monitoring program. However, haem peroxidase levels cannot be used as stand-alone predictors of resistance. They should be used in combination with bioassays or other tests (i.e. molecular) when resistance occurs and the mechanism has been properly identified. The identification of the mechanism responsible for sea lice resistance to EMB, should it occur, will aid in the development of tests for resistance detection and monitoring. Due to the fact that laboratory or field resistance of sea lice towards EMB has yet to be documented, the haem peroxidase test outlined in the current study awaits further validation and application. The utility of the haem peroxidase assay for measurement of total oxidative activity may not be limited to EMB resistance detection only. It may potentially be used to detect resistance to any chemotherapeutant in which oxidative metabolism has been implicated as a resistance mechanism.

6.7 Literature cited

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Table 6.1 Descriptive statistics of the effects of freezing and storage at -80°C, post-mortem enzyme degradation and emamectin benzoate concentration and exposure on haem peroxidase values (μg cytochrome C-equivalents g^{-1} protein) in adult female sea lice (*Lepeophtheirus salmonis*) from New Brunswick salmon farms.

Experiment	Lice group	N	Mean	Median	Std. Dev.	No. Left Censored	No. Right Censored
Freezing	Live/frozen	30	9.36	7.60	7.89	1	0
	Live/non frozen	30	6.18	4.60	5.60	2	0
	Dead/frozen	20	9.78	6.33	9.02	2	0
	Dead/non-frozen	30	9.54	7.57	8.60	1	0
Storage	0 h	10	8.68	5.57	10.55	0	0
	6 h	10	13.55	11.26	11.71	0	0
	12 h	9	10.31	3.40	14.48	0	1
	24 h	10	12.40	4.02	17.12	0	2
	48 h	10	4.66	2.95	4.83	0	0
	1 wk	10	7.84	4.70	4.83	0	0
	2 wk	10	21.08	12.35	22.36	0	2
	1 mo	10	6.17	0.30	9.08	2	0
Post-mortem enzyme degradation	0 h	11	21.65	18.88	22.92	1	1
	1 h	11	14.81	4.82	21.14	1	2
	6 h	10	9.57	0.47	14.86	2	1
	12 h	9	14.72	11.55	16.34	0	0
	24 h	10	8.15	1.70	17.18	0	0
	48 h	10	14.57	3.85	20.34	0	0
	72 h	10	11.10	2.34	27.70	0	0
Concentration and exposure	Source 1	160	5.48	1.67	8.47	18	5
	Source 2	158	5.31	2.27	7.33	12	4
	Source 3	160	7.65	2.29	11.30	19	5

Table 6.2 Descriptive statistics for experiments evaluating the effect of EMB exposure on haem peroxidase activity in adult female sea lice (*Lepeophtheirus salmonis*) from New Brunswick salmon farms.

		Estimated Median (95% CI)		
Factor	**Levels**	**Source 1**	**Source 2**	**Source 3**
concentration	0 ppb	1.45 (0.44,4.73)	1.09 (0.34,3.49)	0.47 (0.13,1.78)
	100 ppb	4.82 (1.67,13.85)	0.39 (0.13,1.19)	0.58 (0.18,1.92)
	200 ppb	1.54 (0.56,4.21)	1.61 (0.61,4.27)	0.74 (0.27,2.01)
	300 ppb	0.39 (0.15,1.03)	1.27 (0.47,3.42)	0.39 (0.14,1.06)
exposure	24 h	2.10 (1.04,4.24)	1.42 (0.64,3.14)	0.78 (0.34,1.79)
	48 h	0.98 (0.50,1.92)	0.66 (0.33,1.33)	0.36 (0.18,0.74)
condition	Dead	5.56 (1.11,27.92)	0.60 (0.11,3.20)	0.08 (0.01,0.51)
	Live	0.72 (0.35,1.48)	1.15 (0.66,2.02)	1.41 (0.73,2.74)
	Moribund	0.73 (0.33,1.63)	10.30 (0.61,174.41)	1.29 (0.48,3.43)

Chapter 7 Summary and Recommendations

7.1 Survey

Sea lice monitoring, sampling methodologies, and the chemotherapeutants available for control vary between Atlantic salmon producing countries. One thing common to all is the need for year-round sea lice monitoring to ensure the strategic timing of treatments. Norway, Scotland, Ireland, and most recently British Columbia, have established control programs and/or area management agreements to ensure the mandatory year-round reporting of the occurrence and severity of sea lice. However, there are currently no regulations for the reporting of sea lice burdens on salmon farms in Atlantic Canada, nor are there officially standardized methods for conducting sea lice counts in the field, although sea lice do receive close attention and management for health and production purposes.

This survey of the Atlantic salmon farming industry was conducted to describe: (1) the different methods of sea lice sampling employed; (2) the types of sea lice treatments being used; and (3) the factors considered when deciding to treat for sea lice. Participants in the survey included 83 of the approximately 95 sites in operation in the Bay of Fundy at the time the survey was conducted.

The results of the survey indicated that salmon farmers in New Brunswick rely on the relationship with their veterinarian for monitoring and timing of appropriate sea lice control measures. Although sea lice monitoring methods vary between sites, similar control strategies are employed by all sites due to the requirements of site veterinarians for reliable information on which to manage fish health and the limited availability of effective treatment options in Canada. Sea lice counting protocols are most commonly

comprised of the following: biweekly sea lice counts during periods of high lice burdens, the strategic selection of cages for sea lice counting of 5 to 10 fish per sampled cage to provide usable information for control decisions, and the recording of the frequency of *L. salmonis* chalimus, mobile and gravid female stages, as well as a total count of *C. elongatus*.

