The Lethal and Sublethal Effects of Anti-sea Lice Chemotherapeutants in Marine Benthic and Pelagic Invertebrates

by
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Declaration of Committee

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Abstract

The salmon aquaculture industry has become a major contributor to the Canadian economy, however, many practices including sea lice pest management strategies have resulted in the contamination of the environment near these operations. Compounds used in sea lice control include Salmosan® (active ingredient [AI] azamethiphos), Paramove®50 (AI hydrogen peroxide), ivermectin (IVM) and SLICE® (AI emamectin benzoate [EMB]). Salmosan® and Paramove®50 are water-soluble formulations applied as bath treatments, whereas IVM and SLICE® are in-feed additives that are hydrophobic and partition to sediment with persistent physicochemical properties. This research assessed both the lethal and sub-lethal effects of these compounds on non-target benthic and pelagic invertebrates at environmentally relevant concentrations. A short-term fertilization success bioassay using the sea urchin *Strongylocentrotus purpuratus* was performed using pest management application-level concentrations of Salmosan® and Paramove®50 in seawater. Paramove®50 significantly inhibited fertilization success with a calculated IC_{50} value of 7.27 mg/L; Salmosan® only marginally inhibited fertilization at the highest concentration (IC_{50} > 100 µg/L). Avoidance behaviour and oxygen consumption were assessed in the benthic amphipod, *Eohaustorius estuarius,* and the polychaete *Nereis virens,* following sub-chronic exposure to environmentally relevant sediment concentrations (< 5 µg/kg) of EMB, IVM and a combination of both (EMB/IVM). *E. estuarius* avoided sediment containing IVM and EMB/IVM ratio concentrations containing 25 and 50 µg/kg IVM, while *N. virens* avoided sediment with 50 and 200 µg/kg IVM and 0.5, 5, 50 and 200 µg/kg EMB/IVM ratio. Impaired burrowing and locomotory behaviour in *N. virens* was also observed with both treatments. Oxygen consumption was significantly decreased in *E. estuarius* and increased in *N. virens* when exposed to EMB, IVM and EMB/IVM at concentrations < 5 µg/kg over a 28-d exposure period. This research provides evidence of impacts to *S. purpuratus*, *E. estuarius* and *N. virens* from anti-sea lice chemotherapeutant exposure at environmentally relevant concentrations and will supplement regulatory decisions and management policies associated with chemicals used in aquaculture in Canada.

Keywords: Aquaculture; sea lice; Salmosan®; Paramove®50; SLICE®; ivermectin; invertebrate; toxicity; sea urchin; amphipod; polychaete

Dedication

This thesis is dedicated to my Dad.

Thinking of you always.

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My journey at Simon Fraser University has been a bit longer than expected thanks to a couple toxicology classes I took in my final year of undergrad. I saw the value in tox from the beginning and I am so glad I chose to continue my education in this field. With that, I would initially like to thank Dr. Chris Kennedy for his insight, support and laughs along the way as my senior supervisor. I am so grateful for all that you do. I would next like to thank my supervisor Dr. Vicki Marlatt – as one of the women in science I admire most, you have kept me inspired throughout both my undergraduate and graduate degree. You instill confidence in your students, while also providing invaluable scientific advice along the way. Thank you to you both for making the MET program so valuable to so many of us.

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List of Acronyms

AI	Active ingredient
ACh	Acetylcholine
AChE	Acetylcholinesterase.
ANOVA	Analysis of variance
BC	British Columbia
CNS	Central nervous system
DFO	Fisheries and Oceans Canada
EC	Effect concentration
EMB	Emamectin benzoate
GABA	γ-aminobutyric acid
GluCl	Glutamate-gated chloride
IC	Inhibitory concentration
IVM	Ivermectin
LC	Lethal concentration
LOEC	Lowest observed effect concentration
MR	Metabolic rate
MS222	Tricaine methane sulfonate
NOEC	No observed effects concentration
SEM	Standard error of the mean
OP	Organophosphate
UV	Ultraviolet

Glossary

EC_{50}	Effect concentration affecting 50% of the test organisms
IC_{50}	Inhibitory concentration affecting 50% of the biological function
LC_{50}	Lethal concentration affected 50% of the test organisms
Log K_{ow}	The log of the ratio of a chemical concentration in octanol and water at equilibrium at a specified temperature. This value is used as a measure of hydrophobicity.
MO_2	Oxygen consumption rate (mg O_2 kg^{-1} h^{-1})

Chapter 1. General Introduction

1.1. Aquaculture in Canada

Seafood is a valuable commodity, providing a source of sustainable food for the growing human population. Global fish consumption has outpaced population growth by approximately 3% and has also exceeded meat consumption from all terrestrial animals combined (Food and Agriculture Organisation of the United Nations [FAO] 2020). With the state of many productive marine ecosystems having observed stock collapses to biologically unsustainable levels from overfishing (FAO 2020), the demand for seafood cannot be met by capture fisheries alone. To accommodate for the increase in per capita demand and a decrease in marine fish stocks worldwide, the aquaculture industry has undergone unprecedented expansion in the last half century. Globally, aquaculture is the fastest growing food producing industry, contributing over 82 million tonnes of seafood annually, accounting for nearly half of global seafood consumption and is worth US $250 billion (FAO 2020). Aquaculture has been conducted on a rural subsistence scale for thousands of years, though in recent decades intensive commercial scale farming of high-value species has become prevalent (Naylor et al. 2000).

Canada has proven to be a substantial contributor to the aquaculture industry worldwide. Operations include marine and freshwater systems, as well as land-based culture technologies, of which numerous species such as Atlantic and chinook salmon, trout, Arctic char and shellfish such as mussels, oysters and clams are produced (Fisheries and Oceans Canada [DFO] 2019a). The aquaculture industry represents about a third of the total fisheries value in Canada, of which 20% is total seafood production (DFO 2019a). Collectively, the economic value of the aquaculture industry nears $1.1 billion CAD in gross domestic product annually with another $1 billion CAD in spin-off impact (DFO 2019a). Many coastal and rural communities have gained economic stability with aquaculture, as nearly 15,000 individuals are employed full-time in the fisheries and aquaculture sector. The farmed Atlantic salmon industry, specifically, provides approximately 10,000 jobs throughout Canada and has the greatest economic value compared to other species produced by aquaculture in the country.

Atlantic salmon (*Salmo salar*) farming originated in Norway during the 1960's, and the practice was adopted by numerous countries including Scotland, England, Japan, Chile, New Zealand, Australia, the United States (US) and Canada. Currently, Norway and Chile are the world leaders in salmon production by tonnes, followed by the United

Kingdom and Canada (FAO 2020, DFO 2019a). Each of these countries have specific licensing and operation policies in place and are understood to be some of the mostly highly regulated food production systems in the world (FAO 2020). The Maritimes saw the birth of successful commercial Atlantic salmon farming in Canada, largely due to its geographic characteristics that provided protective and productive locations for net pens. Today, the Pacific Northwest generates the largest salmon farm production, again largely due to the protected coastal inlets scattered throughout the coastline of British Columbia (BC). The Atlantic salmon farming industry in Canada is regulated by Fisheries and Oceans Canada (DFO), which oversees day-to-day operations, animal husbandry regulations and food safety; BC's provincial government is also involved in licensing and the determination of aquaculture sites (BC Ministry of Environment and Climate Change Strategy [ENV] 2019a). Most of the cultured salmon produced in BC is exported to the US, China and Japan (Ministry of Agriculture and Lands [MAL] 2019). True to the rapid expansion of aquaculture, BC saw a rapid increase in the number of net pen sites from 5 in 1984 increasing to 10 companies that own 147 net pen locations. Three multinational Norwegian companies, Cermaq, Marine Harvest and Grieg Seafood, manage 90% of these aquaculture sites in BC, while the remainder are run by Canadian companies (Living Oceans 2014). As previously mentioned, harvesting and processing provides numerous jobs and economic opportunities for coastal communities and has had successful partnerships with many First Nations groups. Today, the Atlantic salmon aquaculture harvests in BC generate a total farm gate value of $708.7 million CAD from 100,321 tonnes (MAL 2019).

Salmon farms primarily utilize a permeable net pen system that allows a congruent flow of marine water through an enclosed space, providing a natural environment for cultured juvenile fish and reduces costs associated with oxygenation, waste management, salinity and temperature control. Salmon are initially cultured on land and then transported as smolts to the net pens, where they grow for 14 – 18 months in the sea and are then harvested when they reach approximately 4 kg in weight. The salmon are cultured in high densities, and due to the open nature of the pens, nutrients, wastes, chemical inputs and pathogens are able to disperse freely into the surrounding marine environment. Atlantic salmon is the most commonly cultured salmon species in Canada, as previously described, however Pacific salmon that include coho (*Oncorhynchus kisutch*), sockeye (*O. nerka*), and Chinook (*O. tshawytscha*) are also produced in low quantities in net pens (less than 6%) (DFO 2019a, ENV 2019).

The value in seafood as a commercial commodity and rising consumption globally has resulted in an increase in demand for fisheries products, and in response Canada has become one of the world leaders of commercial-scale intensive aquaculture providing product to regional and global markets. However, the associated positives are not without potential problems. Due to the nature of the net pen system, proximal marine ecosystems are exposed to numerous stressors including biological materials such as fish fecal matter and uneaten feed, pests, pathogens and the chemical contaminants used to alleviate them. Specific to this research, sea lice are ectoparasitic copepods that have proven to be detrimental to salmon farms. Although these organisms are natural pests to wild salmon populations, the density of fish in net pens provides an ideal environment for outbreaks of sea lice to occur. Many aquaculture facilities in BC are situated in coastal areas adjacent to juvenile wild salmon migratory routes, therefore wild salmon are at risk of parasitic infestation. Perhaps more pertinent however, is that the chemicals applied to sea lice infested pens, although strictly regulated, are released directly into the marine environment posing risks to non-target organisms across taxa.

1.2. Sea lice management in Canada

Sea lice are marine copepods that exist naturally at low ambient levels and can infect all salmonid species. The species *Lepeoptheirus salmonis* and *Calgus elongatus* are commonly found in the northern hemisphere (Johnson and Albright 1991). *L. salmonis* is unique in that it is a species specific to the Pacific Northwest and only uses salmonids as host organisms. Sea lice attach to the host epidermis and feed on the tissue, mucus and blood, resulting in skin abrasions and lesions that can lead to osmoregulatory issues and increase secondary infections, and can reduce host fecundity, growth and overall survival (Bowers et al. 2000, Sackville 2011, Godwin et al. 2015, 2017). Sea lice are also believed to cause behavioural changes in fish, including leaping by juveniles (to dislodge lice), a behaviour that may attract predators and incur substantial energetic costs (Atkinson et al. 2018). As well, altered feeding behaviours can occur in adults (Dawson et al. 1999). Interestingly, Atlantic salmon have been found to have reduced mucosal and protease defenses, as well as the thinnest epidermal layer of various salmon species which may account for their higher susceptibility to *L. salmonis* (Johnson and Albright 1992, Dawson et al. 1998, Glover et al. 2001, 2005). Pest management strategies used to mitigate negative impacts in salmonid aquaculture caused by sea lice infestations have been estimated to exceed $600 million USD globally (Costello 2009, Abolofia et al. 2017).

The permanent presence of salmon in coastal aquaculture facilities results in a sustained source of sea lice in these ecosystems. Studies in Europe and North America have demonstrated a spatial association between sea lice infected wild fish populations and salmon farms (Mackenzie et al. 1998, Butler 2002, Krkosek et al. 2005, Nekouei et al. 2018). Generally, out-migrating smolts do not encounter parasites due to the temporal difference with adult salmon returning to spawn, however higher loads of sea lice have been documented on numerous species of juvenile Pacific salmon in regions near salmon farms (Nekouei et al. 2018). Political, public, and scientific pressure in BC prompted the implementation of strict management strategies, which include improved animal husbandry, required monitoring of sea lice and mortalities as well as reporting the health status and inventory of the fish to DFO on a monthly basis (DFO 2019b). A regulatory threshold of three motile sea lice per fish permits implementation of management procedures as a means to provide immediate control of infestations. The aquaculture industry relies heavily on the use of chemotherapeutants to reduce sea lice parasitic loads and these methods include topical bath treatments or in-feed preparations.

In Canada, Health Canada oversees federal regulation of chemotherapeutants in which the toxicity, efficacy, and environmental fate of sea lice treatment types is assessed. The topical bath treatments are considered pesticides and are regulated under the *Pest Control Products Act* through the Pest Management Regulatory Agency, while the in-feed treatments are controlled by the Veterinary Drugs Directorate under the *Food and Drugs Act* as they are considered an antibiotic (ENV 2019b, DFO 2019c). Administration of treatments to salmonid aquaculture facilities requires a prescription from a licensed veterinarian. Ultimately following application, all chemical compounds are released into the marine environmental at some capacity, contaminating the water column, benthic sediments or both. Unlike land agriculture pesticides, all anti-sea lice chemotherapeutants lack species specificity, therefore the concern of toxicity to non-target organisms, such as invertebrates, is high.

Anti-sea lice chemotherapeutants are a mechanistically diverse group which include organophosphates, pyrethroids, chitin synthase inhibitors, hydrogen peroxide and avermectins. Resistance and reduced sensitivity to chemical treatments has been found despite efforts to reduce overuse and integrate management strategies. Canadian aquaculture facilities have used numerous chemical strategies since the first reported sea lice outbreak in the early 1990's. These include the pyrethroid formulations Alphamax®

(active ingredient [AI] deltamethrin) and Excis® (AI cypermethrin), ivermectin, Saralect® (AI hydrogen peroxide), and the chitin synthesis inhibitor Calicide® (AI teflubenzuron). Due to low efficacy, threat of resistance or low therapeutic indexes, many of these chemicals were not renewed for registration. Current use treatment options in Canada are limited to three anti-sea lice chemotherapeutants approved by Health Canada (Health Canada 2016, 2017 and 2019):

- SLICE® (AI emamectin benzoate);
- Paramove®50 (AI hydrogen peroxide); and
- Salmosan® (AI azamethiphos).

SLICE® is applied as a medicated fish feed treatment whereas Paramove®50 and Salmosan® are applied in water baths as a topical treatment, either directly into the net pen using a skirt or tarp to enclose the treatment area or by transferring fish into a well-boat. SLICE® is the most common treatment method in Canada.

1.2.1. Azamethiphos (Salmosan®)

Organophosphates are chemicals that impede nervous system functioning through irreversible inhibition of acetylcholinesterase (AChE), the enzyme responsible for the hydrolysis of the neurotransmitter acetylcholine (ACh) into choline and acetic acid in neural synapses (Bajgar 2004). Organophosphates are analogous to ACh, covalently binding to the serine hydroxyl group at the active site of AChE. Under a normal biological state, ACh hydrolysis allows a neuron to return to resting state following excitation. When a competitive inhibitor such an organophosphate irreversibly binds the AChE active site, ACh accumulates in neural synapses causing overstimulation of nicotinic and muscarinic ACh receptors and impedes neurotransmission. Convulsions, ataxia, twitching and eventually paralysis or mortality are common symptoms in individuals affected by acute and severe organophosphate toxicity.

Azamethiphos (S-6-chloro-2,3-dihydro-2-oxo1,3-oxazolo [4,5-b] pyridin-3-ylmethyl 0,0-dimethyl phosphorothioate), the active ingredient in the formulation Salmosan® is currently the only organophosphate used in the aquaculture industry as other compounds were found to have narrow toxicity margins or resistance of sea lice was observed (Urbina et al. 2019). Salmosan® has only been used on the east coast of Canada due to probable toxicity to non-target west coast species such as clams and spot prawns (Health Canada 2017), however due to the threat of resistance, emergency use may be implemented at

facilities located on the Pacific coast. Salmosan® is also registered for use in Chile, Norway and Scotland.

Azamethiphos has a low log octanol-water water partition coefficient (log K_{ow}) value of 1.05 and a high-water solubility (1.1 g/L) (Tomlin 1997). Due to these physicochemical characteristics, azamethiphos will not likely accumulate in sediment and organisms, and will remain in the aqueous phase (Roth et al. 1993, 1996, Ernst et al. 2014). Environmental degradation takes place approximately 6 to 9 d through hydrolysis and photolysis. The formulation Salmosan® is applied as a wettable powder to a target concentration of 100 µg/L of azamethiphos (50% w/w) for a temperature-dependant exposure time of 30 - 60 min at the discretion of the veterinarian (<10°C treated for 60 min and >10°C treated for 30 min). Due to the low efficacy against juvenile and larval sea lice, repeated treatments are warranted during high infestation. Currently, there is a limit of two pulse treatments per d per aquaculture site by the Pest Management Regulatory Agency (PMRA) (Health Canada 2017).

Following application, tides and currents strongly dictate the dilution and distribution of the chemical in the water column. Tarpaulin treatments are usually restricted to weak tidal currents to prevent tarp collapse on the fish, however this may create stagnant plumes of pesticide in the water column. A field study in Atlantic Canada analyzed marine concentrations following the release of Salmosan®-treated baths using rhodamine dye as a tracer in an effort to characterise contaminant plume distribution (Ernst et al. 2014). Azamethiphos concentrations ranged from 1.1 - 11 µg/L and 0.2 - 1 µg/L approximately 1 m and 1000 m from application release areas, respectively, 2 - 3 h after treatment. Generally, the water sample concentrations taken from plumes after well-boat application were one third less than those from tarpaulin treatments.

The sensitivity of sea lice to azamethiphos is variable, as some populations are more sensitive to this chemical than others (Roth et al. 1996, Denholm et al. 2002). Development of resistance to organophosphates is common and has been shown for azamethiphos in insect pests (Levot and Hughes 1989) as well as in aquaculture facilities in Europe (Denholm et al. 2002). Resistance has also been observed in Canada, resulting in the suspension of Salmosan® in 2002 as a result of low efficacy; however, in 2009 it was given emergency registration in New Brunswick and has since undergone full registration in 2017 (Health Canada 2017). Resistance is believed to be due to a mutant allele that reduces the accessibility of azamethiphos to the binding site of AChE (Kaur et

al. 2015 and 2016). In sensitive sea lice populations, azamethiphos is effective in removing > 85 % of adult and pre-adult sea lice but is not effective against the earlier life stages of the parasite (Roth et al. 1996).

Some published literature exists on azamethiphos toxicity to non-target marine species, although research on Pacific marine organisms is limited. Specific groups of crustaceans, including lobster and crab, have been found to be the most susceptible species to azamethiphos. In eastern Canada, lobster aquaculture overlaps geographically with salmonid aquaculture, therefore concerns of cross contamination have led to significant research on species relevant to Atlantic ecosystems. Lethal concentrations affecting 50% of the sample test organisms (LC_{50}) from a 48-h repeated short-term exposure test on the American lobster (*Homarus americanus*) ranged in concentrations of 1.03 µg/L to 3.57 µg/L, depending upon life-stage (Burridge et al. 1999). Of additional concern was that many of the surviving lobsters displayed adverse behavioural effects, becoming agitated, flopping erratically and showing aggressive behaviour. A follow up study by Burridge et al. (2000) supported the previous findings, with a 48-h LC_{50} value of 1.08 µg/L and lobsters again presenting signs of distress at all concentrations following intermittent exposure. Research performed on the Southern rock crab (*Metacarinus edwardsii*) has also shown sensitivity to azamethiphos with a 30-min LC_{50} value of 2.85 µg/L (Gebauer et al. 2017).

Marine invertebrates including bivalves, gastropods, amphipods and echinoderms have been shown to exhibit lethality with azamethiphos exposure, however effects were at concentrations much higher than those prescribed for treatment regimes. Ernst et al. (2001) has performed toxicity tests with Salmosan® on numerous species: the bacterium (*Vibrio fisheri*), the green sea urchin (*Strongylocentrus droebrachiensus*), the painted sea urchin (*Lytechinus pictus*) (assessing fertilization), the threespine stickleback (*Gasterosteus acualeatus*), three amphipod species (*Amphiporeia virginiana, Gammarus* spp, and *Eohaustorius estuarius*), a polychaete (*Polydora cornuta*) and brine shrimp (*Artemia salina*). Lethal concentrations ranged from 5 µg/L (amphipod) to 190 µg/L (stickleback) and > 10,000 µg/L for brine shrimp, whereas sublethal effects (immobilisation) were observed at concentrations as low as 3 µg/L (amphipod). Additional sublethal effects have been shown in mussels, where shell closure rate was reduced at a concentration of 100 µg/L (Burridge and Van Geest 2014). Interestingly, a field study performed by Ernst et al. (2014) did not find toxicity to *E. estuarius* using plume samples,

however this is limited to one species and *E. estuarius* has not displayed marked lethal sensitivity to azamethiphos. There is currently no published work investigating the effects of Salmosan® to planktonic organisms. Table 1-1 in Section 1.6 details a summary of the documented lethal and sublethal effects observed on marine species following exposure to azamethiphos.

1.2.2. Hydrogen peroxide (Paramove®50)

Aquaculture facilities in Canada have also used hydrogen peroxide to treat sea lice outbreaks. Hydrogen peroxide is a strong oxidizing agent that is applied as a bath treatment in the formulation Interox™ Paramove®50 (50% hydrogen peroxide [H_2O_2] w/w), herein referred to as Paramove®50, at a target concentration of 1200-1800 mg/L H_2O_2 (Health Canada 2014). Research suggests that H_2O_2 causes mechanical paralysis in the sea lice through the formation of bubbles in the haemolymph and gut, causing the louse to detach from the fish skin and float (Bruno and Raynard 1994). H_2O_2 is also believed to inactivate enzymes, inhibit DNA replication and cause peroxidation of lipids and membranes from hydroxy radicals (Cotran et al. 1989). In order to fully remove the sea lice from the net pen, the buoyant organisms are removed from the water with a skimmer. Due to the chemical characteristics of H_2O_2, such as its miscibility in water, low log K_{ow} value (-1.6), as well as short half-life (12 h to 7 d) and non-toxic breakdown products (water and oxygen) (Health Canada 2014), there is reduced environmental concern to non-target species as it is unlikely to persist or bioaccumulate.

The use of Paramove®50 was previously limited to areas with severe resistance in sea lice to other approved sea lice chemotherapeutants in Canada, however in 2016 Health Canada fully registered the pesticide under the premise that application would not result in unacceptable risk to human and ecological receptors. As previously stated, H_2O_2 is applied using the liquid formulation Paramove®50 as a bath treatment to a temperature dependant target concentration of 1200 - 1800 mg/L H_2O_2 for a period of 20 to 30 min. Treatments are limited to one application every 7 d, and no more than 5 times per year. In association with bath treatments, cage size, discharge rate, tidal flows, currents and other abiotic factors dictate the dilution and distribution of the chemical. Dye dispersion studies indicate plumes are likely elliptical in shape following release (Okubo 1971). Recently, only well-boat application and subsequent release into the environment is permitted in BC (ENV 2018).

Hydrogen peroxide has demonstrated inconsistent efficacy when used against pre-adult and adult sea lice and has also had reduced effects against larval stages (Mitchell and Collins 1992). Temperature and exposure duration have a large influence on efficacy, as temperature below 10°C and above 14°C are believed to markedly reduce or completely inhibit therapeutic outcomes. Recovery has been observed in laboratory experiments in which adults regained mobility within 30 min to 2 h post-exposure (Hodneland et al. 1993, Bruno and Raynard 1994). Experimental exposures to Atlantic salmon have indicated that temperature also alters toxicity and that there is a narrow therapeutic window before sublethal damage to gills or mortality occurs, at concentrations between 200 and 2000 mg/L if fish are exposed too long (Roth et al. 1993, Thomassen 1993, Keimer and Black 1997). Reduced sensitivity towards H_2O_2 has been observed in Scotland (Treasurer et al. 2000) and recently Norway, in which sensitivity has also been proposed to be hereditary and strain-dependant (Helgesen 2015).

The toxicity information of H_2O_2 to non-target marine organisms is limited. Given the proximity of net pens to bays and inlets, there is a chance that indigenous species, such as crustaceans, could be sensitive to short-term exposures soon after treatment. McCurdy et al. (2013) investigated the effects of 1-h exposures to Paramove®50 on the maritime indigenous mysid shrimp species *Mysis stenolepsis* and *Praunus flexosus* and determined LC_{50} values of 1650 and 1222 mg/L after 24 and 96 h, respectively. Subsequent work performed by Burridge and Van Geest (2014) estimated LC_{50} values of 1637 mg/L for the American lobster *H. americanus* stage I, > 3750 mg/L for adult *H. americanus*, 3182 mg/L for the sand shrimp *Crangon septemspinosa*, and 973 mg/L for the msysid species *M. steolepsis* and *P. flexosus* following a 1-h exposure to Paramove50® and a 95-h post-exposure monitoring period. Additionally, the amphipod *C. volutator* has been found to have a 96-h LC_{50} value of 460 mg/L (Smit et al. 2008) and the brine shrimp *A. salina* had a 24-h LC_{50} value of 800 mg/L (Matthews 1995). Generally, the acute toxicity to non-target species has been observed to be below the application concentrations of 1200 - 1800 mg/L H_2O_2 raising concerns about the effects to marine organisms near salmon farms. Furthermore, there is a lack of data on planktonic species despite their importance in marine ecosystem functioning and potential sensitivity to the chemical. Table 1-2 provides a summary of the toxicity data of Paramove®50 to marine non-target species.

1.2.3. Avermectins: Ivermectin and Emamectin Benzoate (SLICE®)

Avermectins are a group of chemotherapeutants that are widely used in both animal and human medicine for pest and parasite control. Avermectins are 16-membered macrocyclic lactones derived from *Streptomyces avermitilis*, a soil bacterium, that are used at relatively low doses, and have high lipophilicity and chemical stability. Avermectins bind irreversibly to glutamate-gated chloride channels causing an influx of chloride ions, inhibiting nervous system transmission and causing hyperpolarization of nerve and muscle tissue (McKellar and Benchaous 1996, Wolstenholme 2012). Avermectins have systemic action and are therefore effective against endo- and ecto-parasites, which allows a broad use of the chemicals against target parasites (Campbell 1989). The mechanism of action is also unique to invertebrates that allows for selective toxicity and low adverse effects towards host organisms. Formulations range from chewable tablets, oral liquids and topical treatments, to injectable preparations. At aquaculture facilities, salmon are given an in-feed medication in the form of pellets containing the desired avermectin. The chemical is absorbed in the gut and distributed throughout the fish into the plasma, mucus, skin and muscle following consumption (Whyte et al. 2011). Sea lice that are latched onto the skin of the salmon feed on the external tissue and mucus, resulting in paralysis, loss of motor activity and death. The concentration of absorbed avermectins have been reported to be higher in mucus and lowest in skeletal muscle (Sevatdal et al. 2005). Two avermectin products have been used for sea lice treatment in Canada: ivermectin (IVM) and emamectin benzoate (EMB), in which the latter is applied as the formulation SLICE®.

Ivermectin

The discovery of IVM in 1973 resulted in it's widespread use as a chemotherapeutant, primarily in agricultural settings for livestock health following parasitic infections. Veterinary approval and subsequent application is necessary, which is standard protocol regulated by Health Canada for all uses of the chemical. IVM treatment to sea lice impacted fish farms seemed to be a natural segue from terrestrial to aquatic systems. Ireland and Scotland had reportedly used the chemical in the early 1990s (Roth et al. 1993), while in Canada, IVM was available as an "off label" veterinary prescription until 1999. However, following treatment the low therapeutic index of the drug raised concerns regarding fish health as the therapeutic dose is 0.05 mg/kg bw and the lethal dose to salmon is 0.5 mg/kg bw (Davies and Rodger 2000).

Due to the administration of IVM in oral feed, there may be discrepancies between target and measured doses resulting in either under or over exposure to the chemical. As described, IVM is orally administered through an in-feed pellet treatment; the recommended dose is 50 µg ivermectin per kg of food over a 7-d period to achieve therapeutic results (Davies and Rodger 2000). IVM is relatively insoluble in water at 4 mg/L (Tomlin 1997) and has a log K_{ow} value of 3.2 - 3.6 (Campbell 1989), with half-lives in water and sediment greater than 28 d and 200 d, respectively (Campbell 1989, Davies 1998). The low solubility, moderate octanol-water partition coefficient and half-life suggests that IVM will adsorb and persist in sediment, with a slow degradation time. Given the predicted environmental fate following application, in addition to the potential threat of toxicity to farmed salmon due to the low therapeutic index of IVM, residual contamination from fish feces and uneaten feed increase the likelihood of toxicity to non-target biota in the receiving environment, specifically from accumulation in sediments beneath and near treated net pens. In Canada, following the approval SLICE® use in 2000, IVM was discontinued as a sea lice chemotherapeutant largely due to these toxicological concerns (DFO 2019d).

The toxicity of IVM to non-target marine organisms has been quantified in some species. Planktonic organisms, such as mysid shrimp and the water flea, are known to be the most sensitive when exposed in water, with an LC_{50} of 0.026 µg/L and 0.025 µg/L, respectively (Campbell 1989, Grant and Briggs 1998). However, the route of exposure in environmentally relevant situations will likely be through contact with organic matter and unlikely in the dissolved state due to the low solubility of IVM. Unfortunately, there is currently little data regarding the toxicity of IVM associated with sediment exposures. Some oral exposure studies have found toxicity to invertebrate species, in which crustaceans (i.e., amphipods) and marine annelids appear to be the most sensitive. However, information across and within taxa is scarce. Collier and Pinn (1998) investigated effects on the benthic community using sediment cores dosed with IVM. The polychaete *Hediste diversicolor* was the most sensitive species, with 100% mortality within 14 d at a concentration of 8.0 mg/m^2 of sediment. The available literature detailing toxicity to species following sediment exposure to ivermectin is detailed in Table 1-3 in Section 1.6.

Emamectin Benzoate (SLICE®)

Emamectin benzoate (EMB) is a mixture of two avermectin homologues and is the active ingredient of the SLICE® premix feed (0.2% EMB w/w). The remaining ingredients in SLICE® are butylated hydroxyanisole (0.01%), propylene glycol (2.5%), maltodextrin (47.40%) and cornstarch. Butylated hydroxyanisole and propylene glycol have been reported to have negligible risk to the environment (SEPA 1999). The efficacy of EMB is very high immediately following application, causing 98% sea lice disengagement from juvenile and adult Atlantic salmon with no adverse effects to the fish (Stone et al. 2000). The duration of EMB efficacy after oral administration has been observed up to 9 weeks post-treatment (Stone et al. 2000). An application concentration of 50 µg of EMB per kilogram of fish per d for 7-d is recommended for sea lice management practices (Stone et al. 1999).

The simplification of sea lice control using a medicated feed compared to complicated skirted tarpaulin and well-boat treatments with large quantities of fish resulted in licensing of SLICE® in Chile, Canada, Norway, Scotland and Ireland almost immediately after introduction. BC is currently the only province that uses SLICE® for sea lice control in Canada, however as previously stated, the premix feed has been in use for 20 y following the phasing out of IVM. For the first decade, SLICE® was only applied during emergency scenarios under the Health Canada Emergency Drug Release program (Health Canada 2016). In 2009, this chemotherapeutant was approved by Health Canada's Veterinary Drug Directorate and currently residues in fish tissue are monitored by the Canada Food Inspection Agency (CFIA) in a quality management program (CFIA 2018). Despite being the product of choice by many companies and farming locations, challenges still remain regarding toxicity to non-target animals and resistance development.

EMB is a lipophilic compound that has a log K_{ow} value of 5 - 5.9 with a solubility in water of 5.5 mg/L at a pH of 8, and the calculated half-life in marine sediment is 165 - 250 d (McHenery and Mackie 1999, SEPA 2004). These characteristics raise concerns regarding long-term exposure scenarios in the environment; however, the compound is bulky, with a large molecular weight (1000 g/mol) and some polar characteristics that may inhibit its ability to bioconcentrate and bioaccumulate in organisms due to steric hinderance with cell membranes and other cellular components (Nendza and Hermens

1995). Regardless, ecotoxicological data has indicated adverse effects to marine invertebrates following exposure to EMB.

Toxicity tests have been performed on marine species *via* water, sediment and in-feed exposures. Seawater treatments have focused on copepod and small crustacean species, with lethal toxicity observed as low as 0.04 µg/L reported for the water flea *D. magna* after 96-h (Conner et al. 1994). Immobilisation of various copepod species was observed between 0.2 µg/L to 231 µg/L in 48-h experiments (Willis and Ling 2003). As with other in-feed treatments however, SLICE® and the resulting toxicity from EMB exposure will primarily not be in water exposures due to both the application into the environment and it's chemical properties favouring sediment deposition. Toxicity studies using sediment and feed have been performed, however the data is scant. Exposure research in Canada has shown premature molting and loss of eggs in American lobsters, *H. americanus,* fed EMB doses between 220 - 390 µg/kg (Waddy et al. 2007). Amphipods exposed in sediment for 10-d had LC_{50} values ranging from 153 - 193 µg/kg sediment (McHenery and Mackie 1999, Mayor et al. 2008, Kuo et al. 2010). Polychaete sensitivity may be species-dependant, with LC_{50} values ranging from 111 – 1,368 µg/kg for *Arenicola marina* and *H. diversicolor*, respectively (McHenery and Mackie 1999, Mayor et al. 2008). Collectively, like most chemicals, there appears to be a range of toxicity values that depend on the species and exposure scenario. However, it is clear that EMB is not as toxic as other avermectins used for sea lice control as it has a larger therapeutic window with higher LC_{50} values when compared IVM between similar species. A summary of the toxicological parameters performed with EMB through sediment and feed exposures on marine species in given in Section 1.6 in Table 1-4.

Resistance to Avermectins

Resistance to chemotherapeutants is a continuous problem when managing sea lice. There are limited options for chemicals with high efficacy to ensure control of these pests. As mentioned previously, SLICE® has become the chemotherapeutant of choice by many aquaculture facilities as it has a lower toxicity to Atlantic salmon and to non-target species compared to IVM or other drug classes, although overall research investigating non-target animals is limited. Historically, the recurrent use of chemicals often leads to their ineffectiveness over time (Denholm et al. 2002, Haya et al. 2005), which prompts the application of new products or a return to previous methods. Resistance of sea lice to EMB has already been observed in Scotland (Lees et al. 2008), Chile (Bravo et al. 2008)

and in eastern Canada (Wescott et al. 2010, Igboeli et al. 2012). Skilbrei et al. (2008) found that the EMB treatment only protected fish from sea lice for 6 weeks, compared to the 9 and 10 weeks previously observed (Stone et al. 1999 and 2000). The decreased efficacy of EMB observed by Skilbrei et al. (2008) may be due to decreased sensitivity from prolonged application over a 5 - 10 y period. It is important to consider that appetite can vary between individual fish, causing variation in the tissue concentrations of the chemical (Berg and Horsberg 2009), which may be interpreted as resistance if lower efficacy is observed. However, collectively if entire pens lose protection from the chemotherapeutant over time at different treatment locations, including internationally, it is unlikely to be due to differences in diet or application.

As a result of the resistance observed in the laboratory and in practice, reliance on SLICE® as a sea lice treatment has declined. Canadian aquaculture facilities on the west coast have incorporated Paramove®50 into treatment strategies to protect farming stocks in addition to using SLICE® (ENV 2018). Although ivermectin was not used long enough to observe resistance in salmon farms, agricultural use has demonstrated resistance in ruminants that are treated for parasites in countries such as Brazil, New Zealand, Malaysia, Kenya and the United States (Blackhall et al. 1998). The mechanism of resistance has not been fully elucidated but is suggested to be due to enhanced detoxification, increased transcription of drug transport proteins and decreased gated chloride channel expression (Clark et al. 1995, Xu et al. 1998, Tribble et al. 2007, Carmichael et al. 2013).

1.2.4. In-feed treatments and deposition in the marine environment

In-feed treatment for sea lice control provide ease in application and minimal handling of fish compared to bath treatments that require a tarpaulin skirt or well-boat. Unfortunately, during the feeding process only 1 - 17% of feed is consumed by the salmon with the remainder falling through the open net pens into the marine environment (Cubitt et al. 2008). This percentage of feed lost is influenced by fish consumption and excretion, tidal flow/transport, the application method and other factors (Berg and Horsberg 2009, DFO 2012). Approximately 25 - 33% of ingested feed is believed to become feces and destined for the ocean floor (Weston 1986). This combination of food waste and feces production increases the deposition of organic matter in the marine environment, as well as introducing contaminants into the ecosystem. Stucchi et al. (2005) estimated that almost 20% of organic matter beneath net-pens is due to these fish farming by-products.

The extent of organic fallout is typically limited to a 150 m radius, although some net pen residues have been found greater than 300 m away (Weston 1990, Schendel et al. 2004). As previously described, the in-feed treatments active ingredients IVM and EMB are likely to adsorb onto sediment due to their physicochemical properties. These two avermectins also have demonstrated half-lives that exceed 150 d in sediment, which indicates that not only are IVM and EMB likely to bind to sediment but will also remain in the marine environment for extended periods of time with slow degradation.

Studies on leaching and deposition have primarily focused on EMB, likely due it's prevalent use compared to IVM. Due to the similar chemical characteristics and method of application it is probable that deposition and leaching will be comparable. Residues in the environment and the persistence of contaminants are dependant on multiple factors such as application amount, the microorganism community and water chemistry (Hand and Fleming 2007, DFO 2012). Davies et al. (1998) found that approximately 5% of IVM leached from feed over a 4 h period. Similarly, EMB was found to leach from feed up to 5% over a 6-h period, but up to 25% after 7 d (SPAH 2002). The highest amount of accumulation in sediment is generally within 25 - 60 m of the net pens but can be detected hundreds of metres away as a result of seawater hydrodynamics (Tefler et al 2006, DFO 2012). EMB and IVM are both subject to photolysis and may partially degrade once entering the water column (Mustaq et al. 1998), but it does not appear to markedly reduce sediment deposition concentrations due to depth to sediment. EMB concentrations in the water column in the vicinity of a salmon farm undergoing treatment have been found between 0.006 - 0.635 ng/L in Canada (DFO 2012).

Residues of IVM and EMB have been detected in sediment after treatment in various countries, including Norway, Scotland, Chile and Canada. The results of these investigations are described in Table 1-5 in Section 1.6. A majority of studies report detections of EMB beneath net pens after treatment, as is expected given the application of the chemical. Generally, EMB concentrations are between 0.5 and 2 µg/kg of sediment however, some sampling events found concentrations as high as 140 and 366 µg/kg (McHenery and Mackie 1999, Boxall et al. 2002, Lalonde et al. 2012). Modeling studies have also been used to predict concentrations in the environment. McHenery and Mackie (1999) used the DEPOMOD fate model to predict surface sediment concentrations of EMB at 14 - 17 µg/kg and 1.7 - 2.6 µg/kg beneath net pens and 50 m away, respectively. These predictions were later validated in the field, in which EMB was detected at 2.73 and 0.62

µg/kg at 10 and 100 m from the net pen; 12 months later, 1.8 µg/kg was detected 10 m away. There are far fewer reports on the environmental fate and deposition of IVM. Currently, only three reports are available in the literature and all measured concentrations of IVM between 2.6 and 11 µg/kg within 25 m of the net pen (ERT 1997,1998, Canavan et al. 2000). It is important to note that the chemical analysis of sediment contaminated with EMB or IVM reflect only one a brief timepoint of a dynamic chemical mixture in flux. Given the hydrophobic nature of each test compound, both will tend to bind to organic sediments, thus should form highly concentrated aggregates within the substrate. As a result, it is unlikely that sediment will be uniformly distributed with the chemicals. Despite this, the information available indicates a relatively similar distribution from net pens, with average concentrations of approximately 5 µg/kg of each chemical type within the vicinity of fish farms following treatment.

1.3. Non-target species at risk

Due to the nature of application, whether as a bath and subsequently released into the water column or as a feed directly into the open net pen, each of the chemotherapeutants used to treat sea lice outbreaks at Atlantic salmon aquaculture facilities presents some risk to aquatic receptors in the marine environment. Several studies have evaluated adverse effects, and the presence of chemotherapeutants in the environment has also been quantified through modelling and field measurements, as described previously. Of course, the application and physicochemical characteristics of each chemotherapeutant will likely have a larger effect to specific non-target species. In the following sections the vulnerability of pelagic invertebrates to the water-soluble sea lice pesticides (Salmosan® [AI azamethiphos] and Paramove®50 [AI H_2O_2]) and benthic invertebrates to in-feed anti-sea lice chemotherapeutants (IVM and SLICE® [AI EMB]) will be discussed.

1.3.1. Pelagic invertebrates

Pelagic invertebrates, which include various species of phytoplankton, zooplankton, jellyfish, rotifers and cladocerans, dominate the open ocean and are key to the survival of many species as they occupy primary and secondary trophic levels. Adverse effects to these primary producer's effect consumers along the food chain as they are a critical energy source and are heavily preyed upon by fish and some marine mammal species. Consequently, planktonic species are believed to mediate bottom-up food web

dynamics and biogeochemical cycling in the ocean (Armengol et al. 2019). Since these species generally have short generation times and are influenced by local physical factors they are well suited to studying ecosystem responses (Hays et al. 2005, Batten et al. 2018). Within the planktonic species, some remain plankton for the duration of their lifespan (holoplankton), whereas other species are only planktonic for a portion of their life (meroplankton). Species that occupy planktonic life stages include species of fish, squid, octopus, sea urchin, polychaetes and crab (De Senerpont Domis et al. 2013). Collectively, meroplankton and holoplankton contribute to the health of marine ecosystems and are important tools in assessment of environmental health, which includes evaluating potential risks to organisms from contaminants.

Current sea lice treatment regimes include the application of water-soluble pesticide formulations Salmosan® and Paramove®50. There is currently little to no data assessing the toxicity of these formulations and their respective active ingredients to planktonic species. With the myriad of effects that may come about from adverse effects to planktonic communities, it is necessary to gain an understanding if these non-target species are at risk. In BC, the Straight of Georgia, which is a semi-enclosed temperate basin between mainland BC and Vancouver Island, has some of the most seasonally productive surface waters in the northeast Pacific and North America (Harrison et al. 1983, Jackson et al. 2015). Productivity of planktonic species has direct implications to fish stock health, including species such as herring and Pacific salmon. Coincidentally, many Atlantic salmon farms are located within the protective inlets of the coast of BC within the Straight of Georgia. Planktonic species may therefore be at risk of exposure to water-soluble sea lice pesticides if they are within vicinity of treated aquaculture facilities. Current toxicology data does not provide adequate information of the effects of such exposures and does not include potential effects to planktonic species, including those that occupy planktonic life stages, in environmentally relevant scenarios.

1.3.2. Benthic invertebrate communities

Marine infauna occupy lower trophic levels and are vital to ecosystem functioning and well being. The members of benthic invertebrate communities specifically are important contributors to ecosystem processes such as bioturbation, reoxygenation of sediment, remineralisation of waste products, biodeposition and enhance overall biodiversity (Glud 2008, Bertics et al. 2010). Without these organisms, microbial degradation of organic matter would decrease, and marine sediments would become

anoxic and accompanied by toxic hydrogen sulphide formation, potentially causing deleterious effects to various marine species (Glud 2008). The physical properties of bedrock and sediment type drive the benthic habitat, influencing the species present and the resulting interspecific interactions. Consequently, benthic infauna are important indicators of habitat status and change and should therefore be incorporated in assessment of ecological health. Various species such as polychaetes, amphipods, dipterans and mysids are commonly used in monitoring and toxicology studies to assess potential impacts of contaminants.

Waste produced by Atlantic salmon farms are significant contributors the benthic environment, enriching organic matter beneath and in the vicinity of net pens. The contribution of nutrients from un-eaten feed pellets and fecal waste may attract species to these sites. For example, American lobsters have been observed to aggregate near salmon farms in Atlantic Canada (Findlay et al. 1995). Additionally, organically enriched sediment has been found to be dominated by opportunistic species of polychaetes such as those in the genus *Malacoceros* and *Capitalla* (Weston 1990, Black et al. 1997, Tefler et al. 2006, Neofitou et al. 2010). The implications of un-eaten feed and wastes containing avermectins used to treat sea lice at infected farms may therefore not only pose a risk to non-target benthic species but may also amplify adverse effects through attraction. Attraction may be species specific though, as Tefler et al. (2006) found that benthic species diversity increased with distance from salmon farms in Scotland and did not return to a uniform benthos structure until approximately one-year post-treatment.

As described in Section 1.2.3, avermectins have long-half lives between 150 and 200 d, therefore non-target benthic species may be at risk of exposure long after treatment. Davies et al. (1998) found that 100-d old sediment contaminated IVM still exerted toxic effects on the benthic amphipod *C. volutator.* The 100-d LC_{50} was approximately half of the initial 24-h LC_{50} value, while measurements indicated that only 30% of the IVM had degraded during the 100-d period. The half-life of EMB has also been found to exceed 150 d during field investigations of marine intertidal areas (SPAH 2002). Interestingly, Tefler et al. (2006) did not find evidence of toxicity to marine benthic species beneath net pens, and instead attributed observed effects on community structure to organic enrichment deposits. However, considering that Tefler et al. (2006) also found that community diversity increased at 12 months post treatment (thereby suggesting recovery) and that feeding and thus organic deposits would continue post-treatment, it cannot be

assumed that avermectin treatment did not have any adverse effects on certain species following application. The lack of data regarding adverse long-term effects from avermectin exposure is consequently unclear.

Benthic invertebrates live in close association with the top layers of sediment, acting as important indicators of substrate toxicity. Current data available on the effects of the sea lice chemotherapeutants, IVM and EMB (applied as premix SLICE®), is limited to lethal toxicity (see Table 1-3 and 1-4) and a single field study (Tefler et al. 2006). Generally, the concentrations obtained from the lethal toxicity studies are short-term exposures with high doses and are therefore unlikely to be encountered in the environment. Given that avermectins have long half-lives, evaluation of chronic sub-lethal endpoints at low environmentally relevant concentrations is sorely needed. The dose and range of sub-lethal and lethal effects are used by regulatory authorities to establish predicted effect concentrations and associated interim sediment quality guidelines for use and application of chemicals such as pesticides. Therefore, ecologically relevant data can be used to more accurately predict the long-term impacts of in-feed anti-sea lice chemotherapeutants on the marine environment.

1.4. Summary and objectives of study

Anti-sea lice chemotherapeutants are the choice treatment for sea lice infestations at Atlantic salmon farms in Canada. Controlling these parasites is essential to the protection of cultured fish and wild stocks but must be strategically applied to ensure therapeutic outcomes. Understanding the potential effects in the aquatic environment is necessary to ensure the protection of non-target species and marine ecosystems. Currently, limited toxicity data and subsequent risk management criteria are lacking for many anti-sea lice treatments. Specifically, the sub-lethal and behavioural effects of long-term exposure in marine species remain inconclusive and largely unknown. This research addresses the information gaps on the biological effects of anti-sea lice chemotherapeutants in marine benthic and pelagic invertebrates under environmentally realistic conditions. The chemotherapeutants of concern include Salmosan®, Paramove®50, SLICE® and IVM (see Figure 1). This research is necessary to understand the risk that these chemicals pose to non-target species as well as maintain a sustainable aquaculture industry, sustainable commercial and recreational fisheries as well as a healthy coastal marine ecosystem. Future guideline development and risk assessments of contaminated sites will also benefit from this research in Canada.

The sea lice pesticides Salmosan® and Paramove®50 are directly released into the marine environment after application to sea lice infected net pens. Pelagic species that occupy the water column in the vicinity of salmon farms are at risk of exposure. Planktonic species have not been evaluated toxicologically, which includes species that occupy planktonic life stages. On the Pacific coast of BC, sea urchins are important for kelp forest functioning and food sources for marine mammals. As echinoderms, this species uses broadcast spawning of planktonic gametes during reproduction. Therefore, as a meroplanktonic organism found within the coastal inlets of BC where fish farms are also present, they are at risk of exposure to sea lice pesticides in the pelagic zone. Currently, no research has been performed investigating toxicity to this species during this life stage.

The in-feed avermectin chemotherapeutants, SLICE® and IVM, are understood to partition to sediments due to their long-half lives, low solubility and high organic matter partitioning coefficients. SLICE® is the drug of choice due to the low therapeutic index of IVM, however in light of potential onset of drug resistance IVM may be reintroduced as an emergency pest management strategy in Canada. The persistence of avermectins in the environment beneath net pens has been documented at fish farm sites but the data describing sub-lethal environmentally relevant toxicity is poor. Additionally, with the introduction of IVM as an in-feed treatment, sediments beneath net pens may include residues of both contaminants. Benthic invertebrates, such as amphipods and polychaetes, are the ecological receptors most at risk of exposure to avermectins as they occupy and interact with the top layer of marine sediment. In order to understand the potential effects of anti-sea lice chemotherapeutants to these species and the marine ecosystems near treated aquaculture facilities, further research must be performed.

In this thesis two objectives are addressed, providing baseline sublethal data on the biological effects of water-soluble pesticides and in-feed drug treatment chemotherapeutants to benthic and pelagic invertebrate species (crustaceans, annelids, echinoderms) relevant to the Pacific coast of Canada. The two objectives are as follows:

1) To determine the effects of Salmosan® and Paramove®50 on echinoderm bivalve fertilization under realistic exposure concentrations.

2) To determine the sublethal toxicity of SLICE® and IVM and a combination of both, in chronic exposures in sediments representative to sediment dwelling crustacean and annelid species. Sublethal assessments focused on the following two endpoints:

a. Avoidance behaviour to chemotherapeutants.

b. Effects of sublethal chemical exposure on oxygen consumption.

These questions were addressed through several laboratory experiments with field-collected sediment (where applicable) and three marine species. Chapter 2 describes the outcome of the sea urchin fertilization assay for the Pacific purple sea urchin (*Strongylocentrotus purpuratus)* to the sea lice pesticides Salmosan® (AI azamethiphos) and Paramove®50 (AI H_2O_2) at environmentally relevant concentrations. In Chapter 3 and 4, experimental data are presented on the effects of the two avermectins, EMB (applied as SLICE®) and IVM, as well as a 1:1 combination of both, to the benthic invertebrate amphipod (*Eohaustorius estuarius*) and clam worm (*Nereis virens*), at environmentally relevant doses. Chapter 3 describes the avoidance and burrowing behaviour of each species in sediment dosed with the drug through a 48-h (*E. estuarius*) or 7-d period (*N. virens*). Prior to exposure, animals were either not exposed or chronically pre-exposed to the drug(s) for 30 d. Chapter 4 details the sub-lethal evaluation of oxygen consumption, as a measure of stress, throughout a 28-d exposure in sediment for each species. In the final chapter, the results of each of these experiments are discussed, in addition to future research and the application of this work to aquaculture risk management practices in Canada.

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1.6. Tables

Table 1-1.Toxicity test values for various marine species following exposure to azamethiphos via Salmosan® in water. LC_{50} = Lethal concentration affecting 50% of test organisms; EC_{50}= Effect concentration affecting 50% of test organisms; Mysid sp. = *Mysis stenolepsis* and *Praunus flexosus*; * = Mean value.

Species (Organism)	Life Stage	Endpoint	Azamethiphos Concentration (µg/L)	Exposure Time (h)	Reference
Homarus americanus (lobster)	Larval stage II	LC_{50}	0.9 – 37.3	0.5 – 12 + 12 recovery	Pahl and Opitz 1999
	Larval stage I	LC_{50}	3.57	48	Burridge et al. 1999
	Larval stage II	LC_{50}	1.03	48	
	Larval stage III	LC_{50}	2.29	48	
	Larval stage IV	LC_{50}	2.12	48	
	Larval stage I	LC_{50}	> 86.5	1 (96 h observation)	Burridge and Van Geest 2014
	Adult	LC_{50}	24.8	1 (96 h observation)	
Metacarinus edwardsii (crab)	Larva zoea I	LC_{50}	2.84	0.5	Gebauer et al. 2017
		EC_{50} (immobility)	0.94		
Crangon cragon (shrimp)	Adult	LC_{50}	19.2*	24	Ernst et al. 2014
Mysis stenlepsis (mysid)			10.5*		

Species (Organism)	Life Stage	Endpoint	Azamethiphos Concentration (µg/L)	Exposure Time (h)	Reference
Crangon septemspinosa (shrimp)	Adult	LC_{50}	>85.5	1 (96 h observation)	Burridge and Van Geest 2014
Eohaustorius estuarius (amphipod)	Adult	LC_{50}	>20	48	Ernst et al. 2001
		EC_{50} (immobility)	3.0	48	
Artemia salina (brine shrimp)	Adult	LC_{50}	>10 000	24	Ernst et al. 2001
Mysid sp.	Various	LC_{50}	12.5	24	Burridge and Van Geest 2014
			>85.5	1 (96 h observation)	
Polydora cornuta (polychaete)	Juvenile	LC_{50}	2310	96	Ernst et al. 2001
Vibrio fischeri (bacteria)	Logarithmic phase	EC_{50} (luminescence/ cellular respiration)	11 000	0.25	Ernst et al. 2001
Salmo salar (salmon)	Adult	LC_{15}	1000	1	Sievers et al. 1995
Onchorhynchus mykiss (trout)	Adult	LC_{25}	100	4	Intorre et al. 2004
Anguilla anguilla (eel)		LC_{50}	>100		

Table 1-2. Toxicity test values for various marine species following exposure to hydrogen peroxide via Paramove®50 in water.
LC_{50} = Lethal concentration to 50% of test organisms; Mysid sp. = *Mysis stenolepsis* and *Praunus flexosus*.

Species (Organism)	Life Stage	Endpoint	Hydrogen Peroxide Concentration (mg/L)	Exposure Time (h)	Reference
Corophium volutator (amphipod)	Adult	LC_{50}	460	96	Smit et al. 2008
Homarus americanus (lobster)	Larval stage I	LC_{50}	1637	1 (96 h observation)	Burridge and Van Geest 2014
	Adult	LC_{50}	>3750	1 (96 h observation)	
Crangon septemspinosa (shrimp)	Adult	LC_{50}	3182	1 (96 h observation)	
Mysid sp. (shrimp)	Adult	LC_{50}	973	1 (96 h observation)	
Mysid sp.	Adult	LC_{50}	1650	1 (24 h observation)	McCurdy et al. 2013
			1222	1 (96 h observation)	
Artemia salina (brine shrimp)	Adult	LC_{50}	800	24	Matthews 1995

Table 1-3. Toxicity test values for various marine species following exposure in sediment to ivermectin. LC_{50} = Lethal concentration affecting 50% of test organisms; EC_{50} = Effect concentration affecting 50% of test organisms; NOEC = No observed effect concentration.

Species (Organism)	Life Stage	Endpoint	Ivermectin Concentration (µg/kg)	Exposure Time (d)	Reference
Corophium volutator (amphipod)	Adult	LC_{50}	22	10	Allen et al. 2007
	Juvenile	LC_{50}	16.7	28	
	Adult	LC_{50}	180	10	Davies et al. 1998
		NOEC	50	10	
Homarus americanus (lobster)	Juvenile	LC_{50}	212	10	Daoud 2018
		EC_{50} (Abnormal behaviour)	16	15	
Arenicola marina (lugworm)	Adult	LC_{50}	17.9	10	Allen et al. 2007
		LC_{50}	6.8	100	
		LC_{50}	23	10	Thain et al. 1997
		Impaired burrowing	12 - 44	10	
Asterias rubens (starfish)	Adult	LC_{50}	23 600	10	Davies et al. 1998

Table 1-4. Toxicity test values for various marine species following exposure in sediment or feed to emamectin benzoate. LC_{50} = Lethal concentration affecting 50% of test organisms; NOEC = No observed effect concentration.

Species (Organism)	Life Stage	Endpoint	Emamectin Benzoate Concentration (µg/kg)	Exposure Type - Time	Reference
Corophium volutator (amphipod)	Adult	LC_{50}	193.1	Sediment – 10 d	McHenery and Mackie 1999
		NOEC	114.6		
	Adult	LC_{50}	153	Sediment – 10 d	Mayor et al. 2008
Eohaustorius estuarius (amphipod)	Adult	LC_{50}	185	Sediment – 10 d	Kuo et al. 2010
Homarus americanus (lobster)	Adult	LC_{50}	> 69 300	Feed – 8 d	Aufderheide 1999
	Juvenile	LC_{50}	> 589 000	Feed – 7 d	Burridge et al. 2004
	Adult	LC_{50}	> 644 000	Feed – 7 d	
	Adult	Premature Molting	220 – 390	Feed – Fed until molted (max 1 year)	Waddy et al. 2007
	Juvenile	LC_{50}	250	Sediment – 10 d	Daoud 2018
		EC_{50} (abnormal behaviour)	96	Sediment – 15 d	
Arincola marina (polychaete)	Infaunal	LC_{50}	111	Sediment – 10 d	McHenery and Mackie 1999
		NOEC	56		
Hediste diversicolor (polychaete)	Infaunal	LC_{50}	1368	Sediment – 10 d	Mayor et a. 2008

Species (Organism)	Life Stage	Endpoint	Emamectin Benzoate Concentration (µg/kg)	Exposure Type - Time	Reference
Nereis virens (Polychaete)	Adult	Impaired burrowing	400	Sediment – 30 d	McBriarty et al. 2017
Nephrops norvegicus (prawn)	Adult	LC_{50}	> 68 200	Feed – 96 h	McHenery and Mackie 1999
Pandulus platyceros (prawn)	Adult	Mortality	100 – 4800	Sediment – 8 d	Veldhoen et al. 2012
	Adult	LC_{50}	> 100	Feed – 14 d	Park 2007
			735	Sediment – 30 d	

Table 1-5. Concentrations of avermectins, ivermectin and emamectin benzoate, detected in sediment near aquaculture net pens in various countries. Maximum concentrations detected are given in units of µg/kg unless otherwise stated. Near field = ≤ 25 m from net pen; Far field = ≥ 50 m from net pen; * = Geometric mean; ** = Time of sampling unknown. Year reported is the year literature published.

Country	Concentration (µg/kg)	Distance from Net Pen Edge	Year	Reference
Ivermectin				
Ireland	2.6 - 6.8	Near Field	1997	Cannavan et al. 2000
Scotland	5 - 11	Near Field	1998	ERT 1997, 1998
Emamectin Benzoate				
Canada (British Columbia)	35	Near field	2009	DFO 2012 / Ikonomou and Surridge 2013
	0.12	Far field		
Canada (Eastern)	2.2	Near Field	1999*	McHenery and Mackie 1999
	2.73	Near Field		
	0.62	Far Field		
	1.8	Near Field		
	1.8 – 2.5 mg/kg	Near Field	2010	Lalonde et al. 2012
	140	Near Field		
	2.8	Near Field	2016	Hamoutene et al. 2018
Scotland	2.73	Near Field	1997	Tefler et al. 2006
	0.62	Far Field		
	27.9	Near Field	2002	SEPA 2004

Country	Concentration (µg/kg)	Distance from Net Pen Edge	Year	Reference
Scotland	4.60	Near Field	2004	SEPA 2005
	5.38	Near Field	2006	SEPA 2007
	0.6	Near Field	2010	SEPA 2012
	0.6	Far Field	2010	
	3.14**	Near Field	Various	Benson et al. 2017
	1.38**	Far Field		
	12	Near Field	2017	Bloodworth 2019
	366	Near Field	1998	Boxall et al. 2002
	2.73	Far Field		
Norway	2.5 - 6.5	Far Field	2008	Langford et al. 2014
Chile (Patagonia)	8.38	Near Field	2010	Tucca et al. 2017
	9.97	Far Field		

1.7. Figures

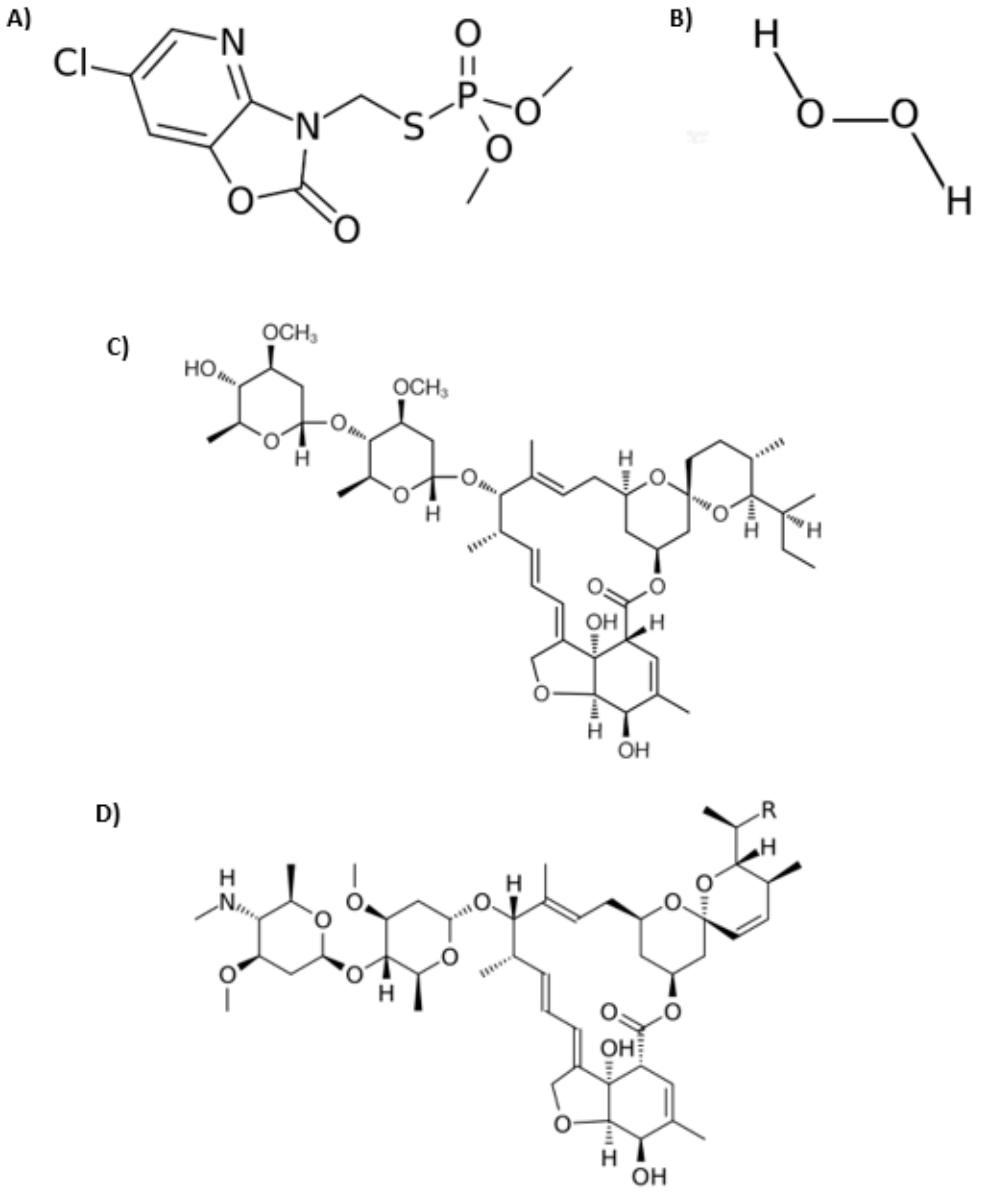

Figure 1-1. Structure of anti-sea lice chemotherapeutants of interest. (A) Azamethiphos, active ingredient in Salmosan®; (B) Hydrogen peroxide, active ingredient in Paramove®50; (C) Ivermectin; and (D) Emamectin benzoate, active ingredient in SLICE®

Chapter 2. Effects of the anti-sea lice pesticides Salmosan® and Paramove®50 on Pacific purple sea urchin fertilization success

2.1. Introduction

The phylum Echinodermata include some of the most familiar marine organisms, which includes sea stars, sea cucumbers and sea urchins, all of which are marine invertebrates with characteristic spiny skin and nearly all occupy the benthic environment. The numerous species occupy various niches and feeding guilds, such as filter feeders and algae scrapers or those that consume animal tissues, and also provide food for higher trophic species (Matranga 2005). Reproduction is typically achieved by external fertilization in which eggs and sperm are released into the water; this is known as broadcast spawning, and these gametes are released by separate sexes. If fertilization occurs, the embryos develop into planktonic larvae before metamorphosing into a final adult form and returning to the ocean floor. Due to this complex life history, echinoderms are understood to be sensitive to environmental changes and population crashes have been associated in areas with contaminated marine waters (Suchanek 1993, Dupont et al. 2010). As a result, these animals can serve as valuable, sensitive test species for toxicological and environmental studies (Iliopoulou-Georgudaki et al. 1997, Coteur et al. 2006).

Sea urchins are meroplanktonic, meaning that they spend a portion of their life as planktonic organisms. Sea urchin gametes, embryos and larvae are excellent experimental organisms because of planktonic characteristics; they are small in size and have transparent tissues which permit the observation of morphological changes associated with embryogenesis and development. Fertilization is initiated when sperm make contact with the exterior of the egg (the egg jelly) and the acrosome, an organelle covering the tip of the sperm that releases hydrolytic enzymes that degrade the jelly coat (Matranga 2005). Numerous cellular cascades that result in microfilament extension, protein binding and membrane depolarization contribute to successful fusion and fertilization. The characteristic cortical granule ring surrounding a fertilized egg (that in part acts as a block to polyspermy) is a visual representation of successful fertilization as well as the complex processes that take place during this sequence of events. Cleavage subsequently occurs until a blastula forms, followed by a gastrula and finally a pluteus, which is the larval planktonic form.

Sea urchins are valuable species in marine ecosystems, reducing algal abundance by their grazing activity, and as stated previously, they also act as an important food source for many higher trophic level species. In the coastal waters of BC, the Pacific purple sea urchin (*Strongylocentrotus purpuratus*) plays a pivotal role in kelp forest health, foraging on the kelp and drift subsidies leading to a dynamic, as well as delicate, population density-dependant relationship between urchins and forest health (Kenner 1992). Kelp forests are vital in ecosystem productivity, regarded as aquatic sanctuaries to numerous species including herring and salmon, and contribute to oxygen production in the atmosphere (Mann 1973, Kenner 1992, Araujo et al. 2013). Sea urchins also play a large role in structuring marine benthic communities (Ebert et al. 1994) and are important kelp processors for other sea floor detritivores that are unable to consume the kelp directly (Yorker et al. 2019). Urchin faecal pellets also contain an assortment of microbes and nitrogen, providing nutrients to benthic dwellers. An overabundance of sea urchins can result in the decimation of kelp forests, known as urchin barrens, whereas reductions in populations are associated with declines in other benthic species (Shelton et al. 2018). Shelton et al. (2018) found that increases in otter abundance, an important sea urchin predator, were correlated with declines for a broad suite of invertebrate species, including bivalves, sea stars, sea urchins and sea cucumbers, and an eventual reduction in kelp growth rates. Recruitment intensity of sea urchins is determined mainly by the supply of sea urchin larvae, which in turn generally depends on the oceanographic conditions that bring the larvae to suitable areas to settle (Ebert et al. 1994). As meroplanktonic organisms, these animals have a wide range of habitat due to the potential for gametes to be transported multiple kilometres after a spawning event.

The coast of BC has 100 open net-pen Atlantic salmon aquaculture farms (Living Oceans 2014), which utilize pesticides and other chemotherapeutants to control pathogen outbreaks. Due to the nature of the application of these chemicals and the open-net pen systems used, treatment regimes can result in the contamination of the marine environment. Potential implications of anti-sea lice treatments have not been assessed for planktonic organisms, including either meroplanktonic and holoplanktonic species. In BC specifically, the water-soluble bath treatment pesticides Salmosan® and Paramove®50 are relevant to animals inhabiting the water column. Salmosan® (active ingredient [AI] azamethiphos) and Parmove®50 (AI hydrogen peroxide) are applied in baths *via* well-boats or tarpaulin skirting to achieve target concentrations of 100 µg/L and 1200 - 1800 mg/L AI, respectively (Health Canada 2014, 2017). The overlap between Pacific purple

sea urchin habitat and aquaculture facilities has prompted concern for nontarget effects in this marine ecosystem, including those on sea urchins themselves and potential downstream effects on kelp forests and the benthic community. Assessing the toxicity of these chemicals to the planktonic life stages of sea urchins will also provide insight into potential effects towards other planktonic species.