The majority of fish farmers, in concert with their veterinarians, practice intensive monitoring and control of sea lice burdens. However, 90% of sites surveyed are using emamectin benzoate (EMB; active chemical in SLICE®) to treat their smolt and premarket salmon for sea lice. Despite the current absence of clinical resistance of sea lice to EMB, continued reliance on it for sea lice control on Atlantic salmon farms in the Bay of Fundy raises concern regarding the potential for sea lice to develop resistance to the drug.

The surveillance study highlighted the importance of the establishment of standardized sea lice monitoring protocols at the industry level in New Brunswick. Standardized protocols should include: (1) mandatory sea lice counting at least biweekly during periods of high lice burdens (e.g. May to October) and prior to the winter period; (2) verification that count methods are comparable between cages and between sites; (3) the number of cages (e.g. one repeated, 2 random) and fish per cage that should be sampled in order to provide veterinarians with accurate and usable information with which to make decisions regarding the strategic timing of treatments on site; (4) a requirement for the staging of sea lice (i.e. attached and mobile stages of *L. salmonis* and a total count of *C. elongatus)*, and (5) mandatory treatment thresholds based on sea lice

counts, particularly at certain times of the year when control measures may be most effective.

7.2 EMB Bioassays

Reports from Europe of reduced sensitivity and resistance of sea lice towards several chemotherapeutants used for their control (Jones *et al.* 1992, Treasurer *et al.* 2000, Tully and McFadden 2000, Sevatdal and Horsberg 2003) necessitates the development of resistance management strategies. An important principle of minimizing resistance development is the avoidance of reliance on single products. This is difficult when a limited range of chemotherapies is available, as is the case in Canada where the only drugs currently available for sea lice control are teflubenzuron (Calicide®) and EMB. The fact that over 90% of sites in New Brunswick are using EMB for sea lice control (Chapter 2) necessitates the development of tools to monitor sea lice sensitivity towards EMB so that changes may be detected at an early stage. In the early stages of resistance selection within a population, the number of resistant individuals may be too few to clinically affect control success. However, if resistance is unchallenged these individuals eventually reach the point at which their contribution to the population will compromise control at the farm level.

Bioassays used to monitor sea lice sensitivity towards the chemotherapeutants used for their control were first developed in Europe (Sevatdal and Horsberg 2003). Most of this research has focused on the development of bioassays for monitoring sea lice sensitivity to chemotherapies applied as bath treatments (e.g. organophosphates). Bioassays for in-feed chemotherapies have not yet been established for the field

monitoring of sea lice. The objectives of this series of studies were: (1) to develop, optimize and validate a simple and suitable bioassay protocol with EMB and field-collected sea lice from Atlantic salmon farms in the Bay of Fundy; and (2) to use the developed protocol to test for differences in the susceptibility of sea lice to EMB in four regions in the Bay of Fundy over a three-year period. Bioassay optimization included an evaluation of the inter-rater reliability of sea lice responsiveness to EMB and an evaluation of gender-related differences in susceptibility. A total of 39 bioassays were completed between 2001 and 2005.

There is an element of subjectivity inherent to bioassay assessment which was apparent in this study. Subjectivity of initial bioassay evaluations experienced by two independent raters led to the adoption of a set of bioassay response criteria. The high level of agreement achieved between the bioassay evaluations of two independent raters provided confidence that the adopted bioassay response criteria were clearly defined, the raters understood consistent definitions of live, moribund, and dead sea lice and were able to consistently apply those definitions to their evaluations. The blinding of raters to treatment dilutions used in the bioassays was essential to remove the raters' expectations of treatment outcomes when performing bioassay evaluations. The results of this study indicated that preadult female *L. salmonis* were more sensitive to EMB compared to preadult males. A laboratory-cultivated population of *L. salmonis*, with no direct exposure to EMB, demonstrated the same gender-related sensitivity.

There were no sea lice with decreased EMB sensitivity in the regions or years monitored with the EMB bioassay used in this study, and the range of EC_{50} values obtained in this three-year study did not appear to be sufficient to affect current clinical

success in the control of sea lice. There was a marginally significant and moderate correlation between measurements taken at the same site for measurements taken 7 days apart. Spatial clustering of the bioassay data from the years 2002 to 2004 was determined to be of no statistical significance for this data set. The results of this study also indicated a seasonal or temperature associated variation in sensitivity to EMB such that season and water temperature were factors in sensitivity (i.e. decreased temperature = decreased sensitivity). The reason(s) for the seasonal effect on bioassay values remains unknown and requires further investigation.

There was difficulty in consistently obtaining the minimum number of 70 sea lice required for several bioassays. There were a number of factors that contributed to limited availability of field collected sea lice during this study. Sampling was dictated by the sporadic availability of sea lice which was due to natural variations in sea lice abundance between the years and regions sampled and the cooperation and participation of sea cage sites in the Bay of Fundy. Furthermore, sea lice used in 2002 to 2003 bioassays were collected during routine sea lice counting on participating sea cage sites. Access to an adequate number of fish for sea lice collection was limited to those being sampled for the purpose of sea lice counting, which was as few as 20 to 30 fish in many cases. We experienced greater success in subsequent sea lice collections during harvesting on sea cage sites where access to a larger number of fish was possible.

There are a number of factors that may have contributed to variations in bioassay results between and within the regions and years samples. The effect(s) of previous sea lice exposure to EMB prior to inclusion in bioassays is unknown and was not an objective of the current study. EMB treatments were regular on some sites and sporadic

on others, thus, the time from EMB treatment to sea lice collection and inclusion in bioassays may have been variable in some instances. Also, variations in the ratio of preadult males to preadult females collected in the field made it difficult to include equal proportions of each sex in the bioassays. Preadult female *L. salmonis* were more sensitive to EMB compared to preadult males; thus, the use of unequal proportions of each sex in a bioassay may have contributed to variability in bioassay results.