In these experiments, sea urchin fertilization success was assessed following exposure to the two anti-sea lice pesticides, Salmosan® and Parmove®50. Gametes were exposed to a range of environmentally relevant concentrations using a standardized protocol.

2.2. Methods

2.2.1. Study organism

Mature and gravid Pacific purple sea urchin (*S. purpuratus)* were collected off the coast of San Diego, US and supplied through Nautilus Environmental Company Inc. (Nautilus Environmental). Following collection, organisms were immediately transported to Nautilus Environmental in Burnaby, BC on the same day, and tests were performed the day of receipt. No mortality occurred during any shipment. *S. purpuratus* were kept in a cool, dry environment and acclimated for approximately 3 h upon arrival. Urchins were inspected for general health and maturation prior to the assay. All adults used to provide gametes for each test were derived from the same batch and source. Animals were euthanized by Nautilus Environmental following completion of the experiment. Nautilus Environmental is a laboratory accredited by the Canadian Association for Laboratory Accreditation Inc. (CALA).

2.2.2. Chemicals

Salmosan® (50% AI azamethiphos w/w) (Fish Vet Group®, Inverness, Scotland) was obtained from Fisheries and Oceans Canada. As a wettable powder, a stock solution was prepared in clean filtered seawater that was subsequently diluted to target concentrations of the AI azamethiphos. Powder was weighed on an analytical scale and was thoroughly mixed in seawater for approximately 1 h until dissolved. Paramove®50 (50% AI hydrogen peroxide w/w) (Solvay, ON) was obtained from Grieg Seafood BC Ltd. (Campbell River, BC). To prevent photolysis and degradation, the stock pesticide solution was transported and stored in a cooled amber glass container covered in aluminum foil to

reduce light exposure and was then stored at 4 ºC (as recommended by the product label and safety data sheet).

Prior to any test, the pesticide was serially diluted to the desired concentrations of AI hydrogen peroxide using clean filtered seawater; mixtures were inverted ten times to ensure complete mixing prior to use in tests, and then used immediately. Potassium chloride, copper(II) chloride and 10% buffered formalin were provided by Nautilus Environmental.

2.2.3. Fertilization test protocol

The seawater source was the Vancouver Aquarium (Vancouver, BC). Water was pumped directly from Burrard Inlet, followed by slow sand filtration and then disinfection with ultraviolet (UV) radiation. In compliance with the standardized test protocol described below, seawater was kept at 13.5 ± 1 °C, with a pH range of 7.5 – 8.5, dissolved oxygen between 90 and 100 % saturation (approximately 7.5 – 8.5 mg/L) and salinity between 28 and 32 ‰.

Test procedures for this bioassay followed Environment and Climate Change Canada's (ECCC) standardized *Biological test method: Fertilization assay using echinoids (sea urchins and sand dollars)* EPS 1/RM/27 (ECCC 2017). All test vessels, equipment and measuring devices were thoroughly cleaned and rinsed with seawater prior to an assay.

Adults were stimulated to spawn by injecting 0.5 mL of 0.5 M KCl through the peristomal membrane. Sea urchins were then gently shaken to distribute the KCl within the animal. Female gametes were collected by placing the organism's aboral surface over a vial filled with seawater, into which eggs were released (see Figure 2-1). Collected eggs were washed three times by mixing with clean seawater and decanting. Male gametes were collected from the animal's surface (see Figure 2-2) using a sterile Pasteur pipet and transferred to a small vial stored on ice. In order to prevent activation of the sperm, all handling was kept dry with no seawater contact. If no spawning occurred in 10 min, a second injection was applied if necessary.

Gametes were checked for quality under a microscope prior to the assay and then pooled to achieve homogeneity of the experimental units. Eggs were inspected for size, shape and vacuolization and sperm were assessed for motility and clumping. Sperm were stored separately on ice following the quality check. Sperm and eggs were pooled from at least 2 or more individuals. ECCC (2017) notes that it is permissible to use one adult if

gametes pass the health check and yields acceptable fertilization success (i.e., >60% success, optimal 80%) from a pre-test, although three or more individuals is optimal.

Eggs were counted on a glass slide and the density of the egg solution was adjusted to achieve approximately 2000 eggs/mL. Sperm were counted using a hemocytometer to quantify a stock concentration of 2 x 10^6 sperm/mL in seawater. Stock concentrations were then used in a 10 mL volume range finding test of 100:1 to 3000:1 ratio of sperm to eggs to determine the ideal sperm: egg fertilization ratio that resulted in 80-90% fertilization success. The Salmosan® assay used a ratio of 800:1, and the Paramove®50 assay used a ratio of 3000:1, which are both within the normal background variation. ECCC (2017) indicates fertilization > 60% and < 98% can be used; however, to avoid under or overestimation of effects, a fertilization success of 80-90% was used in all assays. 1 mL of egg suspension and 0.1 mL of sperm solution were used for both pesticide assays in a vessel filled with seawater to 10 mL.

Experiments followed the 20-min ECCC (2017) protocol, in which sperm were exposed to either pesticide for 10 min, followed by the addition of eggs and incubation for a further 10 min and then termination of the test at 20 min with 10% buffered formalin. All gametes were exposed and then terminated in the test vessels in the same sequence and timing interval to equalize exposure periods. At the end of a test, the sperm-plus-egg exposure was terminated and preserved with five drops of 10% buffered formalin to each vessel. Fertilized eggs were counted immediately after test completion under a light microscope. A fertilized egg was identified if a completed membrane had formed around the embryo. To determine the total percent fertilized, 100 eggs were counted randomly for each replicate as per the protocol.

For each test, various controls were concurrently performed to ensure accuracy in the results. A seawater-only egg and sperm control was used to assess normal fertilization percentages, while an egg-only pesticide control and an egg-only seawater control were used to ensure that no sperm contamination occurred, and to observe background fertilization or potential physical adverse effects to the eggs. A reference toxicant, copper chloride ($CuCl_2$), was used as a positive control; concentrations in tests were 2.5, 5, 10, 20 and 40 µg Cu^{2+}/L.

Pesticide concentrations in the fertilization assays followed a geometric decline from maximum concentrations representative of application amounts used in Atlantic salmon aquaculture facilities during sea lice treatments. The Salmosan® assay used concentrations of 0.50, 1.56, 3.13, 6.25, 12.5, 25, 50 and 100 µg AI azamethiphos/L. The

Paramove®50 toxicity test used concentrations of 18.75, 37.5, 75, 150, 300, 600, 900 and 1200 mg AI H_2O_2/L. All test concentrations are understood to be nominal and three replicates were used for each exposure concentration.

2.2.4. Statistical analysis

Statistical analysis was performed using GraphPad Prism® version 8.0 for Windows (GraphPad Software LLC, LaJolla, California, United States). The percent of eggs fertilized for each concentration were plotted as a mean of each replicate (N = 3) with one standard error of the mean. One-way analysis of variance (ANOVA) followed by Tukey's post-hoc analysis was used to determine significant differences between concentrations within a treatment group. A p-value of < 0.05 was used to infer statistical significance. The no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) was determined if fertilization was statistically the same or different from the control, respectively.

To calculate an inhibitory concentration affecting 50% of fertilization success (IC_{50}) and IC_{25}, treatment concentrations were log-transformed, the data was normalized, and nonlinear regression was performed. Regression curves were plotted with 95% confidence interval bands, and IC_{50} values with 95% confidence intervals, were generated. Using the Hill Slope coefficient, which depicts the steepness of the dose-response curve (Endrenyi et al. 1975, GraphPad Software LLC 2020), and the calculated IC_{50}, the IC_{25} was subsequently calculated using the following equation:

$$IC_F = \left(\frac{F}{100 - F}\right)^{\sqrt{H}} \times IC_{50}$$

Where:

- IC Inhibitory Concentration
- F Fraction of full response
- H Hill Slope coefficient
- IC_{50} Inhibitory concentration affecting 50% of a biological function

2.3. Results

2.3.1. Water quality

Water quality parameters which included temperature, salinity, dissolved oxygen and pH were measured in all experiments and were within standardized protocol

requirements as described in Section 2.2.3. Seawater was kept at 13.5 ± 1 °C, with a pH of 7.8 ± 0.2, dissolved oxygen of 8.5 ± 0.3 mg/L and salinity of 30 ± 1.0 ‰.

2.3.2. Fertilization success

Pacific purple sea urchin gametes were exposed to the formulations Salmosan® and Paramove®50 under a range of AI concentrations, with the maximum concentration used as the application concentration at BC Atlantic salmon farms. The experimental protocol followed the ECCC (2017) 20-min standardized assay and the NOEC, LOEC, IC_{25} and IC_{50} for fertilization were calculated for each AI. The results are described below, and a summary of the results is provided in Table 2-1 and in Figures 2-3 to 2-5.

The reference toxicant copper(II) chloride was used as a positive control to ensure that the inhibition of fertilization success was within known IC_{50} ranges, to assess the relative sensitivity of the batches of gametes that are used under standardized test conditions, and ensure the performance and precision of the test. The test was performed twice, and the calculated IC_{50} values (95% Confidence Interval [CI]) for Cu^{2+} were 23.8 (CI 20.7 – 27.1) and 28.0 (CI 25.6 – 30.8) µg/L. The calculated IC_{50} values within the acceptable range by ECCC are 20 to 26 µg ± 2 SD Cu^{2+}/L, and the percent fertilized at each concentration for each date are within 3 standard deviations of each other, therefore the sensitivity of the gametes and precision of the tests were deemed satisfactory (ECCC 2017). Table 2-1 as well as Figure 2-3 detail the results of the reference toxicant control.

Salmosan® inhibited fertilization by 30% at the maximum concentration tested (100 ug AI azamethiphos/L) (see Figure 2-4), which is the target exposure concentration used at Atlantic salmon aquaculture facilities to treat sea lice infestations. The resulting IC_{50} is therefore > 100 µg/L. When modeled, the IC_{50} was predicted to be approximately 202 µg/L (CI 148.3-360.8 µg/L), however there is uncertainty associated with this value because maximum inhibitory concentration of only 30% was reached. The IC_{25} was calculated to be 74.83 µg/L (CI 59.32 – 90.38), and the NOEC and LOEC were 25 and 50 µg/L, respectively.

Paramove®50 had greater inhibitory effects on fertilization success compared to Salmosan®. Of the eight test concentrations, six (75, 150, 300, 600, 900 and 1200 mg H_2O_2/L) had between 0 and 2% fertilized gametes in replicates (see Figure 2-5). The remaining subsequent two test concentrations and seawater control saw mean fertilization success of 6% (37.5 mg H_2O_2/L), 22% (18.75 mg H_2O_2/L) and 93% (seawater control),

respectively. The calculated IC_{50} for Paramove®50 was 7.27 mg/L (CI 5.96 – 9.53) and the IC_{25} was 1.93 mg/L (CI 1.60 – 2.10). A NOEC could not be determined as the lowest test concentration (18.75 mg/L) was significantly different from the seawater control and was therefore the LOEC, which therefore may not represent a 'true' LOEC due to the concentrations used and observed toxicity. As the target concentration for sea lice treatment with Paramove®50 is 1200-1800 mg H_2O_2/L, the observed inhibitory concentrations are environmentally relevant.

2.4. Discussion

The sea urchin is one of the most investigated model organisms for the study of gamete fertilization and the associated cellular events that take place during this process. During the first phase of fertilization, sperm motility is activated by electrical events and the sperm swims toward the egg. The sodium present in seawater increases the intracellular pH, which activates the flagellum dynein. Cell-to-cell communication, cystoplasmic and skeletal restructuring, and intracellular ion changes associated with sperm motility are likely mediated by neurotransmitter molecules (Falugi 1993). This process may be due to the activation of nicotinic receptors, which is supported by the presence of cholinergic systems in other animal sperm. The activity of acetylcholinesterase (AChE), a carboxyl ester hydrolase responsible for the lysis of acetylcholine (ACh) in the cholinergic system, has been observed in the sperm flagellum of the sea urchin (Cariello et al. 1986). ACh receptors have also been found within sperm cell structures; specifically, muscarinic receptors in the acrosome and nicotinic receptors in the acrosome and flagellar membrane (Baccetti et al. 1995). The muscarinic receptors are associated with G-protein intracellular domains, which may result in signal transduction cascades related to intracellular dynamics involved in fertilization (Falugi et al. 1993). The nicotinic receptors result in Na^+ influx, mediating the pH necessary for movement and propulsion, as described earlier (Nelson 1976, Stroud et al 1990, Falugi et al. 1993). Sperm-egg interaction and membrane fusion at the acrosome may also be mediated by cholinergic events.

The activation and fertilization of the sea urchin egg is also dependant on electrical changes. When the sperm contacts the egg, a depolarisation of the egg's membrane takes place, causing an influx of Na^+ that permits fusion with the sperm. Immediately following, an increase in Ca^{2+} intracellular activates the egg's metabolic activities and initiates the cortical reaction that will block polyspermy (Matranga 2005). The reliance on Na^+ influx is

similar to depolarisation events in neuromuscular synapses, suggesting that cholinergic activity is important during the fusion and subsequent block to polyspermy. The addition of acetylcholine prior to fertilization has been found to significantly increase the percentage of polyspermic eggs as compared to controls (Harrison et al. 2002, Angelini et al. 2004). The presence of nicotinic receptors in the unfertilized sea urchin egg has also been confirmed (Ivonnet and Chambers 1997). It is hypothesized that ACh released by the sperm surface may excite the nicotinic receptors on the egg surface, resulting in the initial depolarisation event responsible for membrane fusion (Angelini et al. 2004).

Azamethiphos is an organophosphate pesticide that irreversibly inhibits acetylcholinesterase, a carboxyl ester hydrolase (Bajgar 2004). In the absence of AChE, nerves will repeatedly fire causing excitation and overstimulation. In consideration of the mechanisms of action of azamethiphos and cellular events involved in sea urchin fertilization (i.e., ACh increasing movement and causing polyspermy), it is reasonable to assume membrane fusion of the egg and sperm was not inhibited, as inhibition of AChE would increase ACh activity. However, as ACh is involved in sperm motility as described previously, inhibition of AChE may also impair mobility of sperm due to over activation. Therefore, the presence of ACh receptors on both egg and sperm and subsequent involvement in gamete activation, fusion and membrane development may explain why azamethiphos had marginal toxicity even at the highest concentration of 100 µg/L. Although mobility of sperm may be inhibited by azamethiphos due to overexcitation, the actual fertilization event may not be impaired. Additional work assessing sperm motility following exposure to azamethiphos could be performed to confirm this potential mechanism of action.

The possible developmental impacts to fertilized eggs from AChE inhibition was not evaluated in this experiment. AChE has been found in the perivitelline space alongside the cortical granules, which suggests a function of the cholinergic system after fertilization and therefore development of the gamete (Angelini et al. 2004). Cholinergic activity is also evident during cleavage of the fertilized egg (Angelini et al. 2004). The development of sea urchin gametes should therefore be assessed in future experiments in order to fully understand the effects of azamethiphos on the early life stages of this species.

Fertilization success was significantly inhibited when gametes were exposed to Paramove®50 (AI H_2O_2). As a by-product of oxygen metabolism, H_2O_2 can result in oxidative stress, however the observed toxicity, especially at low concentrations, suggests

an increased sensitivity to H_2O_2 compared to other toxicity endpoints and marine species. Reported LC_{50}'s to other marine invertebrates, such as amphipods and shrimp, are > 460 mg H_2O_2/L (Smit et al. 2008, McCurdy et al. 2013, Burridge and Van Geest 2014).

During the sperm-egg fusion, the vitelline envelope is raised, creating the fertilization membrane and inactivating antigen receptors (Matranga 2005). The relatively impermeable fertilization membrane that forms around the egg to block polyspermy is due to secretions from cortical granules. Interestingly, a peroxidase-mediated reaction catalyzes the crosslink formations in the fertilization membrane, which is in turn due to the eggs production of hydrogen peroxide (Foerder et al. 1978). It is also suggested that hydrogen peroxide may have spermicidal effects, killing excess sperm in the vicinity of the egg to prevent polyspermy (Foerder et al. 1978, Boldt et al. 1981, Colburn et al. 1981). Treatment of sperm with hydrogen peroxide has resulted in significant decreases in survival and fertilization success (Evans 1947, Boldt et al. 1981, Colburn et al. 1981). Foerder et al. (1978) estimated the maximum concentration of hydrogen peroxide outside of the egg following fertilization is 32 µM, which is approximately 1.1 mg H_2O_2/L. This supports the potency of Paramove®50 observed in this experiment, as polyspermy prevention is paramount for proper development of sea urchin embryos. From an evolutionary perspective, production of H_2O_2 by the egg would be energetically costly if it was inefficient if it had poor spermicidal qualities.

The sea lice pesticides Salmosan® and Paramove®50 are applied as a water bath treatment to infected Atlantic salmon. Following application, the contaminated water is released directly into the marine environment either by removal of a tarpaulin surrounding the net pen or slow release by a well-boat, depending on the application method. Physical dispersion and degradation of the chemicals in the environment is influenced by mechanical and ambient factors such as water temperature, tidal amplitude, currents, depth and length of release as well as physicochemical properties. One of the difficulties in determining potential risk to aquatic organisms is understanding and predicting concentrations of chemicals in the environment. There are limited studies that have investigated the dilution of water bath applied sea lice pesticides into the water column. Burridge et al. (2000) used a scaling analysis to estimate concentrations of the sea lice pesticide Excis® (AI cypermethrin, a pyrethroid) in the field after tarpaulin treatment and found that the pesticide will be likely diluted 100-fold within 3 h of treatment within a 100 m distance. Ernst et al. (2014) used a dye dispersion study in Atlantic Canada to

demonstrate plume spread of Salmosan® *via* tarp and well-boat releases 2 - 3 h after treatment. Concentrations of azamethiphos were higher in the water column when released from tarpaulin treated net pens and was still detectable at approximately at 0.8 µg/L approximately 1000 m away. Within a distance of 10 m from the treatment location, the concentrations of azamethiphos was approximately 7 and 1.5 µg/L for tarp and well-boat, respectively. Recall that 100 µg/L of azamethiphos is the target pest management concentration, therefore assuming dilution of Paramove®50 will follow similar dilution principles as described by Burridge et al. (2000) and Ernst et al. (2004), concentrations 10 - 1000 m from the treatment location may range from 1 - 120 mg H_2O_2/L, which are well within the inhibitory concentrations observed in this experiment.

Pacific purple sea urchin are found in the lower intertidal and sub-tidal zones and typically spawn from January until May. Gonadal indices in individuals start increasing in December until peaking in April and are at a minimum in July and August (Kenner and Lares 1991). The species does well in aerated, churning waters and are prominent members of the kelp forest community, consuming kelp and macroalgae and are an important food source for mammals such as sea otters, as well as provide nutrients for many benthic consumers (Yorker et al. 2019). The meroplanktonic life stage of this animal permits huge dispersal potential, as gametes can spend up to 121 d in the water column (Strathmann 1978). Considering that Atlantic salmon aquaculture facilities are also within coastal inlets along the shoreline of BC, it is reasonable to assume sea urchin populations and their pelagic gametes may be influenced by net pen contaminants. Sea lice outbreaks tend to occur during warmer months during salmon spawning returns in August and September. This time period does not coincide with peak spawning time of the sea urchin, however DFO (2019a,b) has reported use of these pesticides through January and May as farm operators may work to pre-emptively control lice outbreaks as well as treat salmon that exceed the threshold of three sea lice motiles per fish. Sea urchin gametes may therefore be exposed outside of peak sea lice outbreak periods and populations of sea urchins may be at risk if near multiple aquaculture facilities. Paramove®50 specifically should only be applied in scenarios with high dilution due to the sensitivity observed in this assay. Currently, well-boat treatments are the only application method approved in BC as it increases dilution and subsequent degradation (ENV 2018). Alternative strategies to reduce exposures to non-target species in the water column, such as application during high tidal levels and slow release, should be considered.

2.5. References

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2.6. Tables

Table 2-1. Inhibitory concentrations of sea lice pesticides Salmosan® and Paramove®50 on Pacific purple sea urchin (*Strongylocentrotus purpuratus*) fertilization success. Salmosan® active ingredient is azamethiphos, Paramove®50 active ingredient is hydrogen peroxide. The calculated IC_{50} for the reference toxicant copper chloride that was performed concurrently with both pesticides is also provided. NOEC= No observed effect concentration; LOEC = Lowest observed effect concentration; ICx = Inhibitory concentration affecting X% of the biological function (fertilization success); CI = Confidence interval NA = Not applicable; NV = No value.

Chemical	NOEC, LOEC	IC_{25} (95% CI)	Hill Slope (95% CI)	IC_{50} (95% CI)
Anti-sea Lice Pesticide				
Azamethiphos (Salmosan®)	25 µg/L, 50 µg/L	74.83 µg/L (59.32 – 90.38)	0.8169 (0.54 – 1.22)	>100 µg/L (NA)
Hydrogen Peroxide (Paramove®50)	NV, 18.75 mg/L	1.93 mg/L (1.60 – 2.10)	1.456 (1.28 - 1.89)	7.27 mg/L (5.96 – 9.53)
Reference Toxicant $CuCl_2$				
Cu^{2+}	NA	NA	NA	23.78 µg/L (20.67 – 27.06)
	NA	NA	NA	28.01 µg/L (25.62 – 30.63)

2.7. Figures

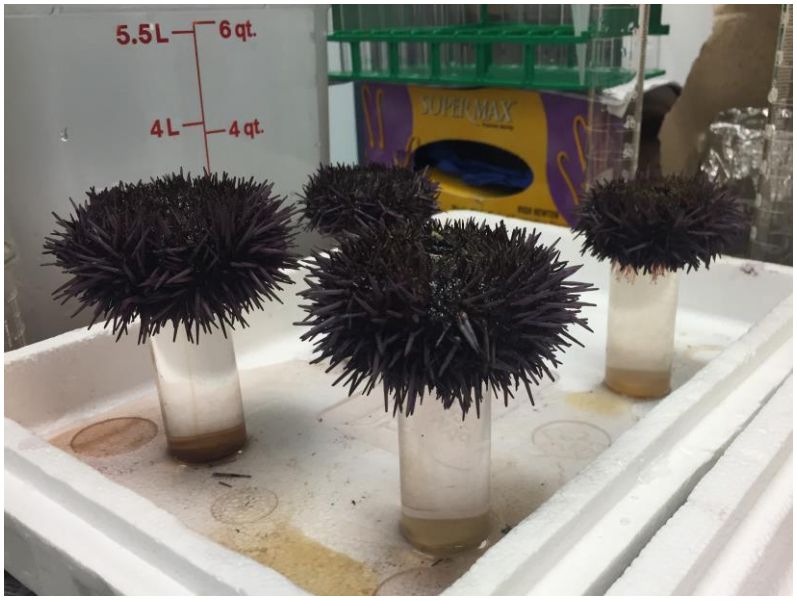
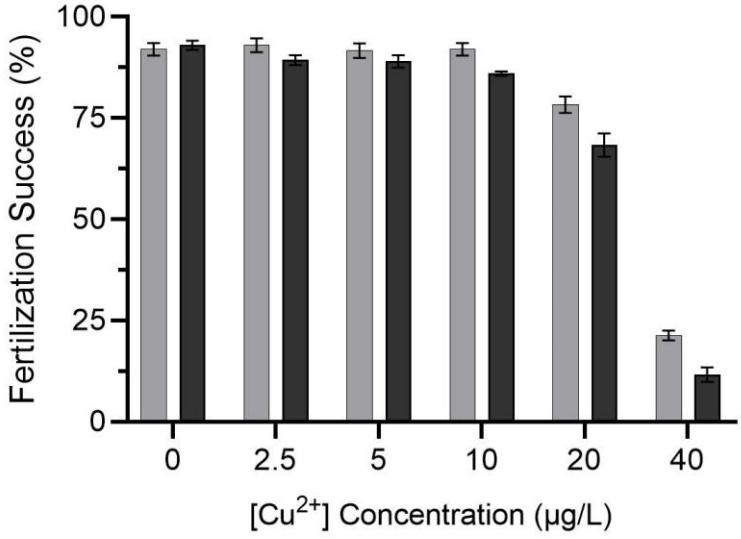


Figure 2-1. Pacific purple sea urchin (*Strongylocentrotus purpuratus*) egg collection. Females are injected with 0.5 M KCl and inverted onto vessels with the aboral surface in contact with clean filtered sea water. Eggs are released and collected in the bottom of the vessels.

Figure 2-2. Pacific purple sea urchin (*Strongylocentrotus purpuratus*) sperm collection. Males are injected with 0.5 M KCl and sperm is subsequently released onto the aboral surface of the animal. Sperm is collected using a sterile pipet into a clean vessel on ice until the start of the assay.

(A)



(B)

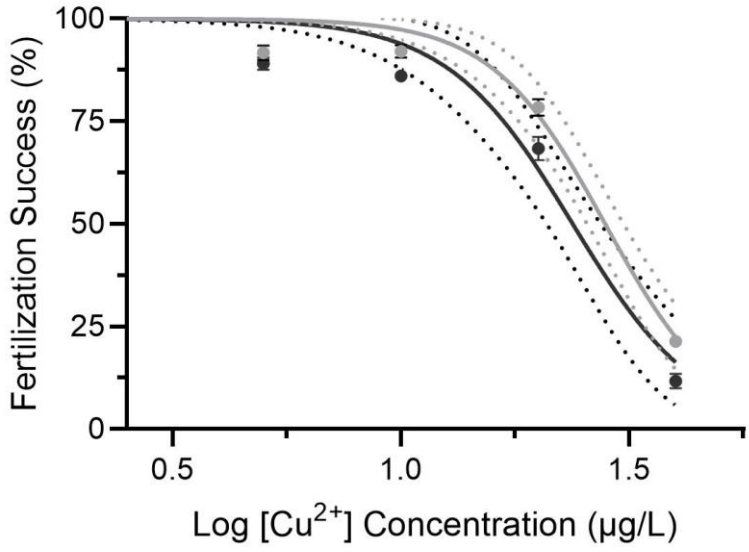
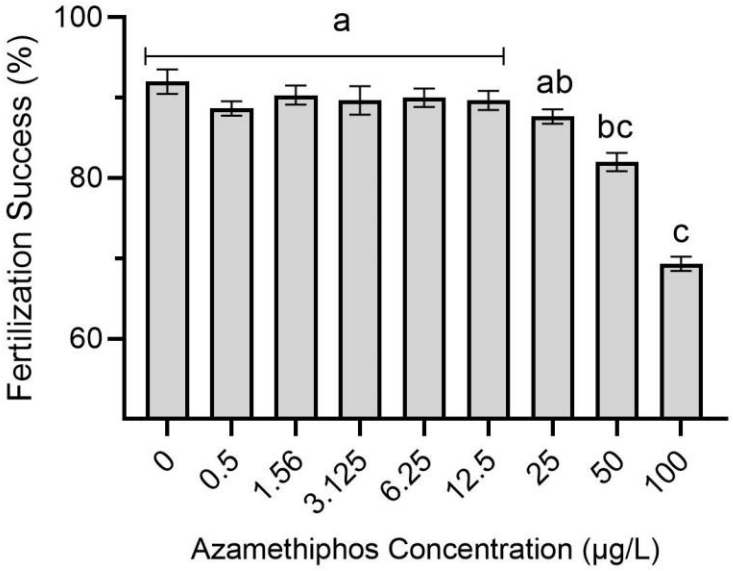


Figure 2-3. Reference toxicant (Cu^{2+}) results from the sea urchin fertilization assays. (A) Fertilization success is plotted as the mean ± 1 standard error of the mean (N=3). (B) the associated dose-response curves on a log concentration scale. Dashed lines are the 95% confidence intervals for each curve. Light grey dots/lines = control test for Paramove®50; Dark grey dots/lines = control test for Salmosan®. The percent fertilized at each concentration for each date are within 3 standard deviations of each other and within the proper range of toxicity concentrations as described by Environment and Climate Change Canada (ECCC 2017), therefore the test results are acceptable.

(A)



(B)

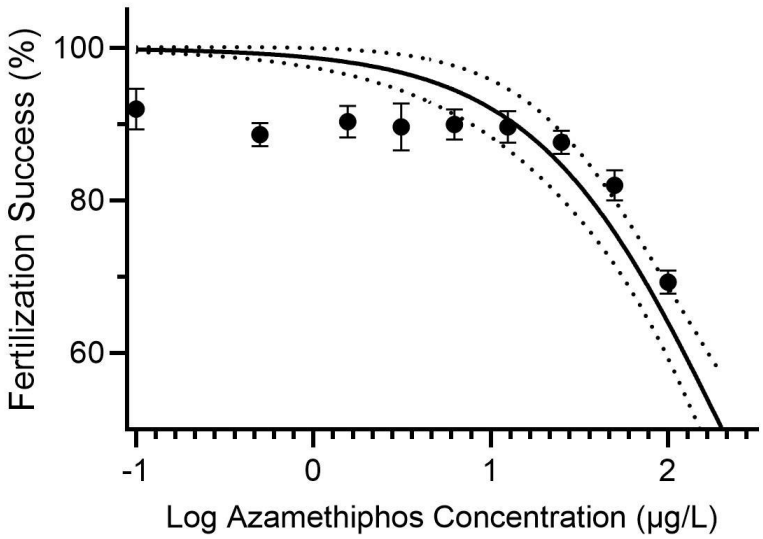
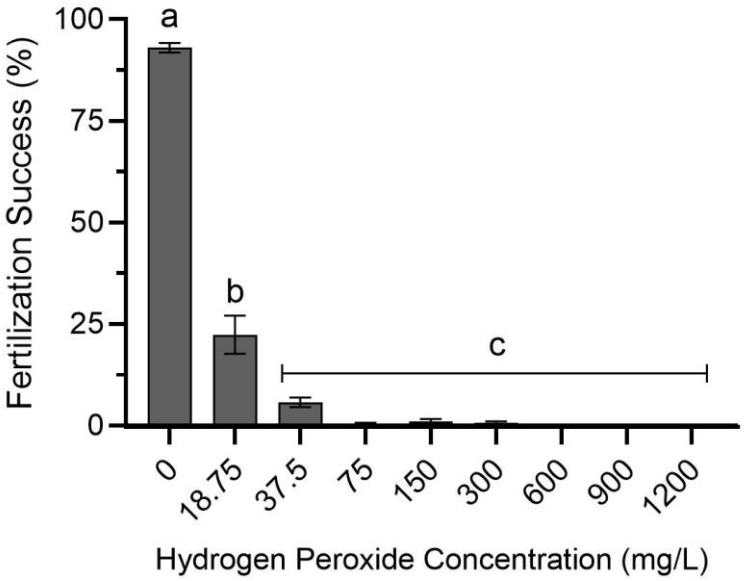


Figure 2-4. Salmosan® (50% azamethiphos w/w) toxicity test results on sea urchin fertilization success. (A) Fertilization success is plotted as the mean ± 1 standard error of the mean (N=3). (B) details the associated dose-response curves on a log concentration scale. Dashed lines are the 95% confidence intervals for each curve. Statistical differences between concentrations were determined using a one-way ANOVA and Dunnett's multiple comparison test. Lowercase letters in (A) represent statistically different groups ($p > 0.05$). Statistical analyses were performed using GraphPad Prism 8.0.

(A)



(B)

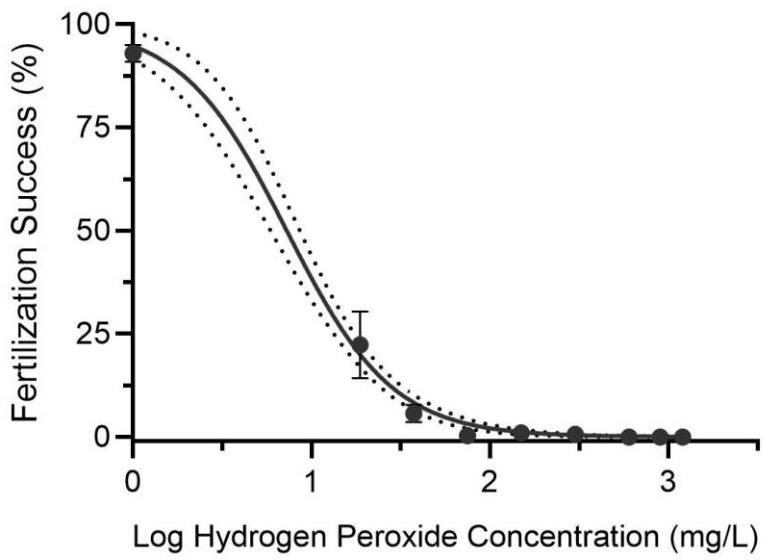


Figure 2-5. Paramove®50 (50% hydrogen peroxide w/w) toxicity test results on sea urchin fertilization success. (A) Fertilization success is plotted as the mean ± 1 standard error of the mean (N=3). (B) details the associated dose-response curves on a log concentration scale. Dashed lines are the 95% confidence intervals for each curve. Statistical differences between concentrations were determined using a one-way ANOVA and Dunnett's multiple comparison test. Letters in (A) represent statistically different groups ($p > 0.05$). Statistical analyses were performed using GraphPad Prism 8.0.

Chapter 3. Avoidance behaviour of marine benthic invertebrates to sediment contaminated with the aquaculture chemotherapeutants SLICE® and ivermectin

3.1. Introduction

In Canada, Atlantic salmon aquaculture operations utilize in-feed medications in the form of pellets containing anti-sea lice chemotherapeutants to control sea lice outbreaks. These treatments may contain one of two avermectins, ivermectin (IVM) or emamectin benzoate (EMB) (applied as the SLICE® Premix [0.2% EMB w/w]) (DFO 2019). At therapeutic doses, the chemical is absorbed in the gut by the fish following consumption and distributed to epithelial tissues (Campbell 1989). Sea lice that are latched onto the surface of the skin, absorb the chemical, resulting in toxicity to the parasitic copepod leading to death (Whyte et al. 2011). These chemicals bind irreversibly the glutamate-gated chloride channels of sea lice; the receptors are broadly found throughout the invertebrate nervous system, resulting in a loss of the control and modulation of locomotion, regulation of feeding, and the mediation of sensory inputs (McKellar and Benchaous 1996, Wolstenholme 2012).

During the treatment process in open net pen systems, both uneaten contaminated feed as well as feces containing the unabsorbed chemicals can settle onto marine sediments. Avermectins have low water solubility with moderate lipophilicity and have long-half lives exceeding 150 d in marine sediments (Campbell 1989, McHenery and Mackie 1999). Due to these physicochemical characteristics, IVM and EMB have moderate persistence in the marine environment and have been found at concentrations ranging from 0.1 – 400 µg avermectin/kg, with a maximum value of 2,600 µg avermectin/kg sediment beneath net pens within a 100 m radius (ERT 1998, Cannavan et al. 2000, Boxall et al. 2002, DFO 2012, Lalonde et al. 2012), with a geomean concentration of 6.38 µg avermectin/kg. The anti-sea lice chemotherapeutants IVM and EMB are therefore present at potentially toxic concentrations, especially given their slow degradation that can result in long-term exposures.

In marine ecosystems, the largest group of animals making up the sediment benthic community are invertebrates. Benthic invertebrates are important members of the marine community, reworking sediments, resulting in oxygen turnover and organic

decomposition, while also acting as a food source for higher trophic level species (Glud 2008, Bertics et al. 2010, Diarte-Plata and Escamilla-Montes 2019). These animals inhabit, graze and ingest sediment particles and are consequently the first organisms potentially affected by contaminated sediment *via* direct contact pathways. Sediment-dwelling species may also promote the release of contaminants at the sediment-water interface from bioturbation activities, affecting exposure, uptake and toxicity (van der Meer et al. 2017). As benthic invertebrates directly influence their own environment through ecological functional traits as previously described, these animals are understood to be valuable contributors to overall ecological health.

Entropy and dilution of a chemical generally creates a uniform concentration of a contaminant in a water column following input; however, sediment chemical distribution can be highly heterogenous and contaminant concentrations will vary spatially (Huang et al. 2003). The presence, abundance and distribution of a species in heterogeneously contaminated benthic environment may be in part a result of their ability to detect a chemical resulting in attraction or avoidance to the chemical. Animal behaviour is often the first line of defence against pollutants, therefore a valuable and potentially sensitive measure. Studies have shown that invertebrate species, such as amphipods, marine worms and clams, are capable of detecting and avoiding metals, hydrocarbon mixtures and the organic enrichment of sediments (Swartz et al. 1986, Lenihan et al. 1995, Rakocinski et al. 1997, Kravitz et al. 1999, Exley 2000, Lopes et al. 2004). Adaptive behaviour has also been observed, as aquatic snails collected from heavy metal contaminated sediment have demonstrated an increased avoidance response to the same sediment compared to snails from a clean, reference site (Lefcort et al. 2004). In some cases, chronic exposure to a chemical may also reduce an organism's ability to respond to the presence of a chemical through attenuation (Gray 1990, Ward et al. 2013a). The inability to sense and avoid certain compounds may result in the loss of a species from an area until contaminant concentrations have decreased to nontoxic levels (Rakocinski et al. 1997, Chariton et al. 2010), whereas mobile chemo-sensing species may be able to avoid toxic effects by relocating to less contaminated habitats ensuring survival (Rakocinski et al. 1997, Lefcort et al. 2004, Lopes et al. 2004, Chariton et al. 2010). The migration of aquatic species from contaminated sites therefore supports avoidance behaviour as a valuable defense mechanism.

It is unknown whether benthic invertebrates are able to sense and avoid avermectin anti-sea lice chemotherapeutants. The concentrations of avermectins beneath Atlantic salmon net pens are generally lower than acute lethal concentrations. For example, a 10-d LC_{50} for the amphipod (*Corophium volutator*) was 180 µg IVM/kg (Davies et al. 1998) and 153 - 193 µg EMB/kg (McHenery and Mackie 1999, Mayor et al. 2008), which is approximately 30x higher than the average avermectin concentration found in marine sediment (ERT 1998, Cannavan et al. 2000, Boxall et al. 2002, DFO 2012, Lalonde et al. 2012), therefore acutely lethal concentrations are not likely present beneath net pens. Given the persistence of these chemicals, determining sublethal effects at low environmentally relevant concentrations is necessary to understand the potential long-term environmental effects of in-feed avermectin anti-sea lice treatment in the marine environment. Assessment of avoidance behaviour specifically will provide information on the ability of benthic species to minimize their exposure to avermectins in sediment, thereby mitigating toxicity.

The objective of this experiment was to assess the chemosensory ability and avoidance behaviour of benthic marine invertebrates to the anti-sea lice chemotherapeutants, IVM and EMB (from SLICE® 0.2% Premix). This was achieved by exposing the amphipod (*Eohaustorius estuarius*) and the clam worm (*Nereis virens*) to sediment contaminated with these drugs at environmentally relevant concentrations and measuring avoidance behaviour to the dosed sediment. A two-chamber static sediment system with no other potential attractants or deterrents (i.e., food or seaweed) was used to determine active responses to the dosed sediment. The effect of pre-exposure to these compounds on avoidance behaviour was also assessed to determine if habituation occurred of if altered behavioural responses occurred following long-term pre-exposure. This was accomplished using two experimental groups; a chronic group, previously exposed to a low concentration, and a naïve group that was not previously exposed to either test chemical before avoidance was assessed.

3.2. Methods

3.2.1. Study organisms

Adult amphipods, *E. estuarius*, from Yaquina Bay, Oregon were provided by Northwestern Aquatic Sciences (Newport, OR). Animals were shipped in 5 x 5 cm containers containing clean-filtered seawater, silica and 100 animals/container. Upon

arrival, each container was placed in a large, aerated seawater bath, held at 11 ± 1°C and a 12:12 h photoperiod. Seawater changes were conducted 2 times per week with 20 - 30% of the water changed at any time. *E. estuarius* were fed ground Cargill® EWOS 1.2 mm farmed fish salmon pellets once a week *ad libitum*. Animals were not size selected for the experiment and were generally 1 - 2 mm in length. *E. estuarius* were acclimated for at least 72 h prior to an experiment.

Adult clam worms (polychaetes), *N. virens*, were collected from sediment flats in New Hampshire by Aquatic Research Organisms Inc. (Hampton, NH). Animals were shipped in styrofoam boxes (300 animals) with cold packs containing damp seaweed and newspaper. Upon arrival, polychaetes were housed communally (50 to 60 animals) in a 38 x 25.5 x 14 cm plastic tub filled to a depth of approximately 7 cm of clean sediment (sediment collection detailed below in Section 3.2.2). Four tubs were then placed within clean, aerated seawater baths (~134 L) held at 11 ± 1°C, under a 12:12 h photoperiod. Water quality was maintained using Hagen® Fluval® FX6 mechanical and biological filters, Coralife® hang-on-back protein skimmers, and Coralife® ultraviolet sterilizers. Seawater changes were conducted 3 - 4 times per week with 20 - 30% of the water changed at any time. Holding densities were approximately 500 - 600 polychaetes per m^2 sediment, well below holding densities recommended for normal health (Safarik et al. 2006). Polychaetes were fed ground Cargill® EWOS 1.2 mm farmed fish salmon pellets 3 - 4 times weekly *ad libitum*. *N. virens* weighed on average 3.75 ± 1.43 g (range 1.5 - 12 g and lengths of 5 - 10 cm). Polychaetes were not size selected for experiments. *N. virens* were acclimated for at least 1 week prior to an experiment.

3.2.2. Sediment and water

The seawater source was the Vancouver Aquarium (Vancouver, BC). Seawater was pumped directly from Burrard Inlet, followed by slow sand filtration and then disinfection with ultraviolet (UV) radiation. Sediment was collected from Centennial beach (Tsawwassen, BC) which is considered an acceptable uncontaminated reference site based on results from the Boundary Bay Assessment and Monitoring Program (BBAMP) (2009 – 2015), completed by Hemmera (2017). Sediment from this region has an organic carbon content of 0.02 – 0.2 % (Hemmera 2014). Sediment was collected from the upper 10 cm, sieved during collection using 1 mm metal sieves to remove debris and dried prior to experimental use.

3.2.3. Chemicals

SLICE® 0.2% Premix (Merck Animal Health, Intervet Canada Corp., Kirkland, QC), which contains 0.2% EMB w/w, was obtained from Fisheries and Oceans Canada (DFO). Target concentrations of EMB were prepared by thoroughly mixing the SLICE® 0.2% Premix in seawater for 30 min to create a stock solution. The stock was subsequently diluted and mixed for 15 min for each additional exposure concentration. All preparations were completed in the dark to reduce photodegradation.

IVM (CAS Number 70299-86-7), which is a solid white powder, was obtained from Sigma-Aldrich (Oakville, ON). Stock solutions were prepared by thoroughly mixing with agitation for 2 h in seawater on ice to prevent degradation (Dorati et al. 2015). All preparations were completed in the dark to reduce photodegradation.

3.2.4. Exposures

Two treatment groups of organisms were used for avoidance assays. A “naïve” group consisted of animals that were only exposed to the chemicals during the avoidance assay. A second “chronic” group were pre-exposed to 5 µg/kg of either EMB, IVM or a combination of both for 30 d before the avoidance assay. The chronic concentration of 5 µg test chemical/kg sediment was deemed to be representative of both EMB and IVM concentrations in sediments beneath net pens based on the literature, as described previously. However, following a 30-d exposure of *E. estuarius* to 5 µg/kg IVM, > 75% mortality occurred, so the experiment was repeated using a lower concentration of IVM at 1 µg/kg for the *E. estuarius* chronic group (< 20% mortality).

For chronic exposures, organisms were placed in 500 mL glass jars containing 300 g of spiked sediment and 400 mL aerated seawater. Sediment spiking followed a protocol similar to methodology described by De Lange et al. (2006) and Burridge and Van Geest (2014). To spike sediments, clean, dry sediment was added to each jar and dosed with chemotherapeutants by creating a sediment-seawater slurry (10 g sediment in 2 mL seawater), and micropipetting 1 mL of the stock solution (described previously) to achieve desired target concentrations. The sediment was thoroughly mixed with a metal spatula for 5 min, then left overnight for 16 h in the dark at room temperature. Following this, filtered seawater was added to each jar, after which animals were introduced. To account for potential behavioural effects due to being housed in a glass jar for 30 d (i.e., potentially increasing or decreasing movements), a chronic negative control was prepared by placing

animals into clean sediment in jars for 30-d. The number of animals exposed in a single jar was species-dependent; 20 *E. estuarius* per glass jar and one *N. virens* per jar were used in the exposures. Animals were fed a pinch of ground fish food weekly *ad libitum* (based on previous pilot feeding trials to be sufficient) and 50% of seawater was changed weekly, complete with water quality checks for salinity, oxygen, pH and temperature. All jars were kept in water baths held at 11 ± 1°C. At the end of the exposure period, animals were removed and used immediately in avoidance assays.

3.2.5. Avoidance assays

Range-finding trials were performed for each species to determine sublethal testing concentrations for use in avoidance assays; concentrations tested were within concentration ranges found beneath treated net pens to determine those that did not result in mortality. *E. estuarius* was found to be more sensitive to IVM, as the initial highest concentration of 200 µg/kg result in 100% mortality after 24 h, therefore the maximum IVM concentration was lowered to 50 µg/kg. The final nominal treatment concentrations in sediment for *E. estuarius* were 0.5, 5, 50 and 200 µg/kg EMB, 0.5, 5, 25 and 50 µg/kg IVM and a 1:1 combination of EMB/IVM of 0.5/0.5, 5/5, 50/25 and 200/50 µg/kg. *N. virens* were exposed to nominal treatment concentrations of 0.5, 5, 50 and 200 µg/kg EMB, 0.5, 5, 500 and 200 µg/kg IVM or a 1:1 combination of EMB/IVM of 0.5/0.5, 5/5, 50/50 and 200/200 µg/kg. Each concentration was tested in triplicate.

Avoidance assays were conducted in glass aquariums of an appropriate size for each species (Hund-Rinke and Wiechering 2001, Loureiro et al. 2005, Ward et al. 2013b). A 7.2-L aquarium (30 cm length x 12 width x 20 height cm) with 2 kg of sediment added (to achieve a depth of 4 cm) was used for the *N. virens* bioassay. For *E. estuarius*, aquaria were modified by inserting a plexiglass barrier that produced a reduced length to 20 cm; 600 g of sediment added to achieve a depth of 2 cm. For the avoidance assay for both species, stiff, removeable plexiglass sheets were used to divide tanks into two equal compartments. This barrier was used during the initiation and termination of experiments to prevent the migration of test organisms between test compartments. One side of the tank contained spiked sediment and the other contained clean, uncontaminated sediment.

The avoidance assay apparatus consisted of aquaria separated into two sections by plexiglass; one side contained clean, dry sediment and the other contained sediment spiked with chemotherapeutants following methodology as described in Section 3.2.4. Clean, filtered seawater was slowly added to the uncontaminated side of the tank, to

reduce disturbance to the spiked sediments, to a volume of 4.5 L. Tanks were left undisturbed to allow for sediments to settle for 4 h prior to test initiation. Avoidance tanks temperature was controlled by water bath and were aerated lightly during the test. Black polyethylene was placed above the tanks to reduce light exposure; however, some light was present throughout the test on a 12 h light: 12 h photoperiod.

The avoidance assay utilized previous methodology conducted for earthworms (Loureiro et al. 2005) and epibenthic deposit feeders (Ward et al. 2013b). At the start of a trial, 20 amphipods or 6 *N. virens* were seeded (placed) onto the contaminated sediment compartment. Time to burrow was recorded for both species for the first 30 s. 10 min following introduction to the tank, the number of animals completely burrowed was recorded; the partition between sides was then removed and animals were allowed access to each side of the tank as desired. No food was added to the tanks throughout the duration of the test, which is defined below for each species. A simplified overview of the methodology used for the avoidance assay is provided in Figure 3-1 in Section 3.6.

The *E. estuarius* avoidance assay was terminated at 48 h. The plexiglass divider was reinserted and the numbers of animals on each side were recorded, as well as the total number burrowed and dead. Amphipods were counted by pipetting swimmers from the water column and carefully sieving amphipods (1 mm sieve) buried on each side.

Parameters for the *N. virens* assay were quantified every 24 h and terminated at 7 d by temporarily reinserting the plexiglass divider and carefully counting animals on the non-seeded/uncontaminated side in order to reduce the disturbance of sediment particles and any potential transfer of contaminated sediment to the clean side. *N. virens* were counted on the seeded side/contaminated at termination of the assay on day 7. The total number of emerged *N. virens* (not burrowed) was recorded each day, in addition to general observations on locomotory behaviour, appearance and mortality. As *N. virens* on the seeded side were only quantified visually (i.e., only if not burrowed) on day 1 - 6, it was assumed that if a polychaete was burrowed on the seeded side it was alive.

Animal euthanizations were performed in seawater mixed with 1 g/L of ethyl 3-aminobenzoate methanesulfonate (MS222) (Sigma Aldrich, Oakville, ON).

3.2.6. Statistical analysis

Statistical analysis for all tests was performed using GraphPad Prism® version 8.0 for Windows (GraphPad Software LLC, LaJolla, California). For *E. estuarius*, avoidance

was defined as a significant increase of at least 10% in the proportion of animals on the non-seeded side compared to the negative control (Ward et al. 2013). A one-way ANOVA followed by Tukey's post-hoc analysis was used to determine significant differences between the mean proportions of amphipods on the seeded v. non-seeded side at each chemical concentration compared to the naïve or chronic control. The two negative controls (naïve and chronic) were statistically assessed by a T-test to determine potential behavioural differences from 30-d exposure. Initial burrowing behaviour (total burrowed within the first 30 s and at 10 min of the assay), mean mortality and the proportion emerged at the termination of the assay were assessed by one-way ANOVA followed by the Dunnett's post-hoc analysis to the naïve negative control only. A p-value < 0.05 was used to determine statistical significance for all tests.

For *N.* virens, the mean proportion on the non-seeded/uncontaminated side and the proportion emerged for each day were assessed using linear regression and two-way ANOVA. Results were assessed for normality prior to testing, and if some or all of the data was non-normal a Friedman test was performed to assess differences. Two-way ANOVA and significant differences over time was determined through linear regression, in which best fit lines over the 7-d period were statistically compared to the negative control within each exposure group. Dunnett's post-test was then performed at each time point within an exposure group compared to the negative control to determine the time point associated with the behavioural response. Statistical tests were only performed within the same exposure group (i.e., the naïve exposed animals were compared to the naïve negative control); however, the naïve and chronic negative control were compared to determine potential effects from 30-d housing in the exposure vessel. A significant increase of at least 20% of *N. virens* on the non-seeded side (to account for the lower number of organisms in a tank) compared to the respective negative control was used to indicate avoidance. Additionally, a significant difference of emerged *N. virens* was used to indicate effects to burrowing behaviour. A p-value < 0.05 was used to determine statistical significance for all tests.

Initial proportions of *N. virens* burrowing and burrowed that was recorded at the start of the assay at 30s and 10 min, respectively, as well as mean mortality for *N. virens* after 7d, was assessed by one-way ANOVA followed by the Dunnett's post-hoc analysis compared to the naïve negative control. A p-value < 0.05 was used to determine statistical significance for all tests.

3.3. Results

3.3.1. Water quality

During the chronic exposures and during the avoidance assay for amphipods, seawater was consistently measured at 11.0 ± 0.5 °C, pH of 7.8 ± 0.2, dissolved oxygen 7.0 ± 0.7 mg/L and salinity 30 ± 1.2 ‰. The polychaete chronic exposures and avoidance assay parameters were temperature of 10.9 ± 0.3 °C, pH 7.7 ± 0.1, dissolved oxygen 6.5 ± 0.5 mg/L and salinity 28 ± 0.4 ‰.

3.3.2. *E. estuarius* mortality, burrowing and avoidance behaviour

At the end of the 30-d chronic pre-exposure period, there were no significant differences in the mean percent mortalities ± standard error of the mean (SEM) between each concentration group: 11 ± 2.2% (for the chronic negative control), 12 ± 0.7% (EMB 5 µg/kg), 16 ± 2.3% (IVM 1 µg/kg), and 13 ± 0.7% (EMB + IVM). The negative control (naïve and chronic negative control) mortality rate for amphipods during the avoidance assay ranged between 0 - 10% in replicates, with a mean percent mortality ± SEM of 2 ± 0.8% and 0% for the naïve and chronic negative control groups, respectively (see Table 3-1).

Amphipods generally burrowed immediately (within 1-2 s) when introduced to sediment and > 98% of control organisms were burrowed at the termination of an avoidance experiment. There was no significant difference between the percent of naïve and chronic control amphipods burrowed at either 30 s or 10 min into the avoidance assay. For the naïve and chronic controls, the distribution behaviour of *E. estuarius* when placed in the aquaria was to remain on the side they were placed into (seeded side) of the test system. As shown in Figure 3-2, the mean percent of *E. estuarius* on the non-seeded side of the tank was low (7 ± 1.4% for both negative control groups), and controls were not statistically different from one another ($p > 0.999$, $F = 1.0$).

When naïve and chronically pre-exposed *E. estuarius* were placed into sediment containing 0.5 to 200 µg/kg of EMB in avoidance assay chambers, no significant differences in the percent of animals that burrowed at the initiation (30 s and 10 min) and termination (48 h) of the assay were seen ($p = 0.48$, $F = 0.97$) (see Table 3-1). As well, no significant differences in the proportions found on the non-seeded/uncontaminated side were seen between EMB concentration groups ($p = 0.96$, $F = 0.35$) (Figure 3-2a). There

were no significant increases in mortality compared to the naïve negative control ($p = 0.0017$, $F = 3.78$).

Naïve *E. estuarius* placed into IVM contaminated sediment showed that there was no significant difference in the percent initially burrowed compared to the naïve negative control, however at the termination of the experiment only 90 ± 2.9% of *E. estuarius* seeded into 25 and 50 µg IVM/kg were burrowed, which was statistically different from the 98% observed for the naïve negative control ($p = 0.0005$, $F = 4.48$) (see Table 3-1). A significantly increased proportion of naïve amphipods were found on the non-seeded sediment side (38 ± 6.0 % and 42 ± 4.4%) in the 25 and 50 IVM µg/kg treatment groups, respectively ($p < 0.0001$, $F = 31$), compared to the naïve negative control (7%) (see Figure 3-2b). There was no significant differences in mortality for naïve *E. estuarius* placed into IVM contaminated sediment compared to the negative control (see Table 3-1).

Chronically pre-exposed *E. estuarius* exhibited no significant differences between any IVM exposure group in the proportion of amphipods initially burrowed at the start of the avoidance assay (both 30 s and 10 min) compared to the chronic negative control. The 50 µg/kg IVM chonic group exhibited a significantly lower proportion of amphipods burrowed at the termination of the assay (89 ± 3.2%) compared to controls (> 98%) ($p = 0.0014$, $F = 5.1$). In the assay, increased proportions of chronic amphipods were found on the non-seeded side at the end of the experiment after seeding into clean sediment as well as sediment with IVM (Figure 3-2b), therefore the concentration-response distribution for the chronically pre-exposed amphipods was U-shaped. There was a significant increase in the proportion of chronic *E. estuarius* on the non-seeded side in the 0, 25 and 50 µg/kg IVM treatment groups (means of 22 ± 6.3%, 44 ± 3.2 % and 41 ± 7.7 %, respectively) compared to the chronic negative control (7 ± 1.4%, $p < 0.0001$, $F = 21.12$). Interestingly, the proportion of animals on the non-seeded side for the chronic 0 µg/kg IVM group was also statistically the same as 0.5 and 5 µg/kg IVM (9 ± 1.7%, 11 ± 3.2 %), and statistically different from the 25 and 50 µg/kg IVM groups. Chronically pre-exposed *E. estuarius* seeded onto sediment containing 25 and 50 µg/kg IVM also had significantly higher mortalities (13 ± 2.0% and 33 ± 8.5%, respectively) compared to controls (< 2 %, $p < 0.0001$, $F = 14.53$) (see Table 3-1).

When naïve *E. estuarius* were exposed to a combination of both EMB and IVM in avoidance assays, no significant difference was noted in the percent burrowed at the beginning (30 s and 10 min) and termination (48 h) of the assay between any treatment

group and the negative control. Significantly higher proportions of animals were found on the non-seeded sediment side for the 50/25 and 200/50 µg/kg EMB/IVM treatment groups (28 ± 3.3% and 28 ± 3.2%) compared to the naïve negative control group ($p < 0.0001$, $F = 29.9$) (Figure 3-2). A significantly higher mortality was also seen in naïve *E. estuarius* seeded onto sediment containing 200/50 µg/kg EMB/IVM (33 ± 4.4 %) compared to the control (1.7 ± 0.8 %) ($p < 0.0001$, $F = 12.03$). Table 3-1 details the mortality and burrowing results at the termination of the assay.

For *E. estuarius* chronically pre-exposed to a combination of EMB/IVM, there were no significant differences between any treatment group and the control with respect to burrowing behaviour at the beginning of the avoidance assay. However, at the termination of the assay, 90 ± 2.0% of chronic *E. estuarius* seeded into 5 EMB/IVM µg/kg were burrowed, which was statistically different than the negative controls (> 98%) ($p = 0.0016$, $F = 5.0$). Regarding avoidance behaviour, significantly higher proportions of animals were found on the non-seeded side at the termination of the assay when compared to the chronic negative control amphipods ($p < 0.0001$, $F = 21.4$) (see Figure 3-2c). The avoidance concentration-response represented a U-shaped distribution with increasing, decreasing, then increasing proportion on the non-seeded side with concentration, as follows; 64 ± 9.5 % (0 µg/kg), 32 ± 1.8 % (0.5 µg/kg), 22 ± 2.3 % (5 µg/kg), 44 ± 4.6 % (50/25 µg/kg) and 59 ± 13.4 % (200/50 µg/kg). A significantly higher mortality at the termination of the assay was also observed for chronic EMB/IVM *E. estuarius* seeded onto sediment containing 50/25 µg/kg EMB/IVM (23 ± 2.9 %) and 200/50 µg/kg EMB/IVM (32 ± 9.0 %) compared to the negative controls (< 2%) ($p < 0.0001$, $F = 14.53$) (see Table 3-1).

3.3.3. *N. virens* mortality, burrowing and avoidance behaviour

At the end of the 30-d chronic pre-exposure period, mean percent mortality ± SEM for each exposure group was 12 ± 2.9% (EMB), 16 ± 2.6% (IVM), 11 ± 2.1% (EMB + IVM) and 9 ± 2.9% for the chronic negative controls. There were no significant differences in mortality following chronic exposure compared to the chronic control. Mortality at the end of the 7-d avoidance assay for *N. virens* was 9 ± 4.0 % and 0 % for the naïve and chronic negative controls, respectively (see Table 3-2).

N. virens burrowed immediately when placed into avoidance chambers, with > 90% of organisms beginning to burrow within 30s and completing burrowing by 10 min (see Figure 3-3). *N. virens* preferred to remain burrowed, with approximately 2 - 8% of naïve

negative control *N. virens* emerged throughout the 7-d assay. The chronic negative control demonstrated the same behaviour as the naïve negative control and there were no significant differences between proportion burrowed at the start of the assay at 30s (>95%) and 10 min (>95%) ($p = 0.56$, $F = 0.73$ and $p = 0.44$, $F = 1.0$), proportions on the non-seeded side (~35%) ($p = 0.07$, $F = 2.1$), and proportions emerged (~3%) ($p = 0.08$, $F = 2.0$). Throughout the 7-d assay, both naïve and chronic control *N. virens* distributed within the tank with a slight preference to remain on the seeded side, in which approximately 35% of organisms were on the non-seeded side, indicating no effect from housing in the exposure vessel for 30-d on baseline behaviour. Figures 3-4 to 3-6 detail the proportion of *N. virens* on the non-seeded side and daily emergence for the negative control for each treatment group.

When naïve and chronic *N. virens* were seeded into sediment spiked with 0.5 to 200 µg/kg of EMB, there were no significant differences in initial burrowing at 30 s and 10 min (see Figure 3-3) and mortality ($p = 0.08$, $F = 2.0$) (see Table 3-2) for any EMB treatment group compared to the naïve negative control. As data was determined to be non-normal (due to observations of zero emergence) Friedman analysis was performed, which indicated no differences in the proportion of polychaetes emerged (not burrowed) between exposure concentrations and applicable controls over 7-d (naïve $p = 0.08$; chronic $p = 0.19$) (Figure 3-4a and b). However, when analyzed for each time point, the naïve 0.5 µg/kg EMB group exhibited a slightly higher proportion of polychaetes emerged compared to the naive negative control ($p = 0.03$). There was no indication of avoidance to EMB; the proportion of *N. virens* on the non-seeded side was not significantly different in treatment groups compared to the appropriate naïve or chronic controls (naïve slopes $p = 0.53$, $F = 0.8$ and intercepts $p = 0.43$, $F = 0.97$; chronic slopes $p = 0.61$, $F = 0.71$ and intercepts $p = 0.63$, $F = 0.69$) (see Figure 3-4a and b). All animals appeared healthy with normal undulated swimming and burrowing ability.

Naïve *N. virens* seeded into IVM contaminated sediment displayed both toxicity and avoidance behaviour. Mortality throughout the assay was not significantly different for any naïve treatment group from the naïve negative control (see Table 3-2). There was no significant difference in initial burrowing behaviour at 30 s and 10 min for naïve polychaetes compared to the naïve negative control (see Figure 3-3). However, there was significantly higher proportion of naive *N. virens* emerged in sediment over the 7-d assay for 50 and 200 µg/kg IVM (slopes $p < 0.0001$, $F = 33.93$, Friedman test $p = 0.0005$) (see

Figure 3-5a). When analyzed at each time point, there was a significantly higher proportion of *N. virens* emerged compared to the naïve negative control on d 4 - 7 for both 50 and 200 µg/kg IVM ($p = 0.03$ to < 0.0001), increasing from approximately 10% (d 1), to 40% (d4) and finally 90% (d 7) above sediment for both concentrations. Polychaetes that emerged displayed impaired locomotion and portions of their tails were severed from the body. Significantly higher proportions of naive *N. virens* on the non-seeded side over 7-d was also observed for 50 and 200 µg/kg IVM (slopes $p = 0.01$, $F = 3.4$) (see Figure 3-5c), indicating avoidance. By d 6 and 7, approximately 80% of naïve polychaetes were found on the non-seeded side at these concentrations and were statistically different ($p = 0.03$ to 0.0047) than the control (~40% on d 7). Proportions on either side of avoidance chambers for other naïve IVM treatment concentrations were not significantly different from the naïve negative control.

N. virens chronically exposed to 5 IVM µg/kg over 30-d did not have significantly increased mortality throughout the assay (see Table 3-2). Chronic polychaetes did however exhibit significantly impaired initial burrowing ability at the start of the avoidance assay, as only 6 - 20% of animals started to burrow within 30 s, and between 30 - 50% were burrowed after 10 min compared to > 90% for the control (see Figure 3-3). During the 7-d avoidance assay, the proportion of chronic *N. virens* emerged was significantly higher in all treatment concentrations compared to the chronic negative control (slopes $p < 0.0001$, $F = 9.8$, Friedman test $p = 0.0005$) in which percent emerged was generally > 25% to a maximum of 100% (see Figure 3-5b). Analysis for each time point indicated significant differences in the proportion emerged on all days for at least 3 or more treatment groups on each day. Emerged polychaetes had impaired locomotory ability, such as poor swimming and irregular undulations, and at higher concentrations some animals were curling with severed tails. Regression analysis of distribution in the tank over time indicated significantly higher proportions on the non-seeded side for 50 and 200 µg/kg IVM (50-90%) compared to the chronic negative control (35-50%) (50 µg/kg intercept $p = 0.0046$, $F = 12.6$; 200 µg/kg slope $p = 0.0036$, $F = 3.7$) (see Figure 3-5d). Analysis by time point indicated significantly higher proportions were found on the non-seeded side for 50 µg/kg IVM on day 6 ($p = 0.0018$) and 200 µg/kg IVM on days 6 and 7 ($p = 0.0002$ and $p = 0.0012$) compared to the control.

N. virens exposed to a combination of both EMB and IVM displayed avoidance and toxicity for both naïve and chronic groups. There was no significant increase in mortality

in any treatment group in the assay when compared to the naïve negative control (see Table 3-2). There was no significant difference in the proportion of naïve individuals starting to burrow and burrowed at 30 s and 10 min, respectively, compared to the control when seeded into sediment containing various concentrations of EMB/IVM at the start of the assay (see Figure 3-3). Burrowing behaviour during the avoidance assay however was impaired, as there was a significantly higher proportion of naïve *N. virens* emerged in the 50 µg/kg and 200 µg/kg EMB/IVM treatment groups (slopes $p < 0.0001$, $F = 25.3$, Friedman test $p = 0.0001$) (see Figure 3-6a). When analyzed for each time point, the proportion emerged was significantly greater than the naïve negative control on d 5 - 7 for 50 µg/kg ($p < 0.0001$) and d 2-7 for 200 µg/kg EMB/IVM ($p = 0.005$ to < 0.0001). At d 7, approximately > 90% of *N. virens* were emerged for both 50 and 200 µg/kg EMB/IVM compared to 0% in the naïve negative control. Distribution within the tank during the avoidance assay showed a significantly higher proportion of naive *N. virens* on the non-seeded side in the 50 and 200 µg/kg EMB/IVM treatment groups ($p = 0.0024$, $F = 4.5$) (see Figure 3-6c). Analysis for each time point found the proportion of naive *N. virens* on the non-seeded side was significantly higher than the naïve negative control at d 5 and 6 for 50 µg/kg ($p = 0.0039$ and 0.0178), and d 2 and 4 - 7 for the 200 µg/kg EMB/IVM treatment group ($p=0.038$ to 0.0004). On d 7, the proportion of naïve *N. virens* on the non-seeded side was 65% and 82% for 50 and 200 µg/kg EMB/IVM, respectively, compared to 30 - 33% for the naïve negative control, 0.5 and 5 µg/kg EMB/IVM treatment concentrations.

During the avoidance assay, it was evident that chronic exposure substantially impaired locomotory ability and overall health, as all chronically exposed polychaetes displayed some level of lethargy and inhibited movement. Chronic polychaetes in the 50 and 200 µg/kg combination treatment concentrations displayed severe toxicity throughout the assay, with severed tails, curling, writhing and almost no locomotory ability. *N. virens* chronically exposed to 5 µg/kg EMB/IVM had significantly impaired burrowing at the beginning of the avoidance assay compared to the control group (see Figure 3-3). Approximately 25% started to burrow within 30s ($p < 0.0001$, $F = 21.8$) and 43% were burrowed after 10 min ($p < 0.0001$, $F = 13.4$) compared to >90% for the control at both 30s and 10 min. All chronic *N. virens* seeded into sediment (0, 0.5, 5, 50 and 200 µg/kg EMB/IVM) had a significant increase in the proportion of emerged, ranging from 13% to 100% over the 7-d assay period, compared to the chronic negative control (5% emerged) (slopes $p = 0.02$, $F = 2.8$, Friedman test $p < 0.0001$) (See Figure 3-6b). The 200 µg/kg

EMB/IVM group specifically, had 75% of polychaetes above sediment at d 1, with 100% emerged on d 4. When analyzed for each time point, significantly higher proportions in the number of *N. virens* emerged compared to the chronic negative control was found on d 5 - 7 for 5 µg/kg EMB/IVM ($p = 0.0083$ and 0.0144) and d 1 - 7 for 50 µg/kg and 200 µg/kg EMB/IVM ($p = 0.045$ to < 0.0001).