There were a number of limitations of this study. The relatively small sample size for this study (i.e. 39 bioassays completed between 2001 to 2005) limited our ability to make conclusions regarding the spatial and temporal clustering of EMB sensitivity within and between years and regions, as well as our ability to evaluate repeated measures for bioassays performed on the same site over time. Due to size variations in sea lice stages, we were limited to using preadult stages only; therefore, we are unable to comment on the sensitivity of field samples of adult sea lice to EMB. The finding of a gender-related difference in EMB susceptibility and a seasonal variation in EMB efficacy may have implications with regards to the strategic dosing and timing of sea lice treatments. Prior to this project, which was a collaboration with European researchers, there were no methods to detect or monitor resistance in sea lice.

Although the bioassay protocol shows promise as a method to verify clinical resistance, it lacks rapidity and simplicity for use as a routine test for a field monitoring program. The time requirement for sea lice collection, bioassay set-up, and the 24 h incubation period, as well as the difficulty in obtaining sufficient numbers of viable sea lice in the field, make this bioassay impractical in many situations.

Future research should include an evaluation of the effect of temperature on bioassay results and the development of successive generations of a laboratory population of sea lice from the Bay of Fundy to use as a comparison to bioassays using field populations. Further studies are required to understand the impacts of the effects of gender and sea lice stage-related sensitivity towards EMB for use in this bioassay. Further investigation of resistance mechanisms would also be greatly facilitated by a known resistant sea lice population maintained in the controlled conditions of the laboratory. Information regarding the timing and frequency of field treatments using EMB should be investigated to determine a correlation between treatment frequency and increased EC_{50} values.

7.3 Haem Peroxidase Assay

Up-regulation of metabolic oxidative enzyme-based systems, such as the CYP monooxygenases, is a well documented form of arthropod resistance. As a potential means of EMB detoxification by sea lice, oxidase enzymes are of particular interest as an alternative to the use of bioassays for resistance detection. Biochemical methods of resistance detection offer a number of advantages over bioassay methods, including the ability to detect sensitivity changes in individual test subjects and confirm resistance with the use of only a small number of subjects (Brogdon and McAllister 1998). The haem peroxidase assay used in the current study was developed for measuring levels of haem-containing enzymes, such as the CYP monooxygenases, in individual mosquitoes

Inherent variability of oxidase levels and the factors influencing oxidase activity within EMB-susceptible populations must be evaluated in order to standardize the haem

peroxidase assay methodology prior to implementation within a resistance monitoring program. In the current study, the haem peroxidase assay was evaluated for potential utility in detecting changes in the sensitivity of field samples of *L. salmonis* to EMB. The objectives of this study were: (1) to quantify total non-specific oxidase activity in individual adult female sea lice from two EMB-susceptible populations; (2) to correlate haem peroxidase values from field samples with EMB bioassay EC_{50} values conducted on preadult stage male and female sea lice from the same sample populations; and (3) to examine several potential factors influencing oxidase activity in EMB-susceptible field collected sea lice.

Some of the haem peroxidase data required censoring because they fell outside the quantification limits of the cytochrome C standard curve used to determine the enzyme activity in individual samples. The frequency distributions for field and laboratory cultivated sea lice exhibited a heavy right skew, suggesting that the haem peroxidase assay was able to detect individuals within both populations expressing elevated oxidative activity. However, due to the fact that the haem peroxidase assay is a non-specific assay that detects total oxidative activity, it is not known whether or not the elevated oxidative activity was an indication of oxidative metabolism. Molecular tests would be required to identify specific CYP enzymes associated with oxidative metabolism of chemotherapeutants.

The results of this study indicated significant effects of region and a seasonal trend on median haem peroxidase values for 2001 to 2004 field sea lice samples. There were no significant effects in the 2005 field data. There was a positive, but not statistically significant, correlation between EMB sensitivity (bioassay EC_{50} values) and

median haem peroxidase activity in field sea lice samples for the years 2001 to 2005. There were no significant time-dependent changes in haem peroxidase activity up to 72 h post mortem for sea lice stored at 3 to 4°C, suggesting that oxidase enzymes in sea lice are remarkably stable after post-mortem delays well within expected delays typically encountered between farm collection and arrival at the laboratory. There was no significant difference in haem peroxidase values for live and dead or frozen and non-frozen sea lice, suggesting that the freezing method used in this experiment offers a convenient and economical alternative to use of liquid nitrogen for freezing of sea lice samples. Storage at -80°C for up to 30 days resulted in significantly lower haem peroxidase activity. Haem peroxidase values were higher following a 24 h versus 48 h EMB exposure period. Effects of condition and concentration were variable in the three sea lice sources. Dead sea lice had the highest haem peroxidase values in source 1 but the lowest in source 3. Sea lice exposed to 100 ppb EMB had the highest haem peroxidase values in source 1 and the lowest in source 2.

The utility of a test measuring total oxidase activity within individual adult female sea lice is difficult to confirm as a diagnostic method for resistance detection due to the absence of sea lice populations known to be resistant to EMB, and the inability of the assay to indicate the specific CYP enzymes responsible for resistance development. However, the ability of the haem peroxidase assay to identify individuals displaying elevated general oxidative activity is encouraging, as field populations are likely to consist of a mixture of susceptible and less susceptible individuals. This study highlights the importance of obtaining data on the parameters influencing oxidase levels in EMB-susceptible sea lice populations in order to standardize the haem peroxidase assay

methodology before implementation in a resistance monitoring program. The haem peroxidase assay should be used in combination with bioassays or other tests (i.e. molecular tests) when resistance is suspected and the mechanism has been identified. The identification of the mechanism responsible for sea lice resistance to EMB will aid in the development of tests for resistance detection and monitoring. The utility of the haem peroxidase assay for measurement of total oxidative activity may not be limited to EMB resistance detection. It may be used to detect resistance to any chemotherapeutant in which oxidative metabolism has been implicated as a resistance mechanism.