All chronic treatment groups exhibited a significantly different proportion of *N. virens* on the non-seeded side compared to the chronic negative control ($p < 0.0001$, $F = 7.25$) (see Figure 3-6d). Interestingly, the chronic *N. virens* seeded into 200 µg/kg EMB/IVM exhibited a clustering behaviour, which resulted in 100% of *N. virens* on the seeded side by d 6, perhaps due to toxicity and not a preference to remain on the seeded/contaminated side. All other sediment treatment concentrations, including 0 µg/kg, exhibited significantly higher proportions on the non-seeded side (ranging from 45 - 90%) in chronically exposed *N. virens* when assessed by linear regression, compared to the chronic negative control (30%). However, only 0.5, 5, 50 and 200 µg/kg EMB/IVM had a greater than 20% difference in proportion compared to the negative control, indicating avoidance behaviour for these treatment concentrations only. Analysis for each time point showed a significant increase in the proportion of animals on the non-seeded side for d 7 for 0.5 µg/kg EMB/IVM ($p = 0.0038$), day 6 and 7 for 5 µg/kg EMB/IVM ($p = 0.0077$ and 0.0001) and d 5 - 7 for 50 µg/kg EMB/IVM ($p = 0.0377$, 0.0002 and 0.0011) compared to the chronic negative control.

3.4. Discussion

Both EMB and IVM have been found in contaminated sediments beneath Atlantic salmon net pens following the implementation of anti-sea lice treatment strategies. Due to their physicochemical characteristics, persistence in marine sediments ranging from months to years has been observed (McHenry and Mackie 1999, Cannavan et al. 2000). Sediments are a heterogenous media and therefore, chemical distribution will likely be sporadic with contaminant hot spots. This can influence the distribution of resident benthic species, their potential exposure, and the resulting toxic effects (Huang et al. 2003). The purpose of these experiments was to determine if the amphipod (*E. estuarius)* and polychaete (*N. virens*) avoid sediments contaminated with EMB, IVM or a combination of both. Through application of a chronic pre-exposure to an environmentally relevant contaminant concentration, attenuation or escalation of the potential avoidance response was also investigated. The assessment of avoidance is adaptively valuable as this

behaviour can limit an organism's exposure to contaminants, reducing uptake and potential toxicity. A bioassay utilizing free access to compartmentalized sides of a chamber with contaminated and uncontaminated sediments was used to test survival, avoidance and burrowing behaviour in these 2 species of naïve and chronic pre-exposed benthic invertebrates.

IVM and EMB are both avermectins, chemicals with systemic action that act on both glutamate-gated chloride (GluCl) and γ-aminobutyric acid (GABA) gated chloride ion channels of nerve and muscle cells in invertebrates (Dudel et al. 1963, Jorgensen 2005, Wolstenholme 2012). These ion channels broadly influence organismal functioning, which includes locomotion, feeding and mediation of sensory inputs (Arena et al. 1995, McKellar and Benchaous 1996). The primary cellular response to avermectin exposure is an increase in plasma membrane permeability *via* agonistic action (Albert et al. 1986), resulting in a decreased membrane input resistance and hence a reduced probability of action potential generation. Direct activation of GluCl channels, specifically, is slow, but once open the channels remain in this state for an extended time, essentially irreversibly in the time frame of electrophysiological recordings. Lethargy, paralysis and death due to overexcitation of these receptors are the most common effects following exposure.

When exposed to sediment containing the anti-sea lice chemotherapeutants SLICE® and IVM, an increase in mortality was only observed for naïve *E. estuarius* exposed to the highest combination exposure concentration, as well as IVM and combination chronic groups. Conversely, no mortality effects were observed for *N. virens* following exposure in sediment. Previous research has found 10-d LC_{50}'s for the amphipods *C. volutator* and *E. estuarius* range from 18 - 180 µg/kg IVM (Thain et al. 1997, Davies et al. 1998, Allen et al. 2007) and 153 - 193 µg/kg EMB (McHenery and Mackie 1999, Mayor et al. 2008, Kuo 2010), as well as a 28-d LC_{50} of 22 µg/kg IVM (Allen et al. 2007). Polychaete species *Arenicola marina* and *Hediste diversicolor* 10-d LC_{50}'s have been reported as 17.9 µg/kg IVM (Allen 2007), 111 µg/kg EMB (McHenery and Mackie 1999) and 1,368 µg/kg EMB (Mayor et al. 2008). Based on these reported toxicity values and the known mechanism of action and associated adverse effects of avermectins, the increased lethality to *E. estuarius* from exposure to avermectins > 50 µg/kg is not surprising. The observed increased mortality for chronic *E. estuarius* (i.e., IVM or combination) compared to the similar concentration naïve groups may be due to cumulative GABA or GluCl receptor binding over time from pre-exposure, which subsequently reached acutely lethal synapse

inhibition once introduced to the higher concentrations. Conversely, the lack of mortality for *N. virens* in this study may be due to their lower comparative sensitivity to IVM and EMB, perhaps due to their larger size, or differences in toxicokinetics; however, significant effects to burrowing behaviour after exposure in sediment were observed, which is subsequently discussed.

When *E. estuarius* was exposed to > 25 µg/kg IVM in sediment and a combination of SLICE® and IVM (either as naïve or chronic groups), burrowing behaviour was affected. Similarly, adverse sublethal effects were observed for *N. virens*, in which significantly altered burrowing behaviour was observed for both naïve and chronically exposed organisms in sediment containing > 0.5 µg/kg IVM and a combination of both. Scant sublethal toxicity information for avermectins and amphipods is available in the literature. However, the polychaetes *A. marina* and *N. virens* showed impaired burrowing when exposed to 12 µg/kg IVM after 10-d of exposure (Thain 1997) and to 400 µg/kg EMB after 30-d (McBriarty et al. 2017). Work by Daoud (2018), also found 15-d impaired behaviour EC_{50}'s of 96 and 15 µg/kg EMB *via* SLICE® and IVM *via* Ivomec®, respectively, for juvenile American lobster (*Homarus americanus)*. The observed behavioural effects seen in both invertebrates exposed to avermectins in the present study is therefore supported by the limited information in the literature. Chronic combination exposures also had reduced time to emergence and an increased the number of *N. virens* emerged compared to IVM alone exposures, which could be due to additive effects on GluCl and GABA receptors (Cully et al. 1994, Menez et al. 2012) from low concentration exposures over time. Conversely, the increase in number of individuals found above the sediment (an abnormal behaviour) for *E. estuarius* did not occur in a consistent concentration-response manner; higher concentrations did not necessarily show altered emergence behaviour, although it is important to note that these same exposure groups had significantly increased mortality, suggesting that emergence is not possible at high concentrations due to the organisms approaching death. Overall, the assay indicated that burrowing behaviour is a valuable indicator of toxicity for *N. virens*, and that emergence from sediment for burrowing species will likely result in mortality.

No available literature exists on avoidance behaviour in these benthic species to anti-sea lice chemotherapeutants; this information is important to fully understand the potential impacts of these chemicals to the benthos. Previous work has shown that amphipods have absent or have reduced populations compared to other species following community

surveys at contaminated sites (Swartz et al. 1982, Kravitz et al. 1999), although this may be species and contaminant dependant (Bach et al. 2010). Conversely, polychaetes do not characteristically avoid contaminated habitat and may even have increased numbers, which may be due to tolerance, chemosensory capability or opportunistic behaviour from absence of competitors (Black et al. 1997, Tefler et al. 2006). In the present experiments, both naive and chronic *E. estuarius* and *N. virens* displayed avoidance behaviour to SLICE® and IVM in sediment. The results here support the typical behaviour observations in the literature; *E. estuarius* avoided low concentrations over a short period of time (48 h) and had significantly higher movement following chronic pre-exposure, whereas *N. virens* displayed a delayed avoidance only after the onset of toxicity (i.e., impaired burrowing, lethargy and tissue discoloration) which suggests that they would not readily avoid sea lice chemotherapeutant sediments the environment. Concentrations of avermectins in sediment beneath Atlantic salmon net pens have generally been found to range between 0.1 to 500 µg/kg (ERT 1998, Cannavan et al. 2000, Boxall et al. 2002, DFO 2012), therefore benthic species may encounter the concentrations applied in this experiment.

As described above, naïve *E. estuarius* seeded into IVM and combination of EMB and IVM had increased movement to the non-seeded side. Interestingly, chronic *E. estuarius* seeded onto IVM and combination contaminated sediments as well as clean sediment displayed a U- or J-shaped hormetic (Calabrese and Baldwin 2001) concentration-avoidance response curve. Chronically pre-exposed amphipods placed into clean sediment as well as those that were exposed to the highest levels of contaminated sediments, had increased proportions on the opposite, non-seeded side compared to the control. Conversely, chronic amphipods did not avoid sediments containing lower to mid-level IVM and combination concentrations most similar to the pre-exposure concentration. Pre-exposed individuals also had higher proportions of individuals on the non-seeded side compared to naïve organisms at similar avermectin sediment concentrations. These results support an adaptive or tolerant response by *E. esturarius*, from low dose avermectin exposure. The increased movement for chronic amphipods when placed into clean sediment and higher contaminant concentrations may be due unexpected changes between the pre-exposure and avoidance vessel chemical environment. Conversely, seeding into the lower concentrations (i.e., 0.5 - 5 µg/kg) may have been too similar to the chronic pre-exposure concentration to result in significant changes to behaviour, and would therefore not be advantageous to for *E. estuarius* to move. Previous work by Lefcort et al. (2004) similarly demonstrated an adaptive avoidance response by aquatic snails

from contaminated sites exposed to metals in sediment, in which an increased avoidance at low and high concentrations of zinc was observed, as well as no change in response to lead when placed into sediment with similar concentrations as the polluted sites.

Mechanistically, the increased movement of chronic pre-exposed *E. esturarius* at 0 µg/kg and higher concentrations of avermectins may be due to a habituated response as a result of repeated stimulation to prolonged low dose agonistic exposure. Although habituation is understood to be a conserved sensory response, it remains poorly understood. The observed behaviour response in this study for *E. estuarius* may be due to changes at the level of sensory transduction, such as receptor desensitization or internalization after extended binding time, dysregulation of negative feedback, downregulation of receptors or downregulation of glutamate (i.e., the innate agonist, via changes in gene transcription rates) (Keramidas and Lynch 2013, Atif et al. 2017). What is important to acknowledge is that that through habituation the animal no longer perceives the contaminant as a stressor, although this does not necessarily mean that other adverse effects from exposure will no longer occur.

The avoidance response of naive *E. estuarius* in this experiment indicates that they can detect avermectins without prior exposure and before the onset of toxicity. Antenular setae are understood to be the primary chemosensory structures in amphipods, however bimodal sensilla are also found on the body (Hallberg and Skog 2011). Sensilla are hair-like structures that contain mechanosensory and/or chemosensory cells that connect the external environment to the CNS, innervating either the olfactory lobe or ganglia along the ventral nerve cord affecting motor control (Hallberg and Skog 2011). Expression of GABA receptors on sensilla of arthropods and arachnids has been found (Panek et al. 2003, Pfeiffer et al. 2013, Pregitzer et al. 2013), which suggests that *E. estuarius* chemosensory avoidance by avermectins may have been due to a GABA-mediated response given that avermectins are understood to be GABA agonists. This is just speculation however, and there are many other possible avenues and integration of signals that contribute to a detection and avoidance response.

As demonstrated by the naïve and chronic control organisms, *E. estuarius* did not readily move throughout the vessel as a baseline behaviour and would typically remain on the seeded chamber. Therefore, if movement of the animals is limited, they may not encounter heterogenic contaminant concentrations that could initiate a behaviour response. It is important to note that behaviours are species dependant however, as a

previous avoidance assay with the amphipod *Melita plumulosa* demonstrated equal distribution of organisms (approximately 50% one each side) when placed into an avoidance tank with clean sediment (Ward et al. 2013b), therefore other Pacific amphipod species may more readily move and encounter various contaminant concentrations. Additionally, environmental factors such as food and predator presence or mating behaviours may increase or decrease movement. Regardless, it is apparent that the amphipod *E. estuarius* is capable of sensing and responding to sediment contaminated with the anti-sea lice chemotherapeutants SLICE® and IVM, including attenuated responses after a long-term low dose exposure period. This could potentially translate to reduction in populations in the benthic community at contaminated sites.

N. virens did not exhibit any avoidance response to avermectins until the onset of sublethal toxicity; this was evident by their emergence from sediment and an impaired locomotor ability prior to significant movement to the non-seeded side. Polychaetes are understood to be equipped with numerous sensory structures, including nuchal organs (which are ciliated pits on annelids) and parapodial cirri, to gain information about their external environment (Lindsay 2009). Therefore, the lack of an immediate avoidance response to avermectins prior to the manifestation of toxic effects in this assay may indicating that *N. virens* did not possess the ability to detect these chemicals, or that *N. virens* do not recognize that avermectins are to be avoided (Kennedy and Tierney 2012). The observed delayed avoidance response by *N. virens* is thus likely due to debilitation (as evident by emergence) rather than a short-term sensory recognition of contaminants (Swartz et al. 1986, Kravitz et al. 1999), essentially indicating that they are emerging and moving due to toxicity only. Emergence by *N. virens* is associated with increased consumption by predators (Kalman et al. 2009, Diarte-Plata and Escamilla-Montes 2019) and is also part of normal breeding behaviour for male *N. virens* (Bass and Brafield 1972). Therefore, premature emergence following exposure to avermectins could have detrimental effects to benthic polychaetes at the population level.

The avoidance assay with polychaetes demonstrated that they are a likely a mobile species as *N. virens* distributed almost equally within the avoidance vessel under the normal conditions. Organism density within a population may also contribute to dispersal and avoidance behaviour, however, as physically larger species may react to overcrowding by actively dispersing more, especially in an area that may be limited by resources (Byers 2000). A density-dependant effect was not investigated in this assay and

may have influenced the response observed for *N. virens* due to the size limitations of the experimental vessel and size of the organisms. However, there is also evidence of polychaetes and other invertebrates converging beneath net pens due to the high organic matter produced from feces and excess feed (Findlay et al. 1995, Black et al. 1997, Tefler et al. 2006, Neofitou et al. 2010), which would result in a higher density of organisms. Considering that SLICE® and IVM are applied as medicated feed and that *N. virens* did not convey a short-term avoidance response to avermectins, attraction to feed beneath net pens may inadvertently result in higher chemotherapeutant exposure to polychaetes.

Behaviour serves as a bridge between physiological processes and ecological consequences. The present study investigated if spatial avoidance following exposure to sediment bound chemotherapeutants is possible for two benthic invertebrate species. As shown, the Pacific amphipod *E. estuarius* avoided IVM and EMB/IVM spiked sediment, with an increased avoidance response when chronically pre-exposed to a low dose, perhaps due to habituation and adaptation. Conversely, the polychaete *N. virens* displayed paralysis and loss of muscular control as evident by emergence from sediment followed by a delayed movement response; this response was intensified when chronically pre-exposed. Both species responded to low environmentally relevant concentrations of avermectins (< 5 µg/kg), although amphipods were more sensitive to lower concentrations of IVM and responded under a shorter time frame. Collectively, the increased sensitivity of *E. estuarius* to lower doses of avermectins indicates their use an indicator of contamination and provides insight into the potential for this species to leave contaminated marine ecosystems through avoidance, reducing species populations. The sublethal toxicity observed for *N. virens* also provides valuable insight into the possible long-term impacts of SLICE® and IVM at the population level, specially for burrowing species that do not readily avoid the anti-sea lice chemotherapeutants and display emergence, which may result in death. To determine the scale and intensity of impacts on marine benthic organisms, community composition measures should be performed at Atlantic salmon farms where the avermectins EMB (*via* SLICE®) and IVM have been used.

3.5. References

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3.6. Tables

Table 3-1. Morality and burrowing behaviour results for the amphipod, *Eohaustorius estuarius*, following a 48-h avoidance assay. Animals were seeded into sediment spiked with anti-sea lice chemotherapeutant (emamectin benzoate [EMB] prepared from SLICE® 0.2% premix, ivermectin [IVM] or a combination of both [EMB/IVM]) and allowed to move freely between the seeded (spiked) and non-seeded side (N=3). Naïve animals were introduced to the chemical at the beginning of the assay, whereas chronic animals were exposed to 1 (IVM) or 5 (EMB) µg/kg of the test chemical for 30 d prior. The chronic negative control included animals exposed to clean sediment for 30 d before the assay. Statistical difference was determined by one-way ANOVA followed by a Dunnett's post-hoc analysis compared to the naïve negative control ($p < 0.05$ *, < 0.01 **, < 0.0001 ****). SEM = Standard error of the mean; NA = Not applicable; NS = Non-significant.

Exposure Group	Chemical and Concentration (µg/kg)	Mean proportion dead ± SEM (N=3)	Statistically different?	Mean proportion burrowed ± SEM (N=3)	Statistically different?
Naïve Negative Control	0	0.02 ± 0.008	NA	0.98 ± 0.008	NA
Chronic Negative Control	0	0.00	NS	1.0	NS
Naïve	EMB 0.5	0.07 ± 0.02	NS	0.98 ± 0.02	NS
	EMB 5	0.05 ± 0.03	NS	1.0	NS
	EMB 50	0.05 ± 0.03	NS	0.98 ± 0.02	NS
	EMB 200	0.05 ± 0.03	NS	0.97 ± 0.03	NS
Chronic	EMB 0	0.00	NS	0.98 ± 0.02	NS
	EMB 0.5	0.00	NS	1.0	NS
	EMB 5	0.00	NS	1.0	NS
	EMB 50	0.00	NS	1.0	NS
	EMB 200	0.00	NS	0.98 ± 0.02	NS
Naïve	IVM 0.5	0.05 ± 0.03	NS	0.98 ± 0.02	NS
	IVM 5	0.02 ± 0.02	NS	0.98 ± 0.02	NS
	IVM 25	0.08 ± 0.02	NS	0.90 ± 0.03	*
	IVM 50	0.05 ± 0.03	NS	0.90 ± 0.03	*
Chronic	IVM 0	0.09 ± 0.05	NS	0.91 ± 0.05	NS

Exposure Group	Chemical and Concentration (µg/kg)	Mean proportion dead ± SEM (N=3)	Statistically different?	Mean proportion burrowed ± SEM (N=3)	Statistically different?
	IVM 0.5	0.08 ± 0.02	NS	0.92 ± 0.02	NS
	IVM 5	0.06 ± 0.00	NS	0.94 ± 0.03	NS
	IVM 25	0.13 ± 0.02	*	0.94 ± 0.03	NS
	IVM 50	0.33 ± 0.09	****	0.89 ± 0.03	**
Naïve	EMB 0.5/IVM 0.5	0.00	NS	1.0	NS
	EMB 5/IVM 5	0.05 ± 0.03	NS	1.0	NS
	EMB 50/IVM 25	0.03 ± 0.03	NS	1.0	NS
	EMB 200/IVM 50	0.33 ± 0.04	****	1.0	NS
Chronic	EMB 0/IVM 0	0.02 ± 0.02	NS	0.98 ± 0.02	NS
	EMB 0.5/IVM 0.5	0.02 ± 0.02	NS	0.98 ± 0.02	NS
	EMB 5/IVM 5	0.1 ± 0.02	NS	0.90 ± 0.02	*
	EMB 50/IVM 25	0.23 ± 0.03	****	0.94 ± 0.04	NS
	EMB 200/IVM 50	0.32 ± 0.09	****	0.93 ± 0.04	NS

Table 3-2. Morality results for the polychaete, *Nereis virens*, following a 7-d avoidance assay. Animals were seeded into sediment spiked with anti-sea lice chemotherapeutant emamectin benzoate (EMB) from SLICE® 0.2% Premix, ivermectin (IVM), or a combination of both (EMB/IVM) and allowed to move freely between the seeded and non-seeded side (N=3 per concentration). Animals were grouped into either a naïve or chronically exposed group. Naïve animals were introduced to the chemical at the initiation of the assay, whereas chronic animals were exposed to 5 µg/kg of the test chemical for 30-d prior. The chronic negative control included animals exposed to clean sediment for 30 d before the assay. Statistical difference was determined by one-way ANOVA followed by a Dunnett's post-hoc analysis for each treatment group compared to the naïve negative control ($p < 0.05$ *). SEM = Standard error of the mean; NA = Not applicable; NS = Non-significant.

Exposure Group	Chemical and Concentration (µg/kg)	Mean proportion dead ± SEM	Statistically different?
Naïve Negative Control	0	0.09 ± 0.04	NA
Chronic Negative Control	0	0.0 ± 0.0	NS
Naïve	EMB 0.5	0.0 ± 0.0	NS
	EMB 5	0.22 ± 0.15	NS
	EMB 50	0.0 ± 0.0	NS
	EMB 200	0.17 ± 0.0	NS
Chronic	EMB 0	0.0 ± 0.0	NS
	EMB 0.5	0.0 ± 0.0	NS
	EMB 5	0.0 ± 0.0	NS
	EMB 50	0.0 ± 0.0	NS
	EMB 200	0.07 ± 0.07	NS
Naïve	IVM 0.5	0.06 ± 0.06	NS
	IVM 5	0.0 ± 0.0	NS
	IVM 50	0.06 ± 0.06	NS
	IVM 200	0.06 ± 0.06	NS
Chronic	IVM 0	0.0 ± 0.0	NS
	IVM 0.5	0.08 ± 0.08	NS
	IVM 5	0.13 ± 0.13	NS
	IVM 50	0.0 ± 0.0	NS
	IVM 200	0.0 ± 0.0	NS
Naïve	EMB 0.5/IVM 0.5	0.22 ± 0.05	NS
	EMB 5/IVM 5	0.0 ± 0.0	NS
	EMB 50/IVM 50	0.0 ± 0.0	NS

Exposure Group	Chemical and Concentration (µg/kg)	Mean proportion dead ± SEM	Statistically different?
	EMB 200/IVM 200	0.22 ± 0.15	NS
Chronic	EMB 0/IVM 0	0.0 ± 0.0	NS
	EMB 0.5/IVM 0.5	0.0 ± 0.0	NS
	EMB 5/IVM 5	0.20 ± 0.12	NS
	EMB 50/IVM 50	0.13 ± 0.07	NS
	EMB 200/IVM 200	0.0 ± 0.0	NS

3.7. Figures

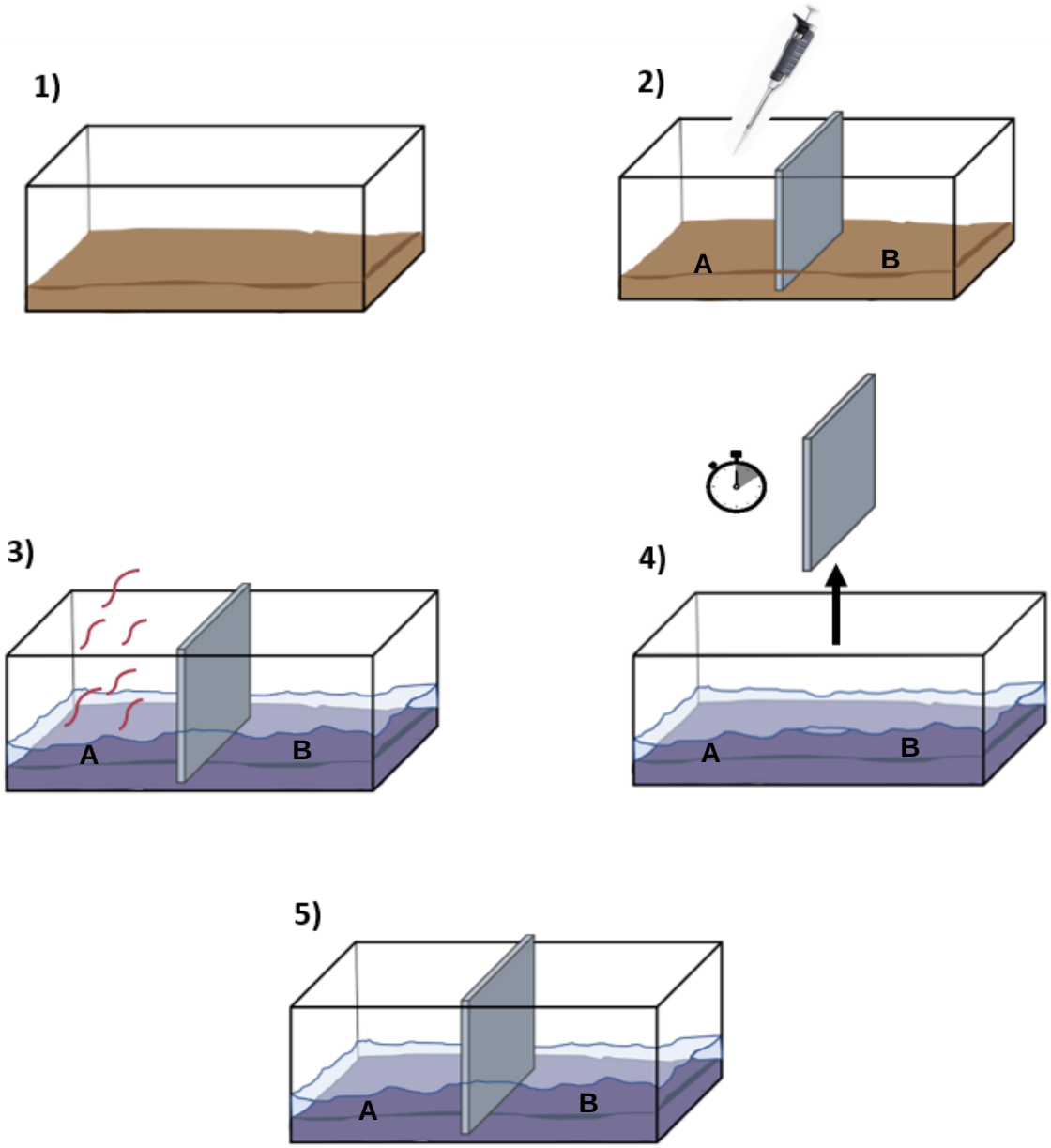

Figure 3-1. Simplified schematic of avoidance assay methodology. Step 1) Add clean, 1 mm sieved sediment to glass tank followed by enough clean, filtered seawater to create a sediment-slurry; Step 2) Insert plexiglass divider. Add contaminant to sediment slurry on one side of the test tank (A) and mix thoroughly. Leave tank covered, overnight; Step 3) Add clean filtered seawater slowly to tank. Add test organisms to the dosed sediment (A); Step 4) After 10 min, remove the plexiglass barrier and allow animals to move freely between the dosed (A) and clean (B) sides; and Step 5) After allotted time (48-h *E. estuarius*, 24-h *N. virens*) insert the plexiglass divider and count the number organisms on each side of the tank (A) and (B).

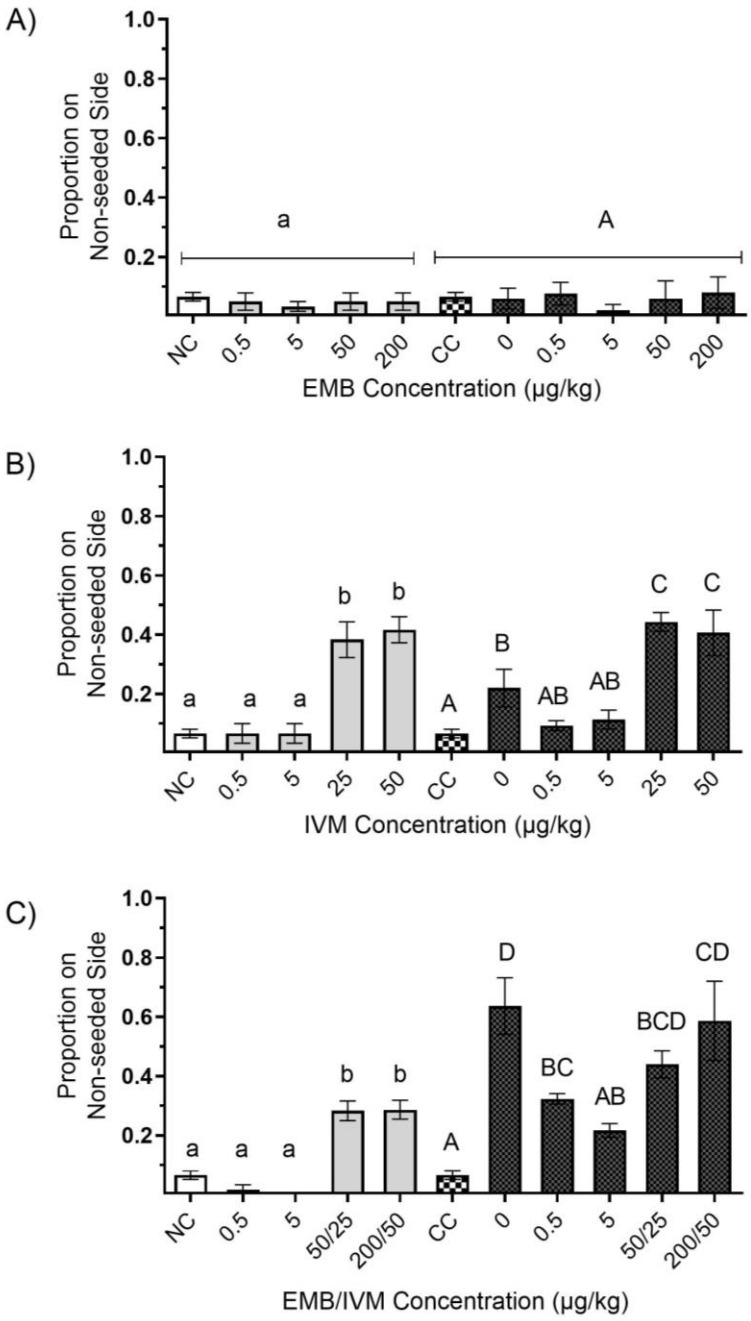

Figure 3-2. Proportion of amphipods, *Eohaustorius estuarius,* on the non-seeded sediment (mean ± 1 SEM, N=3). Sediment was spiked on the seeded side with (A) emamectin benzoate (EMB) prepared from SLICE® 0.2% EMB premix, (B) ivermectin (IVM) or (C) a combination of both (EMB/IVM). Amphipods were introduced to the chemical at the initiation of the assay (naïve, light grey) or chronically pre-exposed to 1 µg IVM/kg, 5 µg EMB/kg or both for 30-d prior (chronic, dark grey). Statistical differences between concentrations were determined using a one-way ANOVA and Tukey's multiple comparison post-hoc test. Lowercase (naïve) and uppercase (chronic) letters represent statistically different groups ($p < 0.05$). NC = Naïve negative control (white); CC = Chronic negative control, animals exposed to clean sediment for 30 d prior (checkered).

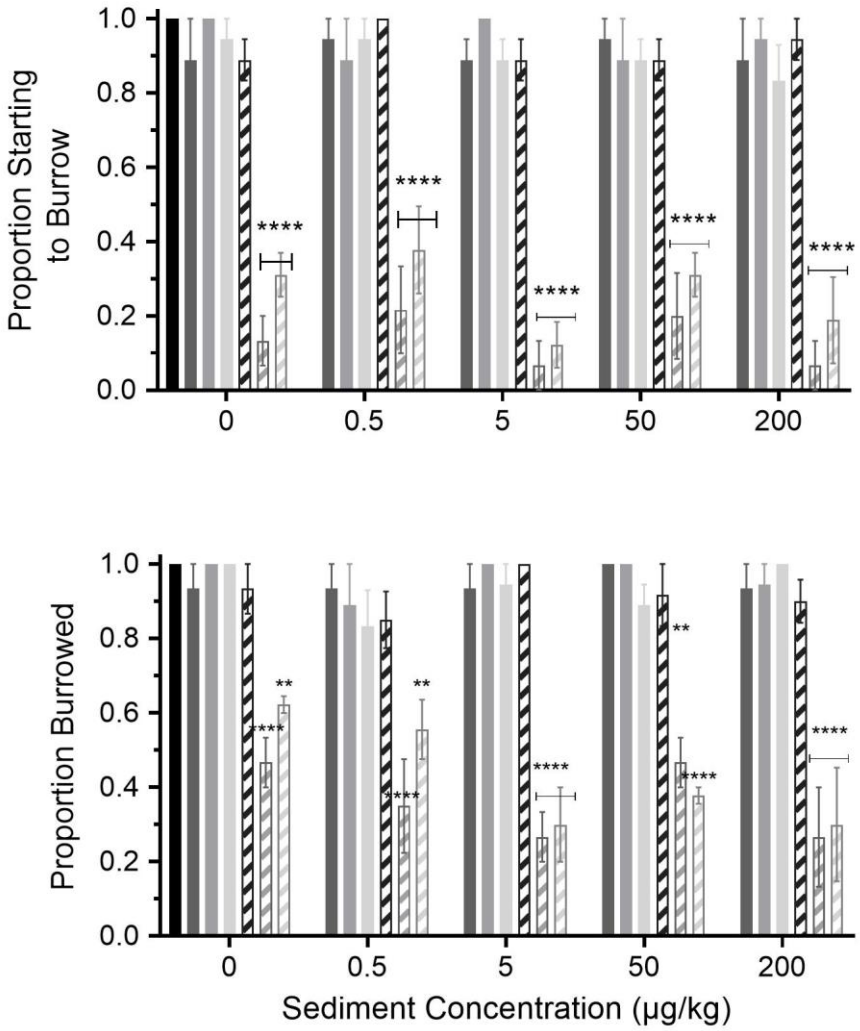

Figure 3-3. Proportion (mean ± 1 SEM, N=3) of polychaetas, *Nereis virens,* starting to (A) burrow at 30 s or (B) burrowed after 10 min. *N. virens* were seeded into sediment containing 0, 0.5, 5, 50 or 200 µg/kg of emamectin benzoate (EMB) prepared from SLICE 0.2% EMB premix, ivermectin (IVM) or a 1:1 combination of both (EMB/IVM). Polychaetes were introduced to the chemical in sediment at the initiation of the assay (naïve) or chronically pre-exposed to 5 µg/kg of the test chemical for 30 d prior (chronic). Chronic negative control animals were exposed to clean sediment for 30 d prior. Chronic negative control ■; Naïve EMB ■; Naïve IVM ■; Naïve EMB/IVM ■; Chronic EMB ▨; Chronic IVM ▨; Chronic EMB/IVM ▨. Statistical differences between treatment groups were determined using a one-way ANOVA and Dunnett's post-hoc compared to the naïve negative controls. Asterisks indicate differences between concentrations and the naïve negative controls ($p < 0.01$ **, $p < 0.001$ ***, $p < 0.0001$ ****).

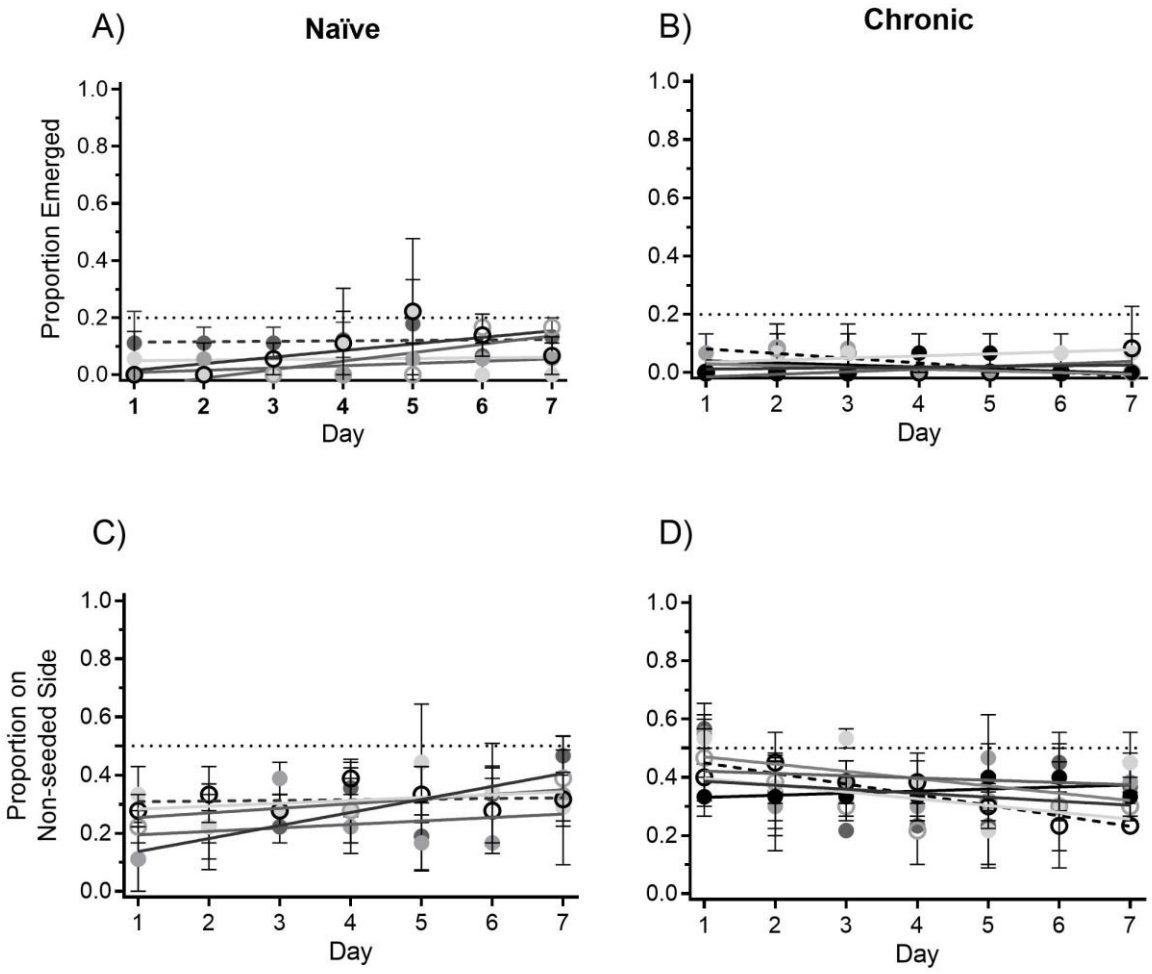

Figure 3-4. Proportion ± 1 SEM of polychaetes, *Nereis virens*, emerged from sediment (A and B) and on the non-seeded side (C and D) of treatment tanks (N=3) containing sediment with the anti-sea lice chemotherapeutant SLICE®. *N. virens* were seeded into sediment containing 0 (○), 0.5 (○), 5 (●), 50 (●) or 200 (●) µg/kg of emamectin benzoate (EMB), prepared from SLICE® 0.2% EMB premix. Behaviour was recorded daily for 7 d. Naïve polychaetes were not pre-exposed to the test chemicals, whereas chronic polychaetes were pre-exposed to 5 µg/kg of EMB or clean sediment (chronic negative control ●) for 30 d before the assay. Mean proportions over time were assessed for statistical differences by Friedman test (A and B) and linear regression (C and D) ($p < 0.05$). Note: The dotted line marking 0.5 and 0.2 is for visual emphasis of normal behaviour only.

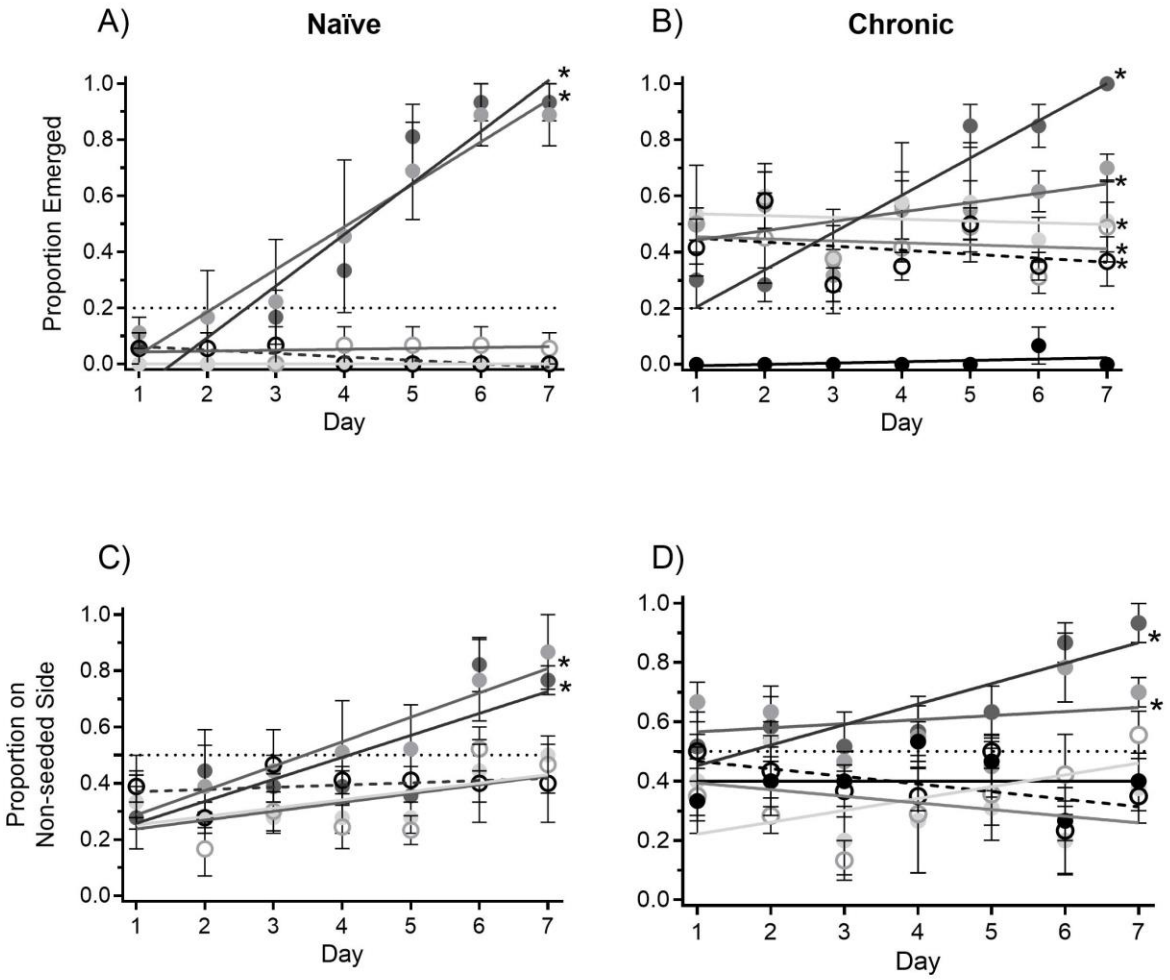

Figure 3-5. Mean proportion ± 1 SEM of polychaetes, *Nereis virens*, emerged from sediment (A and B) and on the non-seeded side (C and D) of treatment tanks (N=3) containing sediment with the anti-sea lice chemotherapeutants ivermectin. *N. virens* were seeded into sediment containing 0 (○), 0.5 (○), 5 (●), 50(●) or 200 (●) µg/kg of ivermectin (IVM) and behaviour was recorded daily for 7 d. Naïve polychaetes were not pre-exposed to the test chemicals, whereas chronic polychaetes were pre-exposed to 5 µg/kg of IVM or clean sediment (chronic negative control ●) for 30 d before the assay. Mean proportions over time were assessed for statistical differences by linear regression ($p < 0.05$). Lines statistically different from the naïve negative control (0 µg/kg) or chronic negative control are indicated by an asterisk (∗). Note: The dotted line marking 0.5 and 0.2 is for visual emphasis of normal behaviour only.

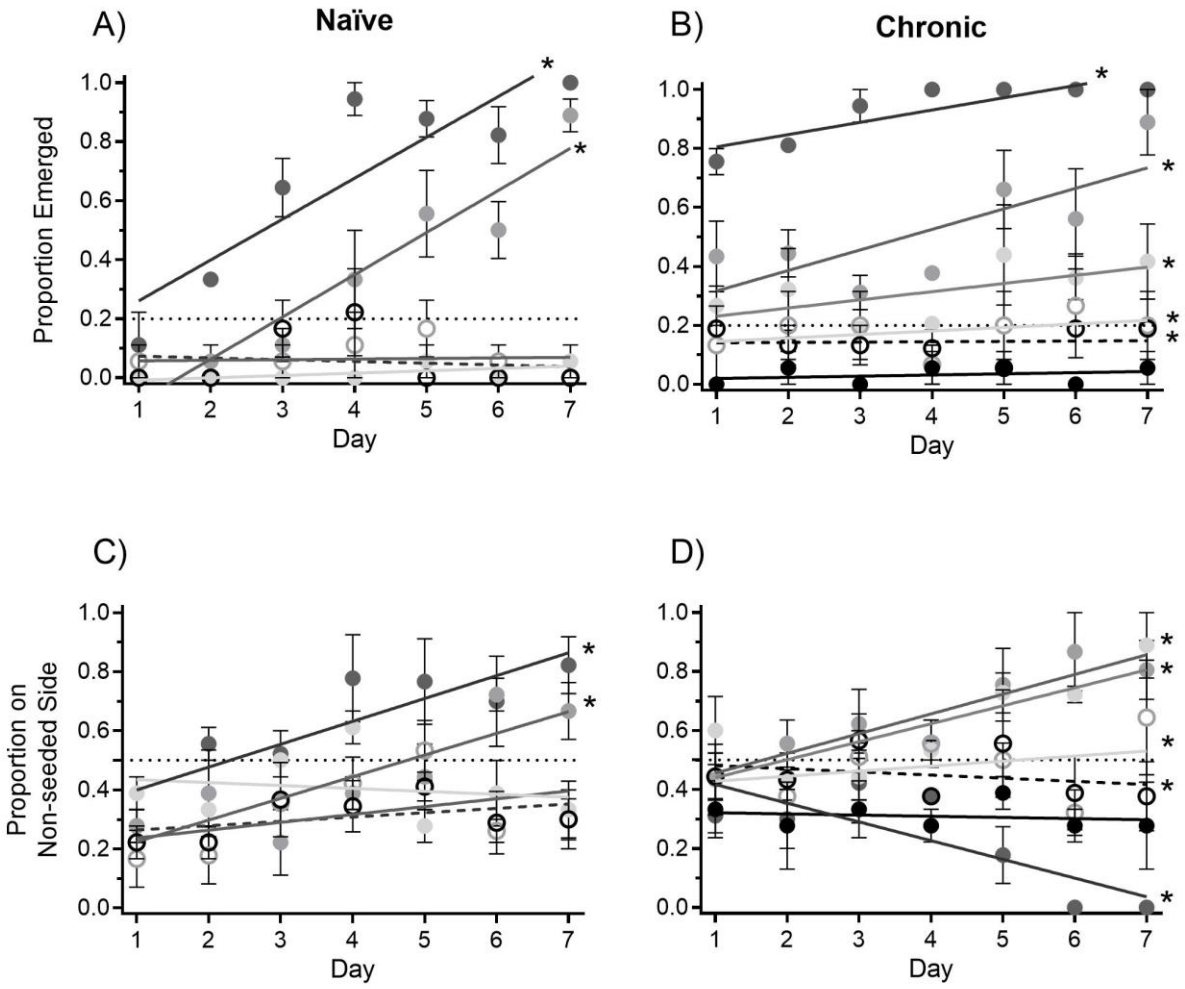

Figure 3-6. Mean proportion ± 1 SEM of polychaetes, *Nereis virens*, emerged from sediment (A and B) and on the non-seeded side (C and D) of treatment tanks containing a combination of the anti-sea lice chemotherapeutants SLICE® and ivermectin. *N. virens* were seeded into sediment containing 0 (○), 0.5 (○), 5 (●), 50(●) or 200 (●) µg/kg of a 1:1 combination of emamectin benzoate (EMB), prepared from SLICE® 0.2% EMB premix and ivermectin (IVM). Behaviour was recorded daily for 7-d. Naïve polychaetes were not pre-exposed to the test chemicals, whereas chronic polychaetes were pre-exposed to 5 µg/kg of EMB and IVM or clean sediment (chronic negative control ●) for 30 d before the assay. Mean proportions over time were assessed for statistical differences by linear regression ($p < 0.05$). Lines statistically different from the naïve negative control (0 µg/kg) or chronic negative control are indicated by an asterisk (*). Note: The dotted line marking 0.5 and 0.2 is for visual emphasis of normal behaviour only.

Chapter 4. The effects of SLICE® and ivermectin on oxygen consumption in marine benthic invertebrates

4.1. Introduction

The anti-sea lice chemotherapeutants emamectin benzoate (EMB) (applied as SLICE® Premix [0.2% EMB w/w]) and ivermectin (IVM) are commonly used to treat pests such as worms and other parasites in medicine and are specifically used to treat sea lice outbreaks at Atlantic salmon aquaculture facilities. These chemicals, which are part of the avermectin chemical family, agonistically bind gated chloride channels causing an influx of chloride ions which results in hyperpolarization of nerve and muscle cells, causing paralysis and death through inhibition of muscle and nerve synapses (Arena et al. 1995, McKellar and Benchaous 1996). At aquaculture facilities, avermectins are applied as an in-feed medication in the form of pellets. Once eaten by the salmon, the chemical is absorbed in the gut and distributed throughout the fish into the plasma, mucus, skin and muscle (Whyte et al. 2011). Sea lice that are latched onto the skin of the salmon are exposed to the chemotherapeutant through feeding on the external tissue and mucus, which subsequently causes paralysis and death of the pests.

Farmed Atlantic salmon are kept in open-net pen systems, that permit the flow of ocean water and other wastes between the net-pen and the surrounding environment. Only 1 - 17% of feed is consumed by farmed salmon (Cubitt et al. 2008) and approximately 25 - 33% of ingested feed is believed to become feces and destined for the ocean floor (Weston 1986). This combination of food waste and feces production increases the deposition of organic matter in the marine environment, as well as introduces contaminants such as avermectin into the ecosystem if applied as a pest management strategy. The in-feed treatments active ingredients IVM and EMB are lipophilic chemicals with long-half lives that exceed 150 d in sediment (Campbell 1989, McHenery and Mackie 1999), which indicates that not only are IVM and EMB likely to sorb to sediment but will also persist in the marine environment.

Benthic invertebrates contribute to the biogeochemical and nutrient cycling in sediments and make up the largest group of animals in benthic communities, thereby significantly influencing the sediment composition and sediment-water dynamics in aquatic environments compared to other species (Glud 2008, Nogaro et al. 2009, Kuntz and Tyler 2017). These animals inhabit, graze and ingest sediment particles and are consequently the first organisms potentially affected by contaminated sediment *via* direct

contact pathways. As previously described, waste produced by Atlantic salmon farms are significant contributors the benthic environment, enriching organic matter both beneath and in the vicinity of net pens. The implications of un-eaten feed and wastes containing avermectins used to treat sea lice at infected farms may therefore pose a risk to non-target benthic species.

EMB and IVM toxicity is poorly characterized despite their known persistence in sediment beneath Atlantic salmon net pens following application. Chemical concentrations ranging from 0.1 - 400 µg/kg, with a maximum of 2600 µg avermectin/kg sediment (geomean of 6.38 µg/kg) have been reported beneath net pens within a 100 m radius following application (ERT 1998, Cannavan et al. 2000, Boxall et al. 2002, DFO 2012, Lalonde et al. 2012). Limited studies have characterized IVM toxicity to several invertebrate species in which marine amphipods and annelids appear to be the most sensitive with 10-d LC_{50} values of 18 - 180 µg/kg, while starfish are the least sensitive with 10-d LC_{50} of 23,600 µg/kg (Davies et al. 1998, Allen et al. 2007). Regarding exposure to EMB *via* SLICE® 10-d LC_{50} values range from 153 - 193 µg/kg for amphipods (McHenery and Mackie 1999, Mayor et al. 2008, Kuo et al. 2010), 250 µg/kg for the lobster *Homarus americanus* (Daoud 2018), 111 - 1,368 µg/kg for the polychaetes *Arenicola marina* and *H. diversicolor*, respectively (McHenery and Mackie 1999, Mayor et al. 2008), and 96-h LC_{50} > 68,200 µg/kg EMB for the prawn *Nephrops norvegicus (*McHenery and Mackie 1999). Based on the reported field concentrations, there is some overlap with lethal concentrations for the more sensitive species, however there is currently little data investigating sublethal low concentration exposures of avermectins. To evaluate the potential ecological effects reflective of the high sediment persistence of these two compounds there is a need to assess long-term low concentration scenarios.

Measures of metabolic rate (MR) through respirometry (measures of oxygen consumption) can be used as simple tools to evaluate stress and toxicity following chemical exposure. Oxygen consumption is vital for optimal physiological function and the survival of aerobic organisms, and provides insight into metabolic activity, health and responses to stimuli. Toxicological studies with various contaminants have demonstrated altered metabolic rates in a variety of different organisms *via* respirometry. Oxygen consumption has been shown to decrease, increase and not change following exposure to many environmental chemicals. For example, the bluegill sunfish had increased metabolic rate when exposed to wastewater effluent (Du et al. 2018), while oysters

exposed to cadmium had decreased respiration (Kurochkin et al. 2011). Decreases in O_2 consumption rate were also found following mercury exposure in in the Paneaid shrimp (Barbieri et al. 2005) and in the nematode *Caenorhabditis elegans* after exposure to dimethyl sulfoxide, zinc and cadmium (Schouest et al. 2009). No change to MR from contaminant exposure has also been found, for example, following cadmium exposure to *Daphnia magna* (Knops et al. 2011) and polycyclic aromatic hydrocarbon exposure to zebrafish (Lucas et al. 2016). Collectively, not all contaminants or species will have similar effects on MR from exposure, however a change in this parameter is indicative of metabolic cost. Redirection of energy toward detoxification, cellular defense, compensatory respiratory improvements, narcosis, cellular necrosis or apoptosis following exposure are examples of mechanisms contributing to changes in MR (Maltby 1999, Lushchak and Bagnyukova 2006, Fan and Bergmann 2008, Kurochkin et a. 2011).

Alterations to respiration rates is an early indication of potential fitness ramifications. There is evidence that MR alterations affect the survival, growth and reproductive output of organisms (Burton et al. 2011, Cooke et al. 2013, Auer et al. 2015). For example, when oxygen consumption is altered reductions in feed intake can occur, resulting in lower growth rates and increased overall stress in fish (Lushchak and Bagnyukova 2006, Bagherzadeh et al. 2013). As described in a review by Maltby (1994), the amphipod *Gammarus pulex* had generally increased respiration and decreased growth through decreased feeding rate and had decreased offspring size when exposed to a range of contaminants. The brown trout *Salmo trutta* was also found to have decreased survival with increased metabolic rate (Álvarez and Nicieza 2005).

Respirometry is also a sensitive endpoint in terms of effective concentrations compared to traditional measures of toxicity, such as lethality, that are commonly used to assess ecological risk at contaminated sites. Schouest et al. (2009) demonstrated *C. elegans* 24-h respirometry EC_{50}'s for metals were 10 to 100-fold less than the 24-h LC_{50}'s and Padmanabha et al. (2015) found that chlorpyrifos was associated with increased respiration rates 12-h after exposure to concentrations 10-fold lower than the 96-h LC_{50} in freshwater fish. Although it is important to note, as previously described, that some organisms do not display changes to MR, however this may be due to limitations of the experiment such as duration of exposure or concentrations applied. Regardless, when physiological knowledge is incorporated into toxicology, it can improve predictions of organism adverse responses to environmental contamination. Respirometry is thus not

only a useful tool in ecotoxicology but may also be valuable for biomonitoring and management of contaminates sites (Samaras 2005).

The objective of this study was to evaluate the sublethal effects of the anti-sea lice chemotherapeutants EMB (using the formulation SLICE® 0.2% Premix) and IVM on benthic marine invertebrates following sediment exposures and using oxygen consumption alteration as a metric of toxicity. Benthic amphipods (*Eohaustorius estuarius)* and polychaetes (*Nereis virens*) were chronically exposed to IVM, EMB or a combination of both at low environmentally relevant concentrations, and oxygen consumption was measured over the course of exposure.

4.2. Methods

4.2.1. Study organisms

Adult amphipods (*E. estuarius*) from Yaquina Bay, Oregon were provided by Northwestern Aquatic Sciences (Newport, OR). Animals were shipped in 5 x 5 cm containers containing clean-filtered seawater, silica and 100 animals/container. Upon arrival, each container was placed in a large, aerated seawater bath, held at 11 ± 1°C and a 12:12 h photoperiod. Seawater changes were conducted 2 times per week with 20 - 30% of the water changed at any time. *E. estuarius* were fed ground Cargill® EWOS 1.2 mm farmed fish salmon pellets once a week *ad libitum*. Animals were not size selected for the experiment and were generally 1 - 2 mm in length. *E. estuarius* were acclimated for at least 72 h prior to an experiment.

Adult polychaetes *(N. virens*) were collected from sediment flats in New Hampshire by Aquatic Research Organisms Inc. (Hampton, NH). Animals were shipped in styrofoam boxes (300 animals) with cold packs containing damp seaweed and newspaper. Upon arrival, polychaetes were housed communally (50 to 60 animals) in a 38 x 25.5 x 14 cm plastic tub filled to a depth of approximately 7 cm of clean sediment (sediment collection detailed below). Four tubs were then placed within clean, aerated seawater baths (~134 L) held at 11 ± 1°C, under a 12:12 h photoperiod. Water quality was maintained using Hagen® Fluval® FX6 mechanical and biological filters, Coralife® hang-on-back protein skimmers, and Coralife® ultraviolet sterilizers. Seawater changes were conducted 3 - 4 times per week with 20 - 30% of the water changed at any time. Holding densities were approximately 500 - 600 polychaetes per m^2 sediment, well below holding densities recommended for normal health (Safarik et al. 2006). Polychaetes were fed ground

Cargill® EWOS 1.2 mm farmed fish salmon pellets 3 -4 times weekly *ad libitum*. *N. virens* weighed on average 3.75 ± 1.43 g (range 1.5 -12 g and lengths of 5 - 10 cm). Polychaetes were not size selected for experiments and were acclimated for at least 1 week prior to an experiment.

4.2.2. Sediment and water

The seawater source was the Vancouver Aquarium (Vancouver, BC) which was pumped directly from Burrard Inlet, followed by slow sand filtration and followed by disinfection with ultraviolet (UV) radiation. Sediment was collected from Centennial beach (Tsawwassen, BC) which is considered an acceptable uncontaminated reference site based on results from the Boundary Bay Assessment and Monitoring Program (BBAMP) (2009 - 2015), completed by Hemmera (2017). Sediment from this region has an organic carbon content of 0.02 - 0.2 % (Hemmera 2014). Sediment was collected from the upper 10 cm, sieved during collection using 1 mm metal sieves to remove debris and dried prior to use.

4.2.3. Chemicals

SLICE® 0.2% Premix (Merck Animal Health, Intervet Canada Corp., Kirkland, QC), which contains 0.2% EMB w/w, was obtained from Fisheries and Oceans Canada (DFO) in September 2017. Target concentrations of EMB were prepared by thoroughly mixing the SLICE® 0.2% Premix in seawater for 30 min to create a stock solution. The stock was subsequently diluted and mixed for 15 min for each additional exposure concentration. All preparations were completed in the dark to reduce photodegradation.

IVM (CAS Number 70299-86-7), which is a solid white powder, was obtained from Sigma-Aldrich (Oakville, ON). Stock solutions were prepared by thoroughly mixing with agitation for 2 h in seawater on ice to prevent degradation (Dorati et al. 2015). All preparations were completed in the dark to reduce photodegradation.

4.2.4. Exposure design

Exposure vessels consisted of organisms in 500 mL glass jars containing 300 g of spiked sediment and 400 mL aerated seawater. Sediment spiking followed a protocol similar to methodology described by De Lange et al. (2006) and Burridge and Van Geest. (2014). To spike sediments, clean, dry sediment was added to each jar and treated with chemotherapeutants by creating a sediment-seawater slurry (10 g sediment in 2 mL

seawater) and micropipetting 1 mL of the stock solution for each chemical to achieve the desired target concentrations. The sediment was thoroughly mixed with a metal spatula for 5 min, then left overnight for 16 h in the dark at room temperature. Following this, filtered seawater was added to each jar after which animals were introduced. The number of animals exposed in a single jar was dependant on species; 7 *E. estuarius* and 1 *N. virens* per jar were used. Animals were fed a pinch of ground fish food weekly *ad libitum* (based on previous pilot feeding trials to be sufficient) and 50% of seawater was changed weekly throughout the 28-d exposure period. Water quality measurements for salinity, oxygen, pH and temperature were performed weekly. All jars were kept in water baths held at 11 ± 1°C.

Range-finding trials were performed for each species to determine sublethal testing concentrations for use in the respirometry assay; concentrations tested were within reported concentration ranges beneath treated Atlantic salmon farm net pens to determine those that did not result in mortality. The concentration of 5 µg test chemical/kg sediment was deemed to be representative of both EMB and IVM concentrations in sediments beneath net pens based on the literature. However, following a 30-d exposure of *E. estuarius* to 5 µg/kg IVM, > 75% mortality occurred, so the experiment was repeated using a lower concentration of IVM at 0.5 µg/kg for *E. estuarius*. The final nominal treatment concentrations in sediment for the 28-d exposure for *E. estuarius* were 0.1, 0.5, 1 and 5 µg/kg EMB, 0.01, 0.05, 0.1 and 0.5 µg/kg IVM and a 1:1 combination of EMB/IVM of 0.1/0.01, 0.5/0.05, 1/0.1 and 5/0.5 µg/kg. *N. virens* were exposed to nominal treatment concentrations in sediment of 0.1, 0.5, 1 and 5 µg/kg EMB, 0.1, 0.5, 1 and 5 µg/kg IVM or a 1:1 combination of EMB/IVM of 0.1/0.1, 0.5/0.5, 1/1 and 5/5 µg/kg. Six replicates were prepared for each treatment concentration.

4.2.5. Respirometry

The respirometry equipment used for oxygen consumption measurements was obtained from Loligo® Systems (Vibord, Denmark). Briefly, a glass mini chamber system with 4 horizontal glass mini chambers (9 mm in diameter x 25 mm length for *E. estuarius,* 28 mm in diameter x 150 mm in length for *N. virens*) was submerged in a temperature control water bath (11.5 ± 1ºC) that was maintained with a programmed water chiller. Oxygen concentration was measured using a multi-channel oxygen meter (Witrox 4) coupled to optical sensors and AutoResp™ automated intermittent respirometry software. A closed respirometry test (Vleck 1987) was performed as intermittent flow and flushing

of seawater in mini chambers harmed the animals. Oxygen sensors were calibrated using the AutoResp™ software prior to measurement according to the user manual (Version 2.3.0). Oxygen saturation was approximately 100% at the beginning of the test and did not fall below 70%. Equipment set up is seen in Figure 4-1.

The small size of *E. estuarius* required that 3 animals be used in a single mini chamber in order to record sufficient oxygen consumption over the recording period. One polychaete was sufficient to determine oxygen consumption in those measurements. An N = 4 (i.e, four individual mini chambers) were used for the amphipod experiment, and the polychaete measurements had an N = 3 chambers. Before measurements, each animal was weighed; the mass was used to calculate consumption of oxygen on a per mass basis. *E. estuarius* were of a similar mass and because multiple animals were evaluated in a mini chamber simultaneously, the average wet weight was calculated from 100 amphipods (0.0054 g ± 0.001), which was universally applied in calculations. *N. virens* oxygen measurements were conducted individually, therefore each individual wet weight was applied in the oxygen consumption calculations.

On d 7, 14, 21 and 28 of exposure, animals were randomly removed from the exposure vessels and placed in a mini chamber with seawater for measurements. Oxygen depletion over time was recorded for each mini chamber simultaneously over a 15-min period, which included a 3-min acclimation period in the mini chamber followed by a 12-min test (to maintain a > 70 % oxygen saturation). At the end of measurements, animals were carefully removed from the mini chambers and returned to their respective exposure vessel or euthanized. Respirometry measurements for all treatment groups and controls were performed on the same day for each animal. Oxygen concentration measurements without any animals in the chamber were also conducted to quantify biological oxygen demand, (i.e., bacterial consumption of oxygen) when no animals were present.

Oxygen consumption values were calculated using linear regressions that were fitted from the decline in oxygen concentrations in the respirometry mini chambers over time. The following equation was used to calculate oxygen consumption rates (see AutoResp™ v 2.3.0 user manual, Loligo®):

$$MO_2 = \frac{(\Delta O_I \times V \times 60)}{BW}$$

Where ΔO_I is the slope of the regression (mg O_2 L^{-1} min^{-1}) due to invertebrate respiration (after subtraction of background microbial respiration), V is the volume of the

chamber (L), units were converted from min to h by multiplying by 60, and BW was the total body weight (kg) of the organism(s) in the chamber. Final units for oxygen consumption rate MO_2 are mg O_2 kg^{-1} h^{-1}.

4.2.6. Statistical analyses

Statistical analyses were conducted using GraphPad Prism® version 8.0 for Windows (GraphPad Software, LaJolla, California). The mean MO_2 for each chemical concentration and associated day (7,14, 21 and 28 d) were plotted and assessed using linear regression and two-way ANOVA. Results were assessed for normality prior to testing. Statistical differences in regression lines for chemical concentrations and the negative control was performed to determine differences in consumption throughout the experiment. Two-way ANOVA and a Dunnett's post-test were then conducted to determine differences for each day between MO_2 for each concentration and the negative control. As all experiments were performed on the same day, the negative control MO_2 is the same for all exposure groups. Statistical analyses were not performed between chemical groups and instead a qualitative analysis was completed to assess similarities and differences. Amphipod mortality was assessed by one-way ANOVA and a Dunnett's post-test; polychaete mortality was not statistically assessed due to an N=1 in exposure vessels, therefore a cumulative mortality > 20% was used as an indication of adverse effects from 28-d exposure. A p-value < 0.05 was used to determine statistical significance for all tests.

4.3. Results

4.3.1. Water quality

During the 28-d exposure period, seawater in exposure vessels was 11.5 ± 0.5 °C, with a pH of 7.9 ± 0.3, dissolved oxygen of 7.3 ± 0.6 mg/L and salinity of 28 ± 1.3 ‰. Seawater during the respirometry assay was 12.03 ± 0.4 °C, pH of 7.9 ± 0.3, and salinity 30 ± 0.9 ‰.

4.3.2. *E. estuarius* mortality

Background mean mortality (± standard error of the mean [SEM]) in controls by the end of the 28-d exposure was 2.4 ± 2.4%. There were significant increases in mortality between treatment concentrations and the control when amphipods were exposed to 0.1

µg/kg IVM (11.9 ± 6.8%), 0.5 µg/kg IVM (14.2 ± 6.4%) and 5/0.5 µg/kg EMB/IVM µg/kg (14.2 ± 5.2%). Table 4-1 details the mortality after the 28-d exposure.

4.3.3. *E. estuarius* oxygen consumption

The mean ± SEM value for oxygen consumption rate (MO_2) in *E. estuarius* in control amphipods was 551 ± 10.7 mg O_2 kg^{-1} h^{-1}, with a minimum and maximum MO_2 of 519 mg O_2 kg^{-1} $h^{-1}$and 563 mg O_2 kg^{-1} h^{-1} on d 14 and 21, respectively. Following linear regression of the MO_2 values with time, no significant change was found to occur over time (i.e., slope was zero) ($p = 0.73$, $F = 0.15$). Figure 4-2 shows the baseline (0 µg/kg negative control group) *E. estuarius* MO_2 through the 28-d exposure period and associated regression line.

Exposure to EMB resulted in significantly lower MO_2 values in amphipods for all treatment concentrations compared to controls ($p = 0.02$, $F = 4.56$) as shown in Figure 4-2a. The 0.1 µg/kg EMB treatment group had a mean MO_2 of 449 ± 18.5 mg O_2 kg^{-1} h^{-1}, and analysis of values for each day indicated that only MO_2 on day 21 was significantly lower than the negative control ($p = 0.04$). Conversely, amphipods exposed to 0.5, 1 and 5 EMB µg/kg had significantly decreased mean MO_2 values that also decreased over time. The 0.5, 1 and 5 EMB µg/kg treatment groups had initial MO_2 values of ~ 470 mg O_2 kg^{-1} h^{-1} on d 7 that decreased to approximately 300 mg O_2 kg^{-1} h^{-1} by d 28 and regression lines were not statistically different from one another ($p = 0.42$, $F = 1.0$). When evaluated for each day, significant decreases in MO_2 compared to the negative control were only found on d 21 and 28 ($p = 0.0082$ to < 0.0001) between the 0.5, 1 and 5 EMB treatment groups and the control.