Future research should involve an evaluation of the effect of temperature on enzyme activity in field samples of sea lice. *In vivo* measurement of the ability of oxygenase inhibitors, such as piperonyl butoxide (PBO), to increase EMB susceptibility in sea lice populations may indicate the involvement of non-specific oxidases if resistance to EMB occurs in the field. Detection of elevated levels of general oxidase activity may then provide a means for the rapid detection of this mechanism as an indication of decreased sensitivity towards a drug, particularly when combined with bioassays.

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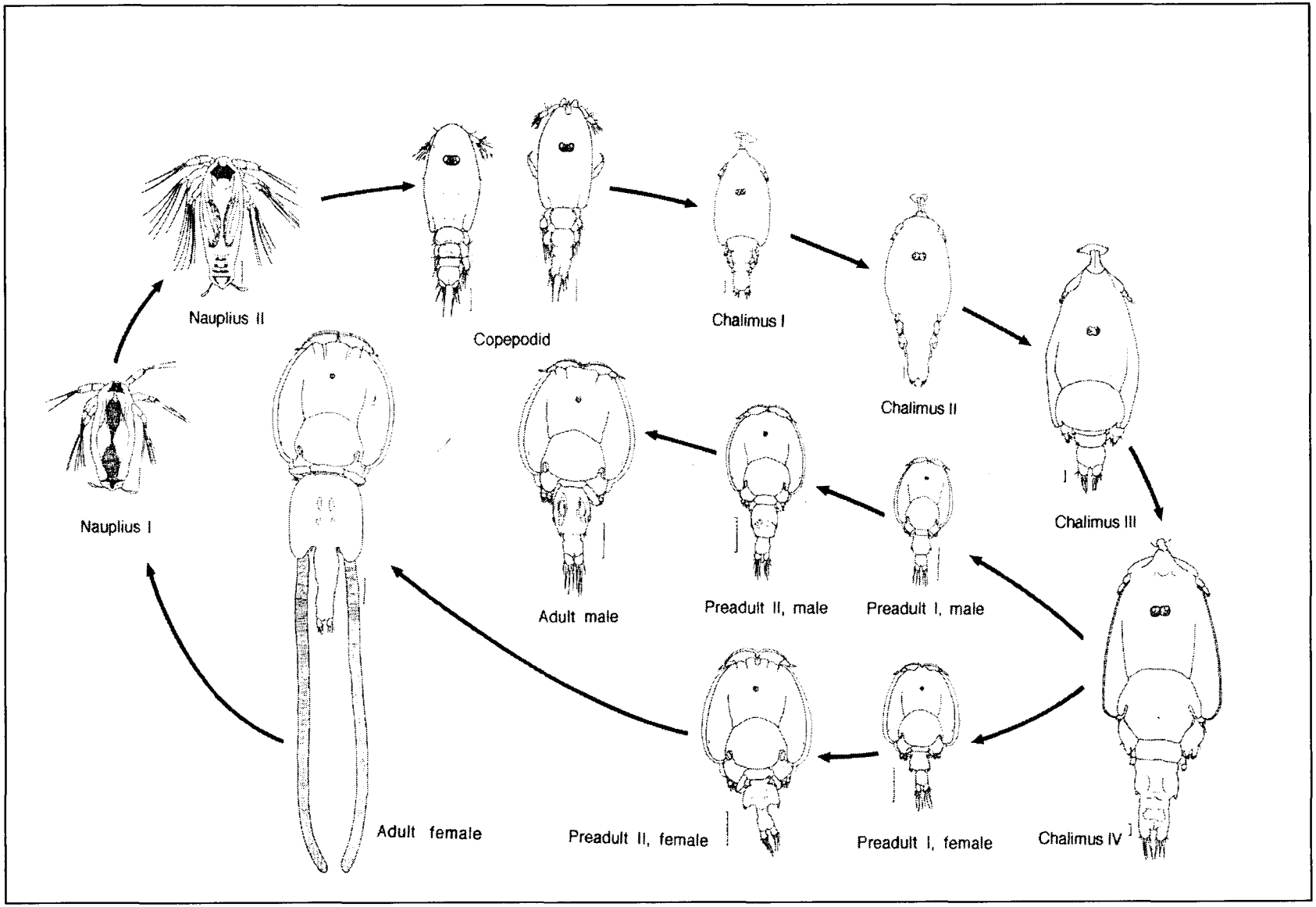
Chapter 8 Appendices

Appendix A: Sea lice (*Lepeophtheirus salmonis*) life cycle

Appendix B: Distinguishing features for identification of sea lice species found in New Brunswick, Canada.

Appendix C: 2002 ISA Risk Factor Study for the New Brunswick Atlantic Salmon Farms



Appendix A. Sea lice (*Lepeophtheirus salmonis*) life cycle (Schram 1993).

Appendix B. Distinguishing features for identification of sea lice species found in New Brunswick, Canada (Margolis and Kabata 1984).