MO_2 in *E. estuarius* decreased over time in all IVM concentrations compared to the negative control ($p = 0.001$, $F = 8.65$) (see Figure 4-2b). The mean MO_2 as measured on d 7 and d 28 decreased from approximately 470 to 300 mg O_2 kg^{-1} h^{-1}, respectively, for all concentrations (0.01, 0.05, 0.1 and 0.5 µg/kg IVM). There were no significant differences in MO_2 between IVM concentrations when analyzed by regression ($p = 0.89$, $F = 0.21$). When analyzed for each day, significant reductions in mean MO_2 compared to the negative control were found on d 14 through d 28 ($p = 0.032$ to < 0.0001).

Exposure to a combination of EMB and IVM resulted in a significantly lower mean MO_2 in the treatment groups for the duration of the exposure when compared to the controls ($p < 0.0001$, $F = 25.3$) (see Figure 4-2c). All combination treatment concentrations

had a zero slope, and therefore did not show a significant decrease over time but were lower than the controls beginning on d 7 of the experiment. The mean ± SEM MO_2 value for combination treatment concentrations ranged from 324 ± 21.2 to 380 ± 17.1 mg O_2 kg^{-1} h^{-1}, compared to a mean value of 551 ± 10.7 mg O_2 kg^{-1} h^{-1} for the negative control. When analyzed for each day, significant reductions in mean MO_2 compared to the negative control were found on d 7 through d 28 ($p = 0.041$ to < 0.0001). There were no statistical differences between MO_2 for each combination chemical concentrations when analyzed by regression ($p = 0.53$, $F = 0.80$).

4.3.4. *N. virens* mortality

Cumulative mortality throughout the 28-d exposure was low for the control *N. virens*, with only 1 polychaete dying in the negative control (16%). An increase in cumulative mortality was observed for *N. virens* when exposed to 0.5 µg/kg IVM (33.3%), 5 µg/kg IVM (33.3%), 0.5/0.5 µg/kg EMB/IVM (33.3%), 1/1 µg/kg EMB/IVM (33.3%) and 5/5 µg/kg EMB/IVM (100%). Note that significant differences in mortality could not be determined, as *N. virens* was exposed individually. 100% mortality occurred in the 5/5 µg/kg EMB/IVM treatment group: this was not expected based on prior range-finding tests. All other treatment concentrations had 0 - 1 deaths (0 - 16%). Table 4-2 in Section 4.5 details the cumulative mortality observed after the 28-d exposure for *N. virens*.

4.3.5. *N. virens* oxygen consumption

The mean ± SEM for MO_2 in the negative control *N. virens* was 123 ± 7.4 mg O_2 kg^{-1} h^{-1} for the duration of the exposure with minimum and maximum values of 108 and 141 mg O_2 kg^{-1} h^{-1}, respectively. Statistical analysis indicated no change in oxygen consumption over time from d 7 to d 28 for the control ($p = 0.73$, $F = 0.12$) . Baseline consumption for the 0 µg/kg negative control and all other treatment concentration MO_2 values throughout the 28-d exposure for *N. virens* can be seen in Figure 4-3.

N. virens exposed to EMB had significantly higher MO_2 values throughout the exposure period in the 1 and 5 µg/kg EMB treatment groups compared to the negative control (see Figure 4-3a) ($p = 0.00012$, $F = 6.83$). MO_2 increased from a mean of 149 and 165 mg O_2 kg^{-1} h^{-1} on d 7 to 278 and 227 mg O_2 kg^{-1} h^{-1} on d 28 for 1 and 5 µg/kg EMB, respectively. The regression lines for 1 and 5 µg/kg EMB treatment groups were not significantly different from each other ($p = 0.57$, $F = 0.38$). A maximum MO_2 of 350 mg O_2 kg^{-1} h^{-1} was also observed on d 14 for the 5 µg/kg EMB treatment group. When analyzed

for each day, *N. virens* had significantly increased mean oxygen consumption values on d 14 ($p < 0.0007$) and d 28 ($p = 0.0043$) compared to the negative control. The MO_2 values in the 0.1 and 0.5 µg/kg EMB treatment groups were not statistically different from the negative control. General observations during the experiment found that most polychaetes appeared healthy throughout the exposure; however, some individuals exposed to the 1 and 5 EMB µg/kg were discoloured and curled in the respirometry chambers on d 14 to 28.

Exposure to IVM similarly showed an increase in MO_2 compared to the negative control over time ($p = 0.003$, $F = 5.13$) (see Figure 4-3b). All IVM treatment concentrations had an increasing trend of MO_2 values from d 7 to d 28 compared to the negative control; however, only the 1 and 5 µg/kg IVM treatment groups were significantly different from the control ($p = 0.0093$, $F = 5.13$). Oxygen consumption for all treatment concentrations increased from approximately 100 to 200 mg O_2 kg^{-1} h^{-1} from d 7 to d 28, respectively. Additional analysis indicated that the 5 µg/kg IVM treatment group was also statistically different from the lower concentration groups of 0.1 and 0.5 µg/kg IVM ($p = 0.0144$, $F = 5.12$), and was not significantly different from 1 µg/kg IVM ($p = 0.05$, $F = 6.52$). Furthermore, *N. virens* had a peak MO_2 of 300 mg O_2 kg^{-1} h^{-1} on d 14 for the 5 µg/kg IVM group. When evaluated for differences on each day, significant differences from the control were found on d 14 to d 28 ($p = 0.02$ to 0.008). Polychaetes displayed abnormal behaviour as early as d 14 for individuals exposed to 1 and 5 µg/kg IVM, which included irregular undulations while swimming, curling in jars, green and swollen tails, inverted bodies (upside down in jars/respirometry mini chambers) and difficulty reborrowing or remaining burrowed in sediment. All other organisms appeared healthy and did not exhibit abnormal locomotory behaviour.

N. virens exposed to a combination of EMB and IVM had significantly increased MO_2 when exposed to 0.5, 1 and 5 µg/kg EMB/IVM compared to the negative control ($p = 0.0022$, $F = 7.58$), while 0.1 µg/kg EMB/IVM was not significant ($p = 0.085$, $F = 4.58$) (see Figure 4-3c). MO_2 in the 0.5 and 1 µg/kg EMB/IVM treatment groups ranged from 200 to 273 mg O_2 kg^{-1} h^{-1} throughout the exposure, compared to the negative control MO_2 value of 120 mg O_2 kg^{-1} h^{-1}. Conversely, the 5 µg/kg EMB/IVM group had MO_2 values of 231 mg O_2 kg^{-1} h^{-1} on d 7, a peak of 384 mg O_2 kg^{-1} h^{-1} on d 14 and 231 mg O_2 kg^{-1} h^{-1} on d 21. Unfortunately, no oxygen consumption was measured on d 28 for the 5 µg/kg EMB/IVM *N. virens* group due to unexpected mortality, as described previously. Regression analysis

of d 7-21 showed no increasing trend for 5 µg/kg EMB/IVM and no differences compared to the 0.5 and 1 µg/kg EMB/IVM best-fit lines. When analyzed for each day, mean MO_2 was significantly different from the negative control on d 14 to d 28. Polychaetes exposed to 0.5, 1 and 5 µg/kg EMB/IVM displayed abnormal behaviour and physical characteristics beginning on d 14 of exposure until the end of the assay. Abnormal behaviour observations included tail discoloration and detachment, swelling of the head and mouth, inverted bodies, as well as impaired locomotion and burrowing activity. All other organisms appeared healthy and did not exhibit abnormal locomotory behaviour.

4.4. Discussion

The aim of the present study was to determine the effects of the anti-sea lice chemotherapeutants SLICE® (AI EMB), IVM and a combination of both on oxygen consumption (MO_2) in the benthic invertebrates *E. estuarius* and *N. virens*. The assays provided a quantitative assessment of relative changes in oxygen consumption resulting from a chronic exposure to avermectin contaminated sediment, in which species-specific changes in MO_2 were observed.

Avermectins are derived from macrocyclic lactones and agonistically bind to both glutamate-gated chloride (GluCl) and γ-aminobutyric acid (GABA) gated chloride ion channels (Arena et al. 1995, McKellar and Benchaous 1996, Wolstenholme 2012). Excitation of chloride channels in the nervous system results in hyperpolarization of nerve cells, which typically results in loss of motor function, paralysis and death. Although avermectins act as ionophores and are understood to have high affinity to GluCl channels, non-specific action can also result in upregulation of a variety other ligand and voltage-gates chloride channels (Zufall 1992, Clark et al. 1995). For example, upregulation of chloride channels can result in apoptosis and osmotic cell death (Heimlich and Cidlowski 2006), therefore necrosis to cells following exposure to IVM and EMB could result in physiological dysfunction, decreasing overall oxygen demand. Novelli et al. (2015) for example, found that Vertimec® (AI abamectin), a drug within the avermectin family, caused gill damage in juvenile zebrafish. Recent research by Juarez et al. (2018) and Park et al. (2020) has also indicated that IVM inhibits the function of mitochondrial complex I, limiting electron movement required for oxidative phosphorylation and ATP generation which would result in cellular stress and/or death potentially also increasing or decreasing overall oxygen demand and MR. Mitochondria are key organelles of eukaryotic cells, best known for their central role in energy homeostasis, however, their failure also affects other cell

functions, such as calcium signaling, lipid, amino acid and steroid metabolism as well as neurotransmitter turnover (Delp et al. 2019). Mitochondrial dysfunctions may consequently have broad effects in different tissues and overall metabolic and physiological functioning. Furthermore, avermectins have been found to inhibit activities of antioxidants, such as super oxide dismutase, induce oxidative stress by inducing generation of reactive oxygen species, as well as inhibit immunological reactions in crustaceans (Huang et al. 2019). Collectively, recent research has shown that avermectins can have broad adverse effects in organisms, resulting in not only paralysis and death, but also sublethal toxicity evident as changes to metabolic functioning and thus respiration.

No significant mortality was observed for *E. estuarius* exposed to the test concentrations of either chemical. Conversely, by d 28 of the experiment, 100% mortality occurred for *N. virens* exposed to the 5 µg/kg EMB/IVM combination, which was not expected based on previous range-finding sub-chronic exposures. It is important to note that *N. virens* were exposed to higher concentrations of IVM (5 µg/kg) compared to *E. estuarius* (0.5 µg/kg), as the amphipods were inherently more sensitive based on previous range-finding tests as well toxicity values reported in the literature. Comparable IVM LC_{50} values in the literature include 22 µg/kg IVM (10-d) and 16.7 µg/kg IVM (28-d) for the amphipod *Corophium volutator* and 17.9 µg/kg IVM (10-d) and 6.8 µg/kg IVM (100-d) for the polychaete *Arenicola marina* (Allen et al. 2007). 10-d EMB LC_{50} values include 153 - 193 µg/kg EMB (McHenery and Mackie 1999, Mayor et al. 2008, Kuo 2010) for amphipods *C. volutator* and *E. estuarius,* and 111 - 1,368 µg/kg EMB for the polychaetes *A. marina* and *Hediste diversicolor* (McHenery and Mackie 1999, Mayor et al. 2008). McBriarty et al. (2017) reported no mortality to *N. virens* after 30-d exposure to 400 µg/kg EMB. The observed 100% mortality for *N. virens* in this experiment to a combination of 5 µg/kg IVM and 5 µg/kg EMB after 28-d is therefore somewhat surprising, as reported values for 28-d exposures or longer had LC_{50} values > 10 µg/kg avermectin. No combination exposures of EMB and IVM have been reported in the literature to compare values; it is possible that IVM and EMB act in an additive or synergistic fashion, which is understandable given that they are both avermectins with a similar mechanism of action.

The measured baseline mean MO_2 for the amphipod *E. estuarius* was 551 mg O_2 kg^{-1} h^{-1} which is similar to reported ranges for MO_2 between 400 and 600 mg O_2 kg^{-1} h^{-1} for other amphipod species, including *Gammarus oceanicus, G. fossarum* and *Bovallia gigantea*, in cold water testing conditions (i.e., 5 - 12 ºC) (Einarson 1993, Simčič T and

Brancelj 2007, Gomes et al. 2014). The baseline mean MO_2 of 123 mg O_2 kg^{-1} h^{-1} observed for *N. virens* is similar to reported ranges for *Nereis sp.* (100 - 150 mg O_2 kg^{-1} h^{-1}) (Kristensen 1983, Nielsen et al. 1995). Differences in species and test conditions does not appear to result in large variations in MO_2 for polychaetes, at temperatures of approximately 8 to 15 ºC. Overall, these previously reported baseline rates validate the baseline rate observed in this experiment under similar test conditions.

E. estuarius displayed a significant decrease in MO_2 when exposed to environmentally relevant concentrations of the anti-sea lice chemotherapeutants SLICE® (AI EMB) and IVM in whole sediment. When exposed to EMB and IVM alone, a significant decreasing trend of MO_2 was observed as the exposure duration lengthened, while exposure to the two chemicals in combination resulted in larger significant decreases in consumption earlier in the exposure with no decreasing trend, indicating that time did not matter once the decrease in MO_2 reached a minimum value. Only EMB exhibited a concentration-dependant relationship for the decline in MO_2. As previously described, LC_{50} values reported in the literature for IVM and EMB are generally between 20 and 200 µg/kg for amphipods, respectively. Therefore, the respirometry measurements proved to be a more sensitive indicator of toxicity since concentrations (ranging from 0.01 - 5 µg/kg avermectin) were well below lethal thresholds. It is important to note that the concentrations of IVM that affected MO_2 were also generally 10-fold lower compared to effective EMB concentrations; *E. estuarius* is clearly more sensitive to IVM in this regard. Additionally, as MO_2 generally did not fall below ~ 300 mg O_2 kg^{-1} h^{-1} for any treatment concentrations through the exposure period, it is suggested that *E. estuarius* may be able to maintain a minimum consumption rate to ensure survival, as no significant increases in mortality were observed with increasing concentrations. Oxygen levels did not fall below 75% saturation in the respirometry mini chambers; therefore, it is unlikely that oxygen availability resulted in the plateau in MO_2 value. The observed decreased and minimum MO_2 for *E. estuarius* were consequently indicative of chemical toxicity. Overall, decreases in respiration rates indicate decreasing metabolism and an inability to tolerate exposure conditions, which is also a predictor for decreased long-term fitness (Maltby 1999, Resgalla et al. 2010).

The observed reduction in MO_2 following exposure to avermectins on *E. estuarius* may be due to inhibition of mitochondrial complexes, cellular necrosis, oxidative damage and inhibited immune response as previously described. Oysters exposed to cadmium,

for example, showed decreased mitochondrial efficiency and decreased respiration (Kurochkin et al. 2011), which supports the association between inhibition of complex I by IVM and observed decreases in MO_2 in this experiment. Amphipod respiration also relies on constant movement of the gills, located on the anterior thoracic legs, for oxygen uptake (Steele and Steele 1991). Thomas et al. (2020) described that the effects of altered GABA receptor function are likely to be widespread invertebrates, including non-behavioral effects, inducing cell differentiation and proliferation, and behavioural effects, such as locomotion as seen in molluscs and crustaceans. Therefore, reduced respiratory rates could also be due to impaired function of the legs *via* paralysis, decreasing gill movement. Further investigation into the effects of avermectins contributing to the decrease in oxygen consumption in *E. estuarius* is required to fully understand the observed respiratory effects.

Compared to amphipods, *N. virens* displayed the opposite effects for MO_2 during the 28-d exposure period. Exposure to the two highest concentrations of EMB and IVM resulted in an increasing MO_2 over time. When exposed in combination, MO_2 remained elevated through the exposure, supporting chemical additivity or synergism when compared to the response from exposure to only one chemotherapeutant. Interestingly, for each of the highest concentrations in each chemical exposure group, a maximum MO_2 was observed on d 14, ranging from 300 - 400 mg O_2 kg^{-1} h^{-1}, which subsequently decreased on d 21. This peak consumption response to exposure may be indicative of a compensatory stress response to cellular and necrotic toxicity following an initial acute exposure (< 14 d), perhaps through stress activated cellular pathways, such as cellular proliferation (Fan and Bergmann 2008) or stress protein production (Ruffin et al. 1994), however, at this time this is only speculation.

N. virens exposed in sediment to SLICE®, IVM and a combination of both displayed an increase in oxygen consumption, as well as adverse locomotory effects at higher concentrations (although these were not quantified). Consequently, the observed sublethal impacts to locomotion and behaviour at higher concentrations is supported by the known mode of action of avermectins. Increased MO_2 rate for *N. virens*, although perhaps contradictory to the observed behaviour impairment and the observed decreased MO_2 for *E. estuarius*, has similarly been observed for polychaetes and other aquatic species in the literature during times of stress (Freitas et al. 2017, Du et al. 2018). Recent work by Freitas et al. (2017) has found that the polychaete *H. diversiscolor* increased

average respiration rate when exposed to mercury compared to control organisms. As whole-body respiration rate is an indirect measure of an organism's maintenance costs, it generally assumed that the metabolic rate should increase with increasing intoxication until irreversible effects impair metabolism itself (Calow 1991). There are thus many potential biochemical and physiological processes that can contribute to an observed increase in MO_2. Increased energy expenditure on defense and repair processes are a common response to aquatic pollutant stress (Maltby 1999) and increasing oxygen uptake may aid in repair of damaged cellular components/tissues (Huang 2018, Bu et al. 2019, Park 2020). Freitas et al. (2017) also found a significant increase in glutathione S-transferase activity, as well as cellular damage measured by lipid peroxidation levels and metabolic activity, assessed by electron transport system activity, suggesting that *H. diversicolor* were initiating defense mechanisms. Polychaetes and other marine invertebrates have also demonstrated glycemic alterations and increased catecholaminergic activity from exposure to pollutants (Carr and Neff 1981, Lacoste et al. 2001), which increase respiration rates during a stress-induced increase in metabolic rate. Production of stress-proteins following exposure to pollutants, such as cadmium, has also been documented for *H. diversicolor* (Ruffin et al. 1994), and zinc exposure to the earthworm *Eisinea andrei* increased overall energy consumption at the cellular level (Świątek and Bednarska 2019), reflecting the high energy demand of the stress response. Given that available energy is a finite resource, increasing production of proteins, metabolic rate and maintenance costs as part of the stress response will mean that fewer resources are available for growth, reproduction and survival (Maltby 1994, Maltby 1999, De Coen and Janssen 2003).

An increase in oxygen consumption may also be due to compensatory changes in oxygen carrier performance (e.g., haemoglobin), ventilation or circulatory adjustments to redistribute or increase blood flow. Previous research by Miller et al. (1976) demonstrated that oxygen consumption in the burrowing shrimp *Callianassa californiensis* is regulated by physiological changes including increased production of respiratory pigments to prolong survival in low oxygen conditions. Du et al. (2018) also found that the bluegill sunfish had increased metabolic rate when exposed to wastewater effluent for 21 d, which was associated with morphological changes to the gill and adjustments in the oxygen transport cascade to increase the gills capacity to unload oxygen to tissues. Polychaetes rely on movement such as swimming, as well as specialized pumping organs in tube dwelling species (e.g., *N. virens*) for oxygen to diffuse through the body wall and parapodia

(gill like structures) into haemoglobin (Rouse and Pleijel 2001). *N. virens* did not display an increase in locomotory activity during the assay, therefore the increase in oxygen consumption would not be due to increased swimming, but could potentially be due to an increased rate of pumping of blood to increase ventilation volume, morphological changes to the parapodia or perhaps an increase in respiratory pigment production. Given that changes to MO_2 occurred generally by d 21 for polychaetes (with a few exceptions) in this study, like Du et al. (2018), this lends some support of increased oxygen demand from the stress response leading to changes in respiratory physiology. Clearly additional work is required to fully understand the molecular and physiological changes associated with increased respiration rates following avermectin exposure.

When exposed to a combination of EMB and IVM, both *E. estuarius* and *N. virens* had enhanced changes to MO_2 (decreases or increases, respectively) and at earlier time points compared to each chemical alone, supporting an additive or synergistic effect. For example, combination exposures exhibited changes to MO_2 for both species as early as d 7, in which *E. estuarius* MO_2 decreased by approximately 200 mg O_2 kg^{-1} h^{-1} (compared to decreases of 100 mg O_2 kg^{-1} h^{-1} for EMB or IVM alone) and *N. virens* increased by 100 mg O_2 kg^{-1} h^{-1} (compared to no change from the control for EMB or IVM alone). As these chemicals are both avermectins, it is reasonable to assume that increased avermectin concentrations in sediment would result in a more toxicity. Avermectin binding to GluCl specifically is understood to be essentially irreversible and non-competitive (Wolstenholme 2012, Cornejo et al. 2014), therefore increased exposure concentrations could simply increase the number of bound receptors until a threshold has been met to illicit toxicity. Avermectin binding is also understood to potentiate GluCl receptors (Cully et al. 1994, Menez et al. 2012), therefore increased avermectin concentrations as IVM and EMB could further increase the extent of hyperpolarization from binding. The differences in magnitude of observed changes are likely due to species differences beyond receptor binding however, especially considering they had opposite respiratory responses from exposure. What remains clear is that exposure to both chemicals resulted in a larger physiological response compared to each chemical alone at similar concentrations, and therefore would likely increase potential risk of toxicity and adverse effects to ecological receptors if both are present in the environment.

In the present study, environmentally relevant concentrations of EMB and IVM that have been found in sediment beneath salmon farm net pens following treatment regimes

were used and are reflective of potential real exposure scenarios. EMB and IVM have high organic partition coefficient values based on their physicochemical properties, therefore these chemicals will bind to organic material and are known to have long sediment half lives in the marine environment (McHenery and Mackie 1999). Concentrations found in the marine environment range between 0.1 - 400 µg avermectin/kg sediment (ERT 1998, Cannavan et al. 2000, Boxall et al. 2002, DFO 2012, Lalonde et al. 2012); in this study, sediment concentrations between 0.01 and 5 µg avermectin/kg were associated with changes in respiration. *N. virens* exposed to a 5 µg/kg combination of EMB and IVM, also had 100% mortality after 28-d exposure. Research has shown that polychaetes are often tolerant of stressful conditions, including changes to temperature, food abundance and presence of pollution (Grassle and Grassle 1974, Chandler et al. 1997, Dean 2008), whereas amphipods may be the most sensitive to toxicity from contaminants and other abiotic changes (Thomas 1993). This experiment demonstrated the sensitivity of both species to avermectins at low concentrations, as well as the potential for consequences to fitness from exposure. As the benthic invertebrate community is important for bioturbation of sediments and influence mobilization and burial of organic matters (Valett et al. 1996, Meysman et al. 2006, Nogaro et al. 2009), adverse effects to the benthic community from avermectin exposure may also impact higher trophic organisms.

The observed adverse effects in this assay supports the inherent toxicity of avermectins, as well as the potential long-term adverse impacts that their presence could have given the toxicity at low concentrations as well as in combination. Physiological parameters are good indicators of the general population health (Maltby 1999, Burton et al. 2011, Cooke et al. 2013, Auer et al. 2015) and prolonged effects on metrics such as oxygen consumption may lead to long-term adverse impacts, such as decreased growth, reproduction and survival from both increases and decreases in MO_2. Consequently, both the decreased MO_2 of *E. estuarius* and increased MO_2 for *N. virens* lends support to potential population/community effects to benthic invertebrates as well as other organisms from sediment contaminated anti-sea lice chemotherapeutants SLICE® and IVM. Further studies are needed to clarify the avermectin concentrations and benthic community health in marine sediment, and whether changes to MO_2 translate to field scenarios.

4.5. References

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4.6. Tables

Table 4-1. Mortality results for the amphipod, *Eohaustorius estuarius*, throughout the 28-d exposure period for the respirometry assay. Animals were placed into jars containing whole sediment spiked with sea lice chemotherapeutant (emamectin benzoate [EMB] from SLICE® premix, ivermectin [IVM] or a combination of both [EMB/IVM]) at environmentally relevant concentrations. N=7 amphipods per jar, N=6 jars per concentration. **Bold** indicates significant increases in mortality between treatment concentrations and the control ($p > 0.05$).

Exposure Group and Treatment Concentration (µg/kg)	28-d Cumulative Mortality (x/42)	% Mean Mortality ± SEM
0	1	2.4 ± 2.4
EMB 0.1	1	2.4 ± 2.4
EMB 0.5	3	7.1 ± 4.9
EMB 1	2	4.7 ± 4.8
EMB 5	4	9.5 ± 4.8
IVM 0.01	2	4.7 ± 3.0
IVM 0.05	0	0
IVM 0.1	**5**	**11.9 ± 6.8**
IVM 0.5	**6**	**14.2 ± 6.4**
EMB/IVM 0.1/0.01	1	2.4 ± 2.4
EMB/IVM 0.5/0.05	3	7.1 ± 4.9
EMB/IVM 1/0.1	4	9.5 ± 4.8
EMB/IVM 5/0.5	**6**	**14.2 ± 5.2**

Table 4-2. Morality results for the polycheate, *Nereis virens*, throughout the 28-d exposure period for the respirometry assay. Animals were placed into jars containing whole sediment spiked with sea lice chemotherapeutant (emamectin benzoate [EMB] from SLICE® 0.2% Premix, ivermectin [IVM] or a combination of both [EMB/IVM]) at environmentally relevant concentrations. N=1 polychaete per jar; N=6 jars per concentration (total of 6); **Bold** = > 20% cumulative mortality.

Exposure Group and Treatment Concentration (µg/kg)	28-d Cumulative Mortality (x/6)	% Cumulative Mortality
0	1	16.7
EMB 0.1	0	0
EMB 0.5	0	0
EMB 1	1	16.7
EMB 5	0	0
IVM 0.1	1	16.7
IVM 0.5	**2**	**33.3**
IVM 1	1	16.7
IVM 5	**2**	**33.3**
EMB/IVM 0.1/0.1	0	0
EMB/IVM 0.5/0.5	**2**	**33.3**
EMB/IVM 1/1	**2**	**33.3**
EMB/IVM 5/5	**6**	**100**

4.7. Figures

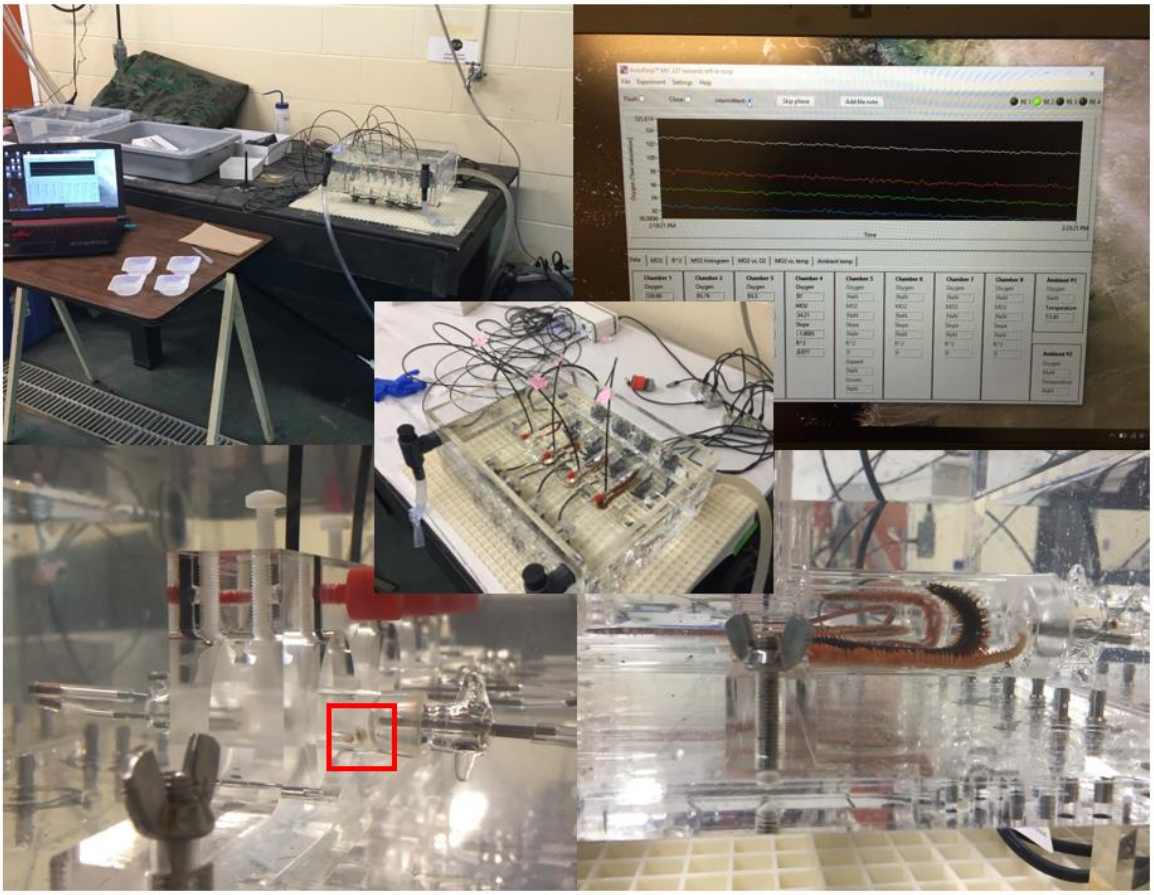

Figure 4-1. Respirometry system overview. Set up with software (top left), software output during test (top right), mini chamber system in water bath (center) and test organisms in mini chambers during tests (amphipods [*Eohaustarius estuarius*] in the red square on bottom left and polychaetes [*Nereis virens]* on bottom right).

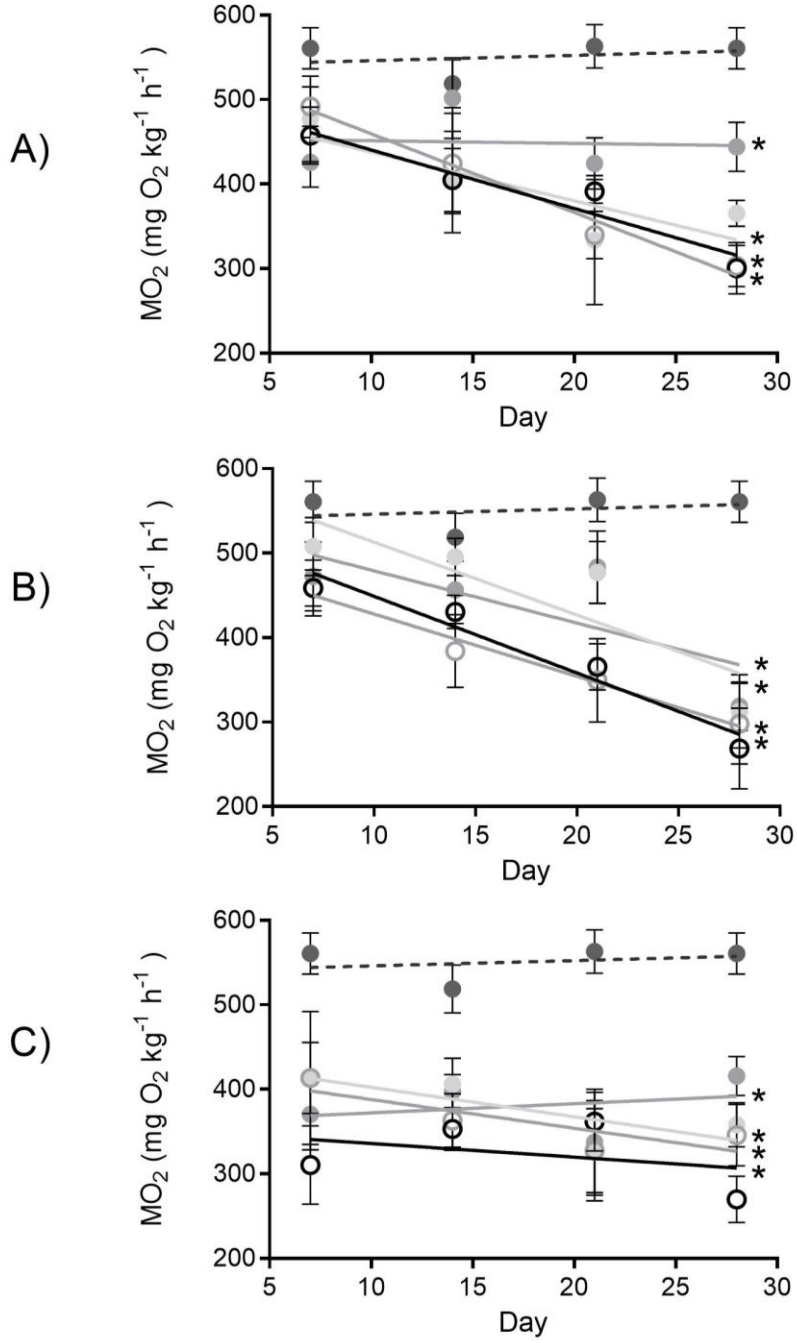

Figure 4-2. Mean oxygen consumption rates (MO_2) (± 1 SEM) of the amphipod, *Eohaustorius estuarius,* as measured on day 7, 14, 21 and 28 following exposure to emamectin benzoate (EMB), prepared from SLICE 0.2% premix, ivermectin (IVM) or a combination of both (N=4). Amphipods were exposed in jars via whole sediment throughout the duration of the test to: **(A)** 0 (●), 0.1 (●), 0.5 (●), 1 (○) or 5 (○) µg/kg EMB; **(B)** 0 (●), 0.01 (●), 0.05 (●), 0.1 (○) or 0.5 (○) µg/kg IVM; or **(C)** 0 (●), 0.1/0.01 (●), 0.5/0.05 (●), 1/0.1 (○) or 5/0.5 (○) µg/kg EMB/IVM. Mean MO_2 was assessed for statistical differences by linear regression ($p < 0.05$). Lines statistically different from the negative control (0 µg/kg, ● and dashed line) are indicated by an asterisk (∗).