Species name	Species description	Distinguishing features for identification
Caligus curtus	▪ dorsal shield subovoid (egg-shaped on underside) ▪ total length of female 5-10 mm; abdomen one-segmented, less than ½ length of genital segment; genital complex of equal length with thoracic zone of dorsal shield ▪ total length of male 5-12 mm; abdomen one-segmented, more than ½ length of genital complex; genital complex roughly circular	▪ lunules present on frontal plate
Caligus elongatus	▪ dorsal shield slightly longer than wide ▪ total length of female 5-6 mm; abdomen one-segmented, more than ½ length of genital complex; genital complex about as long as thoracic zone of dorsal shield ▪ total length of male 4-5 mm; abdomen two-segmented, about as long as genital complex; genital complex oval, slightly shorter than thoracic zone of dorsal shield	▪ lunules present on frontal plate
Lepeophtheirus salmonis	▪ dorsal shield suborbicular (circular or round on underside) ▪ total length of female 7-18 mm; abdomen one-segmented, as long as genital complex; genital complex subquadrangular ▪ total length of male 5-7 mm; abdomen one-segmented, shorter than genital complex	▪ absence of lunules

Appendix C. 2002 ISA Risk Factor Study for the New Brunswick Atlantic Salmon Farms

2002 ISA Risk Factor Study
for the New Brunswick Atlantic salmon farms

Instructions for the interviewer:

Please fill in the information below:
Name of person to be interviewed _____
Position of person to be interviewed _____
Office phone number of person to be interviewed _____
Cell phone number of person to be interviewed_____ Fax number _____
Date planned for interview _____
Time_____
Place to be interviewed _____
What is your current year class? 2001 or 2002
How many cages does your site have? (for the year class of interest) ____
What are/were the cage numbers? _____
This site is a (circle) a) an ISA positive site b) an ISA negative site (for the year class of interest)
If this is a positive site, which cages are positive? _____
Case cages # ____ and ____ or Not applicable
Control cage #'s (randomly picked out of a hat from ISA negative cages) _____ _____ _____ _____
(two cages if only one infected cage, three cages if an ISA negative site, four cages if two case cages)

Please inform the interviewer of these issues:
This questionnaire is designed to identify potential factors that may increase the likelihood of a cage becoming infected by ISA. We feel that identifying risk factors will permit us to make recommendations that will benefit your site and the entire industry.
This project is being supported by the NB Department of Agriculture, Fisheries, and Aquaculture and is being conducted by the Atlantic Veterinary College. We plan to survey all sites.
If the site had 2000 year class fish, information up until harvest will collected. If the site has 2001 year class, information up until the time of the interview will be collected.
All information will be used in a confidential manner that will keep your information from being identified as belonging to a particular company or site.
This study will not cost you any money, just an hour of your time.
Please be prepared to discuss many characteristics of the site of interest including cage types, feeding, smolt history, health concerns, and mortality records

Records that you should bring to our meeting should include:
Smolt history and transfer records
Mortality records for the cages of concern
Health records including medications used to treat fish
Records on weight sampling
Records on net changes
Records on feeding
Records from the processing plants

Information to be collected before the interview:
Site map for the year class of interest
Weekly mortality records for all cages from sea water transfer to harvest or current
Smolt records including total number entered, first thirty day mortalities, and transfer dates for all cages
Medication records (name of drug and dates given) from sea water transfer to harvest or current
Harvest reports for cages of interest

Please fax this information to me at 902-566-0823 and have a copy available for the interviewer

A. Interview information (Please remind interviewee that all questions are for up to the time of harvest of the infected cage)
Date _____
Company _____
Site Number MF_____
Site name? _____
Interviewer _____
Person being interviewed _____
Position _____
DAFA bay management number ____
I have collected weekly mortality records and available harvest records from all three cages [1]Yes or [0]No
I have collected records on when drugs were administered and for what reason for all three or cages [1]Yes [0]No
I have collected smolt records including total number entered, first thirty day mortalities, and transfer dates for all cages. [1]Yes [0]No
I have collected a site map with all cages identified [1]Yes or [0]No
B. Company information
Records are mainly kept by? [1] Superior or a corporately designed or computer spreadsheet [2]Handwritten and placed into files [3]APHIN [4]Other _____
How many total sites does your company have in New Brunswick? _____
How many total sites does your company have in Nova Scotia? _____
How many total sites does your company have in Maine? _____
C. Area and site information
Number of cages at the site? _____
All cages at site:
Type 12 m^2 15 m^2 50 m PC 70 m PC 90 m PC Other: Total # depth of nets (m)
Seawater transfer occurred when? [1]S2000 or [2]F2000 or [3]S2001 or [4]F2001
How close do processing boats (harvest barges) travel past your site when headed to the wharf? [0] They do not pass by our site [1]Within 100 meters

[2]Within 500 meters [3]Within 1 km [4]Within 2 km
Description of boats carrying harvested fish (boat name, owner, type, etc.)?
Name of wharf or wharves to which processing boats are traveling? _____
How close is your nearest neighbor with ISA? <0.5 km[1] <1 km[2] <2 km[3] <5 km[4] >5 km[5] Don't know [-2]
D. Health Parameters
Site Veterinarian? [1]Steve Backman [2]Leighanne Hawkins [3]Dan MacPhee [4]Julia Mullins [5]John O'Halloran
Does the veterinarian or veterinarian's assistant visit the site regularly? [1]Yes or [0]No
How often does the veterinarian or veterinarian's assistant visit the site? [1]once a week [2]once every two weeks [3]once every month [4]once every 6 weeks [5]only when called [6]other _____
Previous to the year class we are investigating, what was the last year the site was diagnosed with ISA? [0]Never or [1]1997 or [2]1998 or [3]1999 or [4]2000
Has BKD (Bacterial Kidney Disease) been detected at this site in the 2000 or 2001 year class? [1]Yes or [0]No
Have there ever been any algal blooms for this year class, if so how many? _____
How many periods of extreme low oxygen has this year class suffered (including algal blooms)? _____
E. Feed History
How are fish fed? [1]Feed Blowers or [2]Hand fed or [3]Combination blower and hand feeding or [4]Automatic feeder on timer
How many weeks were fish on moist feed after seawater transfer? _____
How was moist feed delivered to your site? [1]Delivered by feed company by feed company boat/barge [2]Delivered by farm owned feed delivery boat/barge [3]Delivered by contract feed delivery boat/barge [4]Picked up at wharf by farm boat
Name of boats that bring the moist feed to your site? _____
Did this boat visit more than your site on the days of delivery? [1]Yes or [0]No

Name of wharf that moist feed leaves from? _____
How was dry feed delivered to your site? [1]Delivered by feed company by feed company boat/barge [2]Delivered by farm owned feed delivery boat/barge [3]Delivered by contract feed delivery boat/barge [4]Picked up at wharf by farm boat
Name of boats that bring the dry feed to your site? _____
Did this boat visit more than your site on the days of delivery? [1]Yes or [0]No
Name of wharf that dry feed leaves from? _____
If the feed chart is not convenient, please fill in feed history in space provided making sure that chart's information is included in your write-up.
F. Equipment and Personnel
Is there a policy about not sharing equipment with other sites? [1]Yes or [0]No
Does this site share equipment with other sites? [1]Yes or [0]No
Does it share boats? [1]Yes or [0]No
Does it share a roller/crane barge? [1]Yes or [0]No
What else does it share? _____
Is there a policy about not sharing personnel with other sites? [1]Yes or [0]No
Does this site share personnel with other sites? [1]Yes or [0]No
How many staff would visit other sites in a typical month? _____
What product is used for the foot baths? [1]Iodine based disinfectant [2]Vircon [3]Bleach [4]Other _____
Is there a policy about no visitors allowed on the site? [1]Yes or [0]No
Do visitors (not employed by company) visit the site? [1]Yes or [0]No
G. Diver
Does this site have a contract diver or a staff diver? [1]Contract or [2]Staff
Does this diver dive at other sites? [1]Yes or [0]No
Does the diver have a separate dry suit for each site? [1]Yes or [0]No
Does the diver disinfect between cages? [1]Yes or [0]No
How does the diver disinfect between cages? [0] He does not disinfect between cages [1]Complete submersion in disinfectant [2]Sprayed with disinfectant [3]Pours disinfectant on himself [4]Other _____
Is there a separate mortality bag for each cage? [1]Yes or [0]No
What are the mortality bags disinfected with? [1]Iodine based disinfectant [2]Vircon [3]Bleach

[4]Other _____
How often are mortality dives performed during January? [1]Twice a week [2]Once a week [3]Every two weeks [4]Once a month [5]Other _____
How often are mortality dives performed during August? [1]Twice a week [2]Once a week [3]Every two weeks [4]Once a month [5]Other _____
How often do dives occur in times of consistently elevated mortality? (>20 mortalities per dive in a cage) [1] Every day [2] Three times a week [3] Twice a week [4] Once a week [5] Every two weeks [6]Other _____
How are mortalities disposed of? [1]Brought to Connors fish meal plant [2]Sent to a processing plant for rendering (used to feed other animals) Which one? ____ [3]Brought to compost facility in New Brunswick Which one? _____ [4] Brought to compost facility in Maine Which one? _____ [5]Other _____
H. Smolt History
What was the start date of transfer? _____
What was the end date of transfer? _____
How long was the fallow period? [0]There was no fallow period [1]Two weeks or less [2]One month or less [3]Two months or less [4]Two to four months [5]Greater than four months
When the smolt left the hatchery, were the boxes oxygenated? [1]Yes or [0]No
What was the method of smolt transfer? [1]Well boat or [2]Boxes on a barge or [3]Both
Name(s) of Well boat or barges used for transfer? _____
If the smolt were transferred by well boat, were they oxygenated? [1]Yes or [0]No or [2]N/A
What was the name of the wharf the smolts left from? _____
During well boat haul, where was the water taken for the holding tanks?

[0]Not applicable (smolt were transferred in boxes) [1]Within 100 m of the wharf [2]Within 500 m of the wharf [3]Within 1 km of the wharf [4]Within 2 km of the wharf [5]Greater than 2 km of the wharf
What type of barge was used during transfer? [0]Not applicable (smolt were transferred by well boat) [1]An on-site working barge/scow [2]A harvest barge [3]A feed barge [4]A ferry [5]Other _____
In the two weeks prior to transfer, the barge was used for what other jobs?
The barge was used to pick up feed? [1]Yes or [0]No or [-2]Don't know
The barge was used for harvest? [1]Yes or [0]No or [-2]Don't know
The barge was used at a different site? [1]Yes or [0]No or [-2]Don't know
Other? _____
What was the total % mortality during the immediate post transfer period (30 days) for this site?
How many cages experienced more than 5% loss during the first 30 days of seawater entry? ___
I. Holdovers (fish from previous year class that remain on site after new smolt class have been entered on the site)
How many cages have/had fish heldover from the previous year class? _____
What % of the total fish of that year class were heldover? _____
What was the year class of fish that was held over? There were no holdovers[0] 1998 year class[1] 1999 year class[2] 2000 year class[3]
How long were holdovers on the site after smolt transfer? [0]There were no holdovers [1]Two weeks or less [2]One month or less [3]Two months or less [4]Greater than two months
J. Predators
Have there been any known seal attacks at this site? [1]Yes or [0]No
Which cages were more severely or more often affected? _____
Have there been any otters seen in the cages on this site? [1]Yes or [0]No
Which cages were affected? _____
K. Weight Sampling
How often are weight samples taken at your site? [0]never

[1]once a month [2]once every two months [3]once every three months [4]other _____
How are weight samples conducted when fish are <1 kg? [0]Not applicable (weight samples not done) [1]anesthesia and weighing on a scale [2]video camera and estimates [3]Infrared (AKVAsmart or VAKI) measurements and estimates [4]Other _____
How many fish are usually weight sampled from each cage at this time? _____
How are weight samples conducted when fish are > 1 kg? [0]Not applicable (weight samples not done on this site) [1]anesthesia and weighing on a scale [2]video camera and estimates [3]Infrared (AKVAsmart or VAKI) measurements and estimates [4]Other _____
How many fish are usually weight sampled from each cage at this time? _____
L. Wildfish
When you harvest cages at this site, approximately how many Pollock are in each cage? [1]0-9 or [2]10-99 or [3]100-999 or [4]>999
M. Net Care
Are the nets treated with an anti-fouling agent? [1]Yes or [0]No
Do you clean the nets in the water? [1]Yes or [0]No
Do you use an Idema power washer (underwater disc remover) [1]Yes or [0]No
Do you use manual removal by diver or staff [1]Yes or [0]No
How else do you clean the nets while in the water? _____
P. Site Practices Regarding Sea Lice Counting and Treatment
What is your company policy regarding lice counts? [1] every cage [2] selected cages [3] other _____
What is your company policy for frequency of lice counts conducted? [1] weekly [2] biweekly [3] every three weeks [4] once a month [5] every six weeks [6] other _____
What is the number of fish sampled per cage when conducting lice counts? [1] 5-10 [2] 10-20 [3] 20-30 [4] 30-40 [5] 40-50 [6] 50+

Are fish anesthetized when conducting lice counts? [1]Yes or [0]No
If yes, what type of anaesthetic is used? [1] TMS [Please circle : Definite or Probable] [2] Aquacalm (Metomidate) [3] Clove oil/Eugenol [4] other _____
Are sea lice grouped into categories when sampling (ie. Chalimus, preadult, adult, gravid females)? [1]Yes or [0]No
If No, Why not? Please explain _____
Are there one or more persons at the site trained to identify chalimus vs. preadult stages? [1]Yes or [0]No
If sea lice are grouped into categories when sampling which groups are used? Copepodid [1]Yes or [0]No
Chalimus [1]Yes or [0]No
Preadult [1]Yes or [0]No
Adult [1]Yes or [0]No
Gravid Female [1]Yes or [0]No
Herring Lice (Caligus) [1]Yes or [0]No
Other _____
What is the decision to treat for sea lice based on? [1] lice counts [2] general fish appearance [3] recommendation by the site veterinarian [4] expected problem Explain: _____
Who makes the final decision to treat for sea lice? [1] Site manager [2] Site Veterinarian [3] Site Owner [4] other _____
Have there been any treatments for sea lice on your site in the past two years? [1]Yes or [0]No
In the past 2 years, which treatment methods have been used at this site (choose all that apply) [1] not applicable (no treatment has been given) [2] tarp (full ensclosure) [3] skirted (open bottom) [4] no tarp [5] in-feed [6] other
What is the method of sea lice control generally used on your site to treat smolt? [1] SLICE® (Emamectin Benzoate) [2] Salmosan®(Azamethiphos)

[3] Calicide® (Teflubenzuron)
[4] Hydrogen Peroxide
[5] Ivermectin
[6] Other _____

What is the method of sea lice control generally used on your site to treat pre-market salmon?
[1] SLICE® (Emamectin Benzoate)
[2] Salmosan®(Azamethiphos)
[3] Calicide® (Teflubenzuron)
[4] Hydrogen Peroxide
[5] Ivermectin
[6] Other _____

Site lice count dates: ☐ Check with site vet or applicable counter:
1st _____
2nd _____
3rd _____
4th _____
5th _____
6th _____
7th _____
8th _____
9th _____
10th _____
11th _____
12th _____
other dates: _____

What was the time to first treatment for sea lice on your site (time from smolt transfer to first treatment for sea lice)? _____

O. Unusual circumstances and brief description of events before outbreak

Unusual circumstances and brief description of events occurring before the outbreak

Cage level data (please read "Now I am going to ask you questions regarding the specific cages)
Please continually remind interviewee that information should be collected up until harvest time of the ISA infected cage.

C. Area and site information

Type of cage?
[1]12 m^2 [2]15 m^2 [3]50 m PC
[4]70 m PC [5]90 m PC [6]Other: _____

How many meters are under the bottom of the net at low tide? _____
How deep is the net (in meters)? _____
D. Health Parameters *("up until ISA infected cage removal...")*
How many times had the fish been treated for lice since seawater entry?___
How many times was Ivermectin used? _____
How many times was Emamectin (Slice) used? _____
How many times were bath treatments used? _____
Had the fish in this cage ever been treated with antibiotics? [1]Yes or [0]No
How many times had the fish been treated for skin sores? _____
How many times did you treat the skin sores with TM Aqua (oxytetratcycline)? _____
How many times did you treat the skin sores with Aquaflor (florfenicol)? _____
How many times did you treat the skin sores with Romet 30 (sulphadimethoxine:ormetoprim)? _____
How many times did you treat the skin sores with Tribrissen (sulphadiazine:trimethoprim)? _____
How many times did you treat the skin sores with Amoxicillan? ____
Had BKD been detected or diagnosed in this cage? [1]Yes or [0]No
How many times had the fish been treated for BKD? _____
Had there been any gill parasites since seawater entry? [1]Yes or [0]No
How many times had the fish been treated for Gill disease since seawater entry? _____
Had this cage been graded for grilse (sexual maturity)? [1]Yes or [0]No
If yes, when was this cage graded for grilse (sexual maturity)? (dd/mm/yy) _____
H. Smolt History
Saltwater transfer year class? [1]S00 or [2]F00 or [3]S01 or [4]F01
Total # smolts transferred into cage? _____
Was this cage [1]Single stocked or [2]Double stocked or [3] Other (specify)_____?
Date(s) transferred to sea? _____
Hatchery source _____
Hatchery Manager (or contact) and phone number _____
Type of hatchery? [1]Flow through [2]Recirculation hatchery [3]Reuse (some water reused without a biofilter)
If a flow through or reuse hatchery, what is the water source? [1]Lake/River water [2]Well water [3]Spring (Artesian well) water

Average weight of smolt when stocked? _____(in grams)
Condition of smolts at transfer? Healthy, normal? [1]Yes or [0]No
Unusually small? [1]Yes or [0]No
Ungraded (wide range of sizes)? [1]Yes or [0]No
Weak? [1]Yes or [0]No
High degree of fin rot? [1]Yes or [0]No
High degree of spinal or jaw deformities? [1]Yes or [0]No
Other _____
What was the last day the smolts were vaccinated? _____(dd/mm/yy)
What company supplied the vaccine? [1]Aqua Health [2]Bayotek (Microtek) [3]Other _____
Was ISA virus included in the vaccine? [1]Yes or [0]No
Full name of Vaccine? _____
Was BKD bacterin (Renogen vaccine) included in the vaccine? [1]Yes or [0]No
Number of degree days from the time of vaccination until the sea water entry? _____
Were fish vaccinated by immersion as pre-smolt or fry? [1]Yes or [0]No or [-2]Don't know
If Yes, which disease was the vaccine for? _____
J. Predators ("up until ISA infected cage removal...")
Had there been any known seal attacks at this cage? [1]Yes or [0]No
How many days were the seals a problem at this cage? _____
Had there been any otters seen in the cages in this cage? [1]Yes or [0]No
How many days were the otters a problem in this cage? _____
K. Weight Sampling ("Prior to ISA infected cage removal...")
When was the last weight sample done on this cage? _____(dd/mm/yy)
How many fish were sampled? _____
What was the average weight on that sample? _____
What was the standard deviation on that sample? _____
L. Net Care ("Prior to ISA infected cage removal...")
How many times were the nets changed on this cage?
Was the net changed on this cage in last 90 days prior to disease outbreak of infected cage? [1]Yes or [0]No
Was the net cleaned during the last 90 days prior to disease outbreak of infected cage? [1]Yes or [0]No

M. Wild Fish *("Prior to ISA infected cage removal... ")*

How many wild fish or were present in this cage:

Pollock [1]0-9 or [2]10-99 or [3]100-999 or [4]>999

Other (specify) _____

[1]0-9 or [2]10-99 or [3]100-999 or [4]>999

Had there been wild fish mortalities removed from this cage by the diver?
[1]Yes or [0]No

Pollock [1]0-9 or [2]10-99 or [3]100-999 or [4]>999

Other (specify) _____

[1]0-9 or [2]10-99 or [3]100-999 or [4]>999

Which month had the most mortalities? _____

Q: Information regarding sea lice treatment for specific cages

Were these cages treated in the hatchery with emamectin prior to smolt transfer? [1]Yes or [0]No

What was the date of the first sea lice treatment for these cages post transfer? (dd/mm/yy) _____

What method(s) of treatment application have been used on these cages since smolt transfer?
[1] tarp (full ensclosure)
[2] skirted (open bottom)
[3] no tarp
[4] in-feed
[5] other _____
[6] not applicable (no treatment has been applied)

What method(s) of sea lice control have been used on these cages since smolt transfer?
[1] SLICE® (Emamectin Benzoate)
[2] Salmosan®(Azamethiphos)
[3] Calicide® (Teflubenzuron)
[4] Hydrogen Peroxide
[5] Ivermectin
[6] other _____
[7] not applicable (no treatment has been applied)

What was the water temperature at the time(s) of application? _____

What was the length of the treatment(s) applied (in minutes, hours, days)?
_____

R: Information regarding sea lice counts for specific cages

NOTE: If the site does not have information regarding lice counts indicate whom we should contact to obtain such information:
☐ Site Veterinarian _____
☐ Site Personnel _____
☐ other _____

What was the date of the first sea lice count for this cage?_____
1st sea lice count information:
# of Chalimus _____
# of Pre-adults _____
# of Adults _____
# of Gravid Females _____
# of Herring Lice (Caligus) _____
What was the date of the second sea lice count for this cage?_____
2nd sea lice count information:
# of Chalimus _____
# of Pre-adults _____
# of Adults _____
# of Gravid Females _____
# of Herring Lice (Caligus) _____
What was the date of the third sea lice count for this cage?_____
3rd sea lice count information:
# of Chalimus _____
# of Pre-adults _____
# of Adults _____
# of Gravid Females _____
# of Herring Lice (Caligus) _____
What was the date of the fourth sea lice count for this cage?_____
N. Unusual circumstances and brief description of events before ISA outbreak
Unusual circumstances and brief description of events occurring before the outbreak (ie: anything that distinguished this cage from others at site or anything happen at this site?)
Final result of the cage
Date cage diagnosed with ISA? _____
Date of letter from the Minister ordering slaughter? _____
Beginning date cage was harvested? _____ (dd/mm/yy)
Ending date cage was harvested? _____ (dd/mm/yy)
Where were the fish slaughtered? [1]On a barge specifically used for harvest [2]On a site working barge/scow [3]On a feed barge or boat used for feed delivery [4]On a boat used for fishing or non-farm activities [5]Other _____
Who slaughtered the fish? [1]Site staff or [2]Contract company
How were the fish slaughtered? [1]CO_2 and gilled

[2]Chilled and gilled [3]Other _____
Was the blood water contained on the harvest barge? [1]Yes or [0]No
What percent of the blood water was actually contained? (estimate) [1]>95% [2]>90% [3]>75% [4]<75%
What was the name of the boat used for harvest? _____
Where were the fish processed? [1]Heritage Salmon in Black's Harbour [2]Limekiln fisheries (Ocean Legacy) in Limekiln [3]Stolt Sea Farms (Sterling) in St. George [4]Jail Island Salmon in St. George [5]Cooke Aquaculture (True North) in St. George [6]Atlantic Silver in St. George [7]Deer Island Salmon (DIS) [8]Other _____