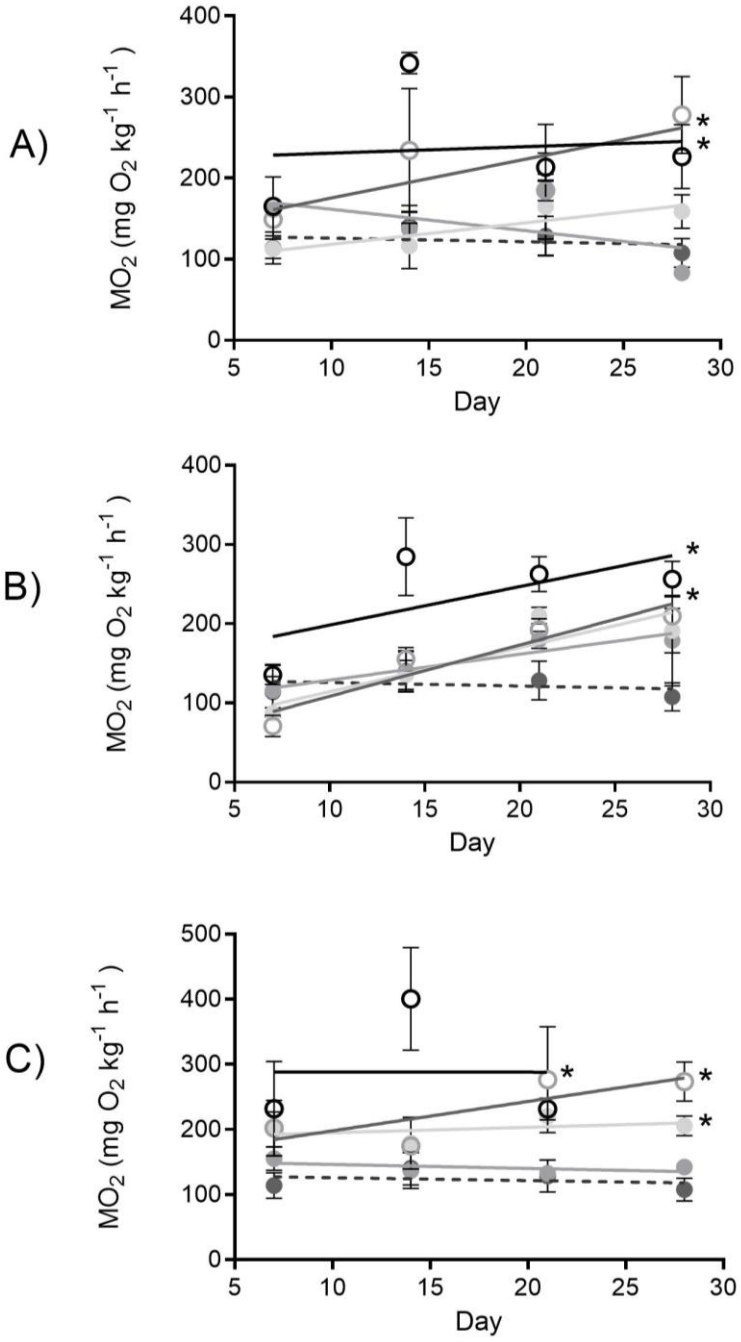

Figure 4-3. Mean oxygen consumption rates (MO_2) (± 1 SEM) of the polychaete, *Nereis virens,* as measured on day 7, 14, 21 and 28 following exposure to emamectin benzoate (EMB), prepared from SLICE 0.2% premix, ivermectin (IVM) or a combination of both (N=3). Polychaetes were exposed in jars via whole sediment throughout the duration of the experiment to 0 (●), 0.1 (●), 0.5 (●), 1 (○) or 5 µg/kg (○) of **(A)** EMB, **(B)** IVM, or **(C)** a 1:1 combination of both. Mean MO_2 was assessed for statistical differences by linear regression ($p < 0.05$). Lines statistically different from the negative control (0 µg/kg, ● and dashed line) are indicated by an asterisk (∗).

Chapter 5. Conclusions and Future Research

This thesis examined the effects of two anti-sea lice chemotherapeutants on marine benthic and pelagic invertebrates using environmentally relevant concentrations. Three experiments were conducted: first, evaluation of the effects on fertilization success in Pacific purple sea urchin (*Strongylocentrotus purpuratus*) gametes following exposure to Salmosan® and Paramove®50; second, an assessment of avoidance behaviour and sublethal behavioural toxicity in the amphipod *Eohaustorius estuarius* and polychaete *Nereis virens* exposed to ivermectin (IVM) and emamectin benzoate (EMB) (from SLICE® 0.2% Premix) in whole sediment; and third, measurement of oxygen consumption rates in *E. estuarius* and *N. virens* during a subchronic exposure to IVM and EMB in whole sediment.

Paramove®50 was found to be acutely toxic (success of sea urchin gamete fertilization) at very low concentrations, whereas Salmosan® was only marginally toxic at the highest concentration tested. In an avoidance assay, *E. estuarius* and *N. virens* displayed avoidance to IVM and a combination of IVM and EMB; however, *N. virens* only avoided contaminated sediment after adverse effects to behaviour were observed. This suggests that avoidance was a secondary response to chemical exposure in this species. Exposure to low concentrations of IVM and EMB had significant effects on oxygen consumption rates (MO_2), with a decrease and increase in MO_2 for *E. estuarius* and *N. virens*, respectively. Collectively, these experiments demonstrated adverse lethal and sublethal effects to marine invertebrates at environmentally relevant concentrations of anti-sea lice chemotherapeutants.

5.1. Future research

Information gaps remain in the assessment of anti-sea lice chemotherapeutant toxicity to indigenous non-target species in the Pacific Northwest marine environment of Canada. Sensitivity as a result of different life stages could be assessed for these species. Although a portion of the planktonic life stage of the Pacific purple sea urchin was assessed, this did not include the larval planktonic form, which would have markedly different physiological traits, likely resulting in different toxicological outcomes compared to gametes. Reproduction and developmental assays could also be performed for these benthic invertebrate species, to further evaluate potential long-term population impacts.

As SLICE® and IVM are applied as in-feed anti-sea lice treatments, it may be worthwhile to conduct experiments using a feed-coated exposure method to be more realistic and given that organic enrichment is understood to attract some benthic species (Findlay et al. 1995, Tefler et al 2006, Neofitou et al. 2010). Recovery experiments, where organisms are exposed and then transferred to clean sediment, would also aid in understanding if avoidance behaviour even after toxicity could result in prolonged effects. Ultimately, field studies such as benthic surveys beneath active aquaculture facilities or perhaps plankton tows in the water column at near-field and far-field locations would be the best method of quantifying the impacts associated with anti-sea lice chemotherapeutic treatment. Characterization of chemical concentrations and their distribution in sediment would also provide valuable information regarding the extent of contamination in what is understood to be a heterogenous media.

5.2. Ecological implications and relevance to risk assessment

There are concerns that pest management practices at Atlantic salmon aquaculture facilities may result in adverse effects to the surrounding marine environment, largely due to the open net pen systems used to house the fish. The results of this thesis provide evidence for potential adverse effects to non-target species from both water bath and in-feed treatments based on realistic exposures. However, it is important to consider the factors outside of a laboratory setting that can also influence chemical concentrations and toxicity, such as ocean currents, temperature, organic carbon content and acid volatile sulfides in sediment. With respect to persistence, the water bath treatment pesticides Salmosan® and Paramove®50 will primarily decrease in concentration from dilution and are understood to degrade after approximately 1 week (Burridge and Van Geest 2014). Field and modeling studies have indicated that concentrations are approximately 100-fold less within a 1000 km radius of release a few hours after treatment (Burridge et al. 2000, Ernst et al. 2014). As demonstrated in this thesis however, adverse acute effects are possible even at concentrations following dilution. Given the strict regulatory control over anti-sea lice chemotherapeutant application, such as two pulse treatments per day per aquaculture facility (Health Canada 2017), it is unlikely that long-term adverse impacts from Salmosan® or Paramove®50 to aquatic species would take place as chemical concentrations would be low and degrade quickly (Burridge and Van Geest 2014).

Conversely, the same physicochemical principles and nature of dilution do not apply to the in-feed treatments, IVM and SLICE® (AI EMB), which have been found at measurable concentrations in sediment 1 year after treatment (Tefler et a. 2006). In this thesis, adverse effects including changes to MO_2, locomotory behaviour and mortality, were observed at environmentally relevant concentrations in sediment. These results support the likelihood of toxicity to non-target benthic species within the vicinity of aquaculture sites. The ecological implications could therefore extend beyond the species investigated in this thesis and result in population or community level effects, as well as impacts to higher trophic species.

The assessment of risk to the environment from the use of avermectins as a treatment for sea lice infestation in Atlantic salmon farms is complex and requires further investigation from field studies to fully assess the potential long-term effects. Currently in Canada, only SLICE® is applied as an in-feed anti-sea lice treatment. Therefore, to fully understand the risk to non-target species, sediment EMB concentrations and benthos community metrics beneath net pens in BC should be quantified. Additionally, Canada does not have ecological guidelines developed for any of the current use anti-sea lice chemotherapeutants at this time. Therefore, even under strict treatment regimes, given that a reference concentration protective of most marine species is not available, assessment of ecological risk cannot be fully elucidated at active aquaculture facilities. Scotland, which is one of the worlds largest farmed Atlantic salmon producers, has derived an ecological standard for EMB of 0.012 µg/kg, which is actively applied during monitoring programs (SEPA 2017). Derivation of ecological guidelines in Canada would ultimately aid in maintaining a sustainable aquaculture industry and healthy coastal marine ecosystems. The sub-lethal concentrations that resulted in adverse effects in this thesis for each of the anti-sea lice chemotherapeutants could be used in support of the development of aquaculture guidelines specific to the Canadian marine ecosystems.

5.3. References

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Appendices

Appendix I: Avoidance Assay Statistical Analyses – Polychaetes Multiple Comparisons

Appendix II: Oxygen Consumption two-way ANOVA Statistical Analyses

Appendix I. Avoidance Assay Polychaete Statistical Analyses

Polychaetes Multiple Comparisons

Emamectin Benzoate

Naïve EMB Avoidance

Table Analyzed	EMB Avoidance				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	14.42	0.9470	ns	No	
Day	5.884	0.4985	ns	No	
Concentration	3.603	0.5114	ns	No	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.4315	24	0.01798	F (24, 70) = 0.5529	P=0.9470
Day	0.1760	6	0.02933	F (6, 70) = 0.9021	P=0.4985
Concentration	0.1078	4	0.02695	F (4, 70) = 0.8287	P=0.5114
Residual	2.276	70	0.03251		
Data summary					
Number of columns (Concentration)	5				
Number of rows (Day)	7				
Number of values	105				
Row 1 vs. Row 7	-0.1478	-0.3210 to 0.02544	No	ns	0.1226

Within each row, compare columns (simple effects within rows)					
Number of families	7				
Number of comparisons per family	4				
Alpha	0.05				

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Row 1					
0 vs. 0.5	0.05556	-0.3123 to 0.4235	No	ns	0.9871
0 vs. 5	-0.05555	-0.4235 to 0.3124	No	ns	0.9871
0 vs. 50	0.1667	-0.2012 to 0.5346	No	ns	0.6191
0 vs. 200	0.1667	-0.2012 to 0.5346	No	ns	0.6191
Row 2					
0 vs. 0.5	-3.333e-008	-0.3679 to 0.3679	No	ns	>0.9999
0 vs. 5	0.1111	-0.2568 to 0.4790	No	ns	0.8646
0 vs. 50	0.1111	-0.2568 to 0.4790	No	ns	0.8647
0 vs. 200	0.1111	-0.2568 to 0.4790	No	ns	0.8647
Row 3					
0 vs. 0.5	1.110e-005	-0.3679 to 0.3679	No	ns	>0.9999
0 vs. 5	2.110e-005	-0.3679 to 0.3679	No	ns	>0.9999
0 vs. 50	-0.1111	-0.4790 to 0.2568	No	ns	0.8648
0 vs. 200	0.05556	-0.3123 to 0.4235	No	ns	0.9871

Row 4					
0 vs. 0.5	0.1111	-0.2568 to 0.4790	No	ns	0.8647
0 vs. 5	0.1111	-0.2568 to 0.4790	No	ns	0.8647
0 vs. 50	0.1667	-0.2012 to 0.5346	No	ns	0.6191
0 vs. 200	0.03333	-0.3346 to 0.4012	No	ns	0.9980
Row 5					
0 vs. 0.5	0.000	-0.3679 to 0.3679	No	ns	>0.9999
0 vs. 5	-0.1111	-0.4790 to 0.2568	No	ns	0.8647
0 vs. 50	0.1667	-0.2012 to 0.5346	No	ns	0.6191
0 vs. 200	0.1444	-0.2235 to 0.5123	No	ns	0.7249
Row 6					
0 vs. 0.5	1.233e-006	-0.3679 to 0.3679	No	ns	>0.9999
0 vs. 5	-0.06666	-0.4346 to 0.3012	No	ns	0.9749
0 vs. 50	0.1111	-0.2568 to 0.4790	No	ns	0.8647
0 vs. 200	-0.05556	-0.4235 to 0.3123	No	ns	0.9871
Row 7					
0 vs. 0.5	-0.07221	-0.4401 to 0.2957	No	ns	0.9668
0 vs. 5	0.02777	-0.3401 to 0.3957	No	ns	0.9990
0 vs. 50	-0.01663	-0.3845 to 0.3513	No	ns	0.9998
0 vs. 200	-0.1500	-0.5179 to 0.2179	No	ns	0.6989

Naïve EMB Burrowing

Table Analyzed	EMB Burrowing
Friedman test	
P value	0.0838
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	5
Friedman statistic	8.220

Chronic EMB Avoidance

Table Analyzed	Chronic EMB Avoidance				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant ?	
Interaction	18.63	0.8057	ns	No	
Day	9.498	0.0864	ns	No	
Concentration	2.737	0.6509	ns	No	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.6370	30	0.02123	F (30, 84) = 0.7547	P=0.8057
Day	0.3247	6	0.05411	F (6, 84) = 1.923	P=0.0864
Concentration	0.09357	5	0.01871	F (5, 84) = 0.6652	P=0.6509
Residual	2.363	84	0.02813		
Data summary					
Number of columns (Concentration)	6				
Number of rows (Day)	7				
Number of values	126				
Within each row, compare columns (simple effects within rows)					
Number of families	7				
Number of comparisons per family	5				
Alpha	0.05				
Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant ?	Summary	Adjusted P Value
Row 1					
CC vs. 0	-0.06667	-0.4175 to 0.2842	No	ns	0.9847

CC vs. 0.5	-0.1333	-0.4842 to 0.2175	No	ns	0.7913
CC vs. 5	-0.2000	-0.5509 to 0.1509	No	ns	0.4519
CC vs. 50	-0.2167	-0.5675 to 0.1342	No	ns	0.3755
CC vs. 200	-0.2333	-0.5842 to 0.1175	No	ns	0.3070
Row 2					
CC vs. 0	-0.1167	-0.4675 to 0.2342	No	ns	0.8634
CC vs. 0.5	-0.05000	-0.4009 to 0.3009	No	ns	0.9957
CC vs. 5	-0.03333	-0.3842 to 0.3175	No	ns	0.9996
CC vs. 50	0.03333	-0.3175 to 0.3842	No	ns	0.9996
CC vs. 200	-0.01667	-0.3675 to 0.3342	No	ns	0.9998
Row 3					
CC vs. 0	-0.05000	-0.4009 to 0.3009	No	ns	0.9957
CC vs. 0.5	0.03333	-0.3175 to 0.3842	No	ns	0.9996
CC vs. 5	-0.2000	-0.5509 to 0.1509	No	ns	0.4519
CC vs. 50	-0.05000	-0.4009 to 0.3009	No	ns	0.9957
CC vs. 200	0.1167	-0.2342 to 0.4675	No	ns	0.8634
Row 4					
CC vs. 0	-0.05000	-0.4009 to 0.3009	No	ns	0.9957
CC vs. 0.5	0.1167	-0.2342 to 0.4675	No	ns	0.8634
CC vs. 5	-0.03333	-0.3842 to 0.3175	No	ns	0.9996
CC vs. 50	0.03333	-0.3175 to 0.3842	No	ns	0.9996
CC vs. 200	0.1000	-0.2509 to 0.4509	No	ns	0.9207
Row 5					
CC vs. 0	0.1000	-0.2509 to 0.4509	No	ns	0.9207
CC vs. 0.5	0.08333	-0.2675 to 0.4342	No	ns	0.9609
CC vs. 5	0.1833	-0.1675 to 0.5342	No	ns	0.5351
CC vs. 50	-0.06667	-0.4175 to 0.2842	No	ns	0.9847

CC vs. 200	0.1667	-0.1842 to 0.5175	No	ns	0.6220
Row 6					
CC vs. 0	0.1667	-0.1842 to 0.5175	No	ns	0.6220
CC vs. 0.5	0.1000	-0.2509 to 0.4509	No	ns	0.9207
CC vs. 5	0.1000	-0.2509 to 0.4509	No	ns	0.9207
CC vs. 50	0.000	-0.3509 to 0.3509	No	ns	>0.9999
CC vs. 200	-0.05000	-0.4009 to 0.3009	No	ns	0.9957
Row 7					
CC vs. 0	0.1000	-0.2509 to 0.4509	No	ns	0.9207
CC vs. 0.5	0.03333	-0.3175 to 0.3842	No	ns	0.9996
CC vs. 5	-0.1167	-0.4675 to 0.2342	No	ns	0.8634
CC vs. 50	-0.05000	-0.4009 to 0.3009	No	ns	0.9957
CC vs. 200	-0.03333	-0.3842 to 0.3175	No	ns	0.9996

Chronic EMB Burrowing

Table Analyzed	Chronic EMB Burrowing
Friedman test	
P value	0.1851
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	6
Friedman statistic	7.515

Ivermectin

Naïve IVM Avoidance

Table Analyzed	Ivermectin Avoidance				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant ?	

Interaction	15.07	0.4240	ns	No	
Day	27.23	<0.0001	****	Yes	
Concentration	15.72	0.0002	***	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.7279	24	0.03033	F (24, 70) = 1.047	P=0.4240
Day	1.315	6	0.2191	F (6, 70) = 7.566	P<0.0001
Concentration	0.7592	4	0.1898	F (4, 70) = 6.553	P=0.0002
Residual	2.028	70	0.02897		
Data summary					
Number of columns (Concentration)	5				
Number of rows (Day)	7				
Number of values	105				
Within each row, compare columns (simple effects within rows)					
Number of families	7				
Number of comparisons per family	4				
Alpha	0.05				
Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant ?	Summary	Adjusted P Value
Row 1					
0.5 vs. 0	1.333e-005	-0.3472 to 0.3473	No	ns	>0.9999
5 vs. 0	-0.05555	-0.4028 to 0.2917	No	ns	0.9840
50 vs. 0	-0.05555	-0.4028 to 0.2917	No	ns	0.9840
200 vs. 0	-0.1111	-0.4583 to 0.2362	No	ns	0.8400
Row 2					
0.5 vs. 0	-0.1111	-0.4584 to 0.2361	No	ns	0.8400
5 vs. 0	-1.000e-005	-0.3473 to 0.3472	No	ns	>0.9999
50 vs. 0	0.1111	-0.2361 to 0.4584	No	ns	0.8400
200 vs. 0	0.1667	-0.1806 to 0.5139	No	ns	0.5715
Row 3					
0.5 vs. 0	-0.1668	-0.5140 to 0.1805	No	ns	0.5710
5 vs. 0	-0.1889	-0.5362 to 0.1583	No	ns	0.4627
50 vs. 0	-0.02222	-0.3695 to 0.3250	No	ns	0.9994

200 vs. 0	-0.07780	-0.4250 to 0.2694	No	ns	0.9477
Row 4					
0.5 vs. 0	-0.1667	-0.5139 to 0.1806	No	ns	0.5718
5 vs. 0	-0.1334	-0.4806 to 0.2139	No	ns	0.7395
50 vs. 0	0.1000	-0.2472 to 0.4472	No	ns	0.8827
200 vs. 0	-0.02224	-0.3695 to 0.3250	No	ns	0.9994
Row 5					
0.5 vs. 0	-0.1778	-0.5250 to 0.1695	No	ns	0.5162
5 vs. 0	-0.1333	-0.4806 to 0.2139	No	ns	0.7395
50 vs. 0	0.1110	-0.2362 to 0.4582	No	ns	0.8404
200 vs. 0	-0.05557	-0.4028 to 0.2917	No	ns	0.9840
Row 6					
0.5 vs. 0	0.1223	-0.2249 to 0.4696	No	ns	0.7913
5 vs. 0	0.04454	-0.3027 to 0.3918	No	ns	0.9931
50 vs. 0	0.3668	0.01953 to 0.7140	Yes	*	0.0352
200 vs. 0	0.4223	0.07509 to 0.7696	Yes	*	0.0120
Row 7					
0.5 vs. 0	0.06667	-0.2806 to 0.4139	No	ns	0.9692
5 vs. 0	0.1000	-0.2472 to 0.4472	No	ns	0.8827
50 vs. 0	0.4667	0.1194 to 0.8139	Yes	**	0.0047
200 vs. 0	0.3667	0.01942 to 0.7139	Yes	*	0.0353

Naïve IVM Burrowing

Table Analyzed	Ivermectin Burrowing				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant ?	
Interaction	27.24	<0.0001	****	Yes	

Day	16.18	<0.0001	****	Yes	
Concentration	42.73	<0.0001	****	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	3.269	24	0.1362	F (24, 70) = 5.733	P<0.0001
Day	1.942	6	0.3237	F (6, 70) = 13.62	P<0.0001
Concentration	5.127	4	1.282	F (4, 70) = 53.96	P<0.0001
Residual	1.663	70	0.02376		
Data summary					
Number of columns (Concentration)	5				
Number of rows (Day)	7				
Number of values	105				
Within each row, compare columns (simple effects within rows)					
Number of families	7				
Number of comparisons per family	4				
Alpha	0.05				
Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Row 1					
0 vs. 0.5	0.000	-0.3145 to 0.3145	No	ns	>0.9999
0 vs. 5	0.05556	-0.2589 to 0.3700	No	ns	0.9771
0 vs. 50	-0.05556	-0.3700 to 0.2589	No	ns	0.9771
0 vs. 200	-1.000e-006	-0.3145 to 0.3145	No	ns	>0.9999
Row 2					
0 vs. 0.5	1.000e-006	-0.3145 to 0.3145	No	ns	>0.9999
0 vs. 5	0.05556	-0.2589 to 0.3700	No	ns	0.9771
0 vs. 50	-0.1111	-0.4256 to 0.2034	No	ns	0.7897
0 vs. 200	0.05556	-0.2589 to 0.3700	No	ns	0.9771
Row 3					
0 vs. 0.5	0.06667	-0.2478 to 0.3811	No	ns	0.9565
0 vs. 5	0.06667	-0.2478 to 0.3811	No	ns	0.9565
0 vs. 50	-0.1556	-0.4700 to 0.1589	No	ns	0.5460

0 vs. 200	-0.1000	-0.4145 to 0.2145	No	ns	0.8428
Row 4					
0 vs. 0.5	-0.06667	-0.3811 to 0.2478	No	ns	0.9565
0 vs. 5	0.000	-0.3145 to 0.3145	No	ns	>0.9999
0 vs. 50	-0.4556	-0.7700 to -0.1411	Yes	**	0.0021
0 vs. 200	-0.3333	-0.6478 to -0.01882	Yes	*	0.0344
Row 5					
0 vs. 0.5	-0.06667	-0.3811 to 0.2478	No	ns	0.9565
0 vs. 5	0.000	-0.3145 to 0.3145	No	ns	>0.9999
0 vs. 50	-0.6889	-1.003 to -0.3744	Yes	****	<0.0001
0 vs. 200	-0.8111	-1.126 to -0.4966	Yes	****	<0.0001
Row 6					
0 vs. 0.5	-0.06667	-0.3811 to 0.2478	No	ns	0.9565
0 vs. 5	0.000	-0.3145 to 0.3145	No	ns	>0.9999
0 vs. 50	-0.8889	-1.203 to -0.5744	Yes	****	<0.0001
0 vs. 200	-0.9333	-1.248 to -0.6189	Yes	****	<0.0001
Row 7					
0 vs. 0.5	-0.05557	-0.3700 to 0.2589	No	ns	0.9771
0 vs. 5	0.000	-0.3145 to 0.3145	No	ns	>0.9999
0 vs. 50	-0.8889	-1.203 to -0.5744	Yes	****	<0.0001
0 vs. 200	-0.9333	-1.248 to -0.6189	Yes	****	<0.0001

Chronic IVM Avoidance

Table Analyzed	Chronic IVM Avoidance				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant ?	
Interaction	20.02	0.1064	ns	No	

Day	6.502	0.0409	*	Yes	
Concentration	34.10	<0.0001	****	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	1.252	30	0.04173	F (30, 84) = 1.423	P=0.1064
Day	0.4067	6	0.06778	F (6, 84) = 2.312	P=0.0409
Concentration	2.133	5	0.4266	F (5, 84) = 14.55	P<0.0001
Residual	2.463	84	0.02932		
Data summary					
Number of columns (Concentration)	6				
Number of rows (Day)	7				
Number of values	126				
Within each row, compare columns (simple effects within rows)					
Number of families	7				
Number of comparisons per family	5				
Alpha	0.05				
Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant ?	Summary	Adjusted P Value
Row 1					
0 vs. CC	0.1667	-0.1915 to 0.5249	No	ns	0.6400
0.5 vs. CC	0.01667	-0.3415 to 0.3749	No	ns	0.9998
5 vs. CC	0.06667	-0.2915 to 0.4249	No	ns	0.9861
50 vs. CC	0.3333	-0.02486 to 0.6915	No	ns	0.0769
200 vs. CC	0.1833	-0.1749 to 0.5415	No	ns	0.5544
Row 2					
0 vs. CC	0.03333	-0.3249 to 0.3915	No	ns	0.9996
0.5 vs. CC	-0.1167	-0.4749 to 0.2415	No	ns	0.8726
5 vs. CC	0.1333	-0.2249 to 0.4915	No	ns	0.8039
50 vs. CC	0.2333	-0.1249 to 0.5915	No	ns	0.3257
200 vs. CC	0.1833	-0.1749 to 0.5415	No	ns	0.5544
Row 3					
0 vs. CC	-0.03333	-0.3915 to 0.3249	No	ns	0.9996

0.5 vs. CC	-0.2667	-0.6249 to 0.09153	No	ns	0.2122
5 vs. CC	-0.2000	-0.5582 to 0.1582	No	ns	0.4719
50 vs. CC	0.06667	-0.2915 to 0.4249	No	ns	0.9861
200 vs. CC	0.1167	-0.2415 to 0.4749	No	ns	0.8726
Row 4					
0 vs. CC	-0.1833	-0.5415 to 0.1749	No	ns	0.5544
0.5 vs. CC	-0.2444	-0.6026 to 0.1138	No	ns	0.2841
5 vs. CC	-0.2667	-0.6249 to 0.09153	No	ns	0.2122
50 vs. CC	0.01667	-0.3415 to 0.3749	No	ns	0.9998
200 vs. CC	0.03333	-0.3249 to 0.3915	No	ns	0.9996
Row 5					
0 vs. CC	0.03333	-0.3249 to 0.3915	No	ns	0.9996
0.5 vs. CC	-0.1111	-0.4693 to 0.2471	No	ns	0.8923
5 vs. CC	-0.1567	-0.5149 to 0.2015	No	ns	0.6912
50 vs. CC	-0.01667	-0.3749 to 0.3415	No	ns	0.9998
200 vs. CC	0.1667	-0.1915 to 0.5249	No	ns	0.6400
Row 6					
0 vs. CC	-0.03333	-0.3915 to 0.3249	No	ns	0.9996
0.5 vs. CC	0.1556	-0.2026 to 0.5138	No	ns	0.6969
5 vs. CC	-0.06667	-0.4249 to 0.2915	No	ns	0.9861
50 vs. CC	0.5167	0.1585 to 0.8749	Yes	**	0.0018
200 vs. CC	0.6000	0.2418 to 0.9582	Yes	***	0.0002
Row 7					
0 vs. CC	-0.05000	-0.4082 to 0.3082	No	ns	0.9960
0.5 vs. CC	0.1556	-0.2026 to 0.5138	No	ns	0.6969
5 vs. CC	-0.02333	-0.3815 to 0.3349	No	ns	0.9997
50 vs. CC	0.3000	-0.05820 to 0.6582	No	ns	0.1309

200 vs. CC	0.5333	0.1751 to 0.8915	Yes	**	0.0012

Chronic IVM Burrowing

Table Analyzed	Chronic Ivermectin Burrowing				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	17.40	0.0110	*	Yes	
Day	6.517	0.0033	**	Yes	
Concentration	50.59	<0.0001	****	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	1.637	30	0.05458	F (30, 84) = 1.911	P=0.0110
Day	0.6131	6	0.1022	F (6, 84) = 3.579	P=0.0033
Concentration	4.760	5	0.9520	F (5, 84) = 33.34	P<0.0001
Residual	2.399	84	0.02855		
Data summary					
Number of columns (Concentration)	6				
Number of rows (Day)	7				
Number of values	126				
Within each row, compare columns (simple effects within rows)					
Number of families	7				
Number of comparisons per family	5				
Alpha	0.05				
Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Row 1					
0 vs. Jar Control	0.4167	0.06319 to 0.7701	Yes	*	0.0147
0.5 vs. Jar Control	0.5000	0.1465 to 0.8535	Yes	**	0.0023
5 vs. Jar Control	0.5333	0.1799 to 0.8868	Yes	**	0.0010
50 vs. Jar Control	0.5000	0.1465 to 0.8535	Yes	**	0.0023
200 vs. Jar Control	0.3000	-0.05348 to 0.6535	No	ns	0.1231
Row 2					

0 vs. Jar Control	0.5833	0.2299 to 0.9368	Yes	***	0.0003
0.5 vs. Jar Control	0.4500	0.09652 to 0.8035	Yes	**	0.0072
5 vs. Jar Control	0.6000	0.2465 to 0.9535	Yes	***	0.0002
50 vs. Jar Control	0.5667	0.2132 to 0.9201	Yes	***	0.0005
200 vs. Jar Control	0.2833	-0.07015 to 0.6368	No	ns	0.1588
Row 3					
0 vs. Jar Control	0.2833	-0.07015 to 0.6368	No	ns	0.1588
0.5 vs. Jar Control	0.3778	0.02429 to 0.7312	Yes	*	0.0318
5 vs. Jar Control	0.3778	0.02429 to 0.7312	Yes	*	0.0318
50 vs. Jar Control	0.3167	-0.03681 to 0.6701	No	ns	0.0943
200 vs. Jar Control	0.3667	0.01319 to 0.7201	Yes	*	0.0393
Row 4					
0 vs. Jar Control	0.3500	-0.003481 to 0.7035	No	ns	0.0533
0.5 vs. Jar Control	0.4148	0.06132 to 0.7683	Yes	*	0.0153
5 vs. Jar Control	0.5777	0.2242 to 0.9311	Yes	***	0.0003
50 vs. Jar Control	0.5500	0.1965 to 0.9035	Yes	***	0.0007
200 vs. Jar Control	0.5667	0.2132 to 0.9201	Yes	***	0.0005
Row 5					
0 vs. Jar Control	0.5000	0.1465 to 0.8535	Yes	**	0.0023
0.5 vs. Jar Control	0.4889	0.1354 to 0.8424	Yes	**	0.0030
5 vs. Jar Control	0.5777	0.2242 to 0.9311	Yes	***	0.0003
50 vs. Jar Control	0.5500	0.1965 to 0.9035	Yes	***	0.0007
200 vs. Jar Control	0.8500	0.4965 to 1.203	Yes	****	<0.0001
Row 6					
0 vs. Jar Control	0.2833	-0.07015 to 0.6368	No	ns	0.1588
0.5 vs. Jar Control	0.2444	-0.1090 to 0.5979	No	ns	0.2726
5 vs. Jar Control	0.3777	0.02419 to 0.7311	Yes	*	0.0319

50 vs. Jar Control	0.5500	0.1965 to 0.9035	Yes	***	0.0007
200 vs. Jar Control	0.7833	0.4299 to 1.137	Yes	****	<0.0001
Row 7					
0 vs. Jar Control	0.3667	0.01319 to 0.7201	Yes	*	0.0393
0.5 vs. Jar Control	0.4889	0.1354 to 0.8424	Yes	**	0.0030
5 vs. Jar Control	0.5110	0.1575 to 0.8645	Yes	**	0.0018
50 vs. Jar Control	0.7000	0.3465 to 1.053	Yes	****	<0.0001
200 vs. Jar Control	1.000	0.6465 to 1.353	Yes	****	<0.0001

Combination (Ivermectin and Emamectin Benzoate)

Naïve Combo Avoidance

Table Analyzed	Combo Avoidance				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	23.34	0.0107	*	Yes	
Day	14.96	0.0002	***	Yes	
concentration	28.54	<0.0001	****	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	1.183	24	0.04930	F (24, 70) = 2.052	P=0.0107
Day	0.7586	6	0.1264	F (6, 70) = 5.263	P=0.0002
concentration	1.447	4	0.3617	F (4, 70) = 15.06	P<0.0001
Residual	1.682	70	0.02402		
Data summary					
Number of columns (concentration)	5				
Number of rows (Day)	7				
Number of values	105				

Within each row, compare columns (simple effects within rows)					
Number of families	7				

Number of comparisons per family	4				
Alpha	0.05				
Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Row 1					
0.5 vs. 0	-0.05556	-0.3718 to 0.2607	No	ns	0.9776
5 vs. 0	0.1666	-0.1496 to 0.4829	No	ns	0.4910
50 vs. 0	0.05553	-0.2607 to 0.3718	No	ns	0.9777
200 vs. 0	0.05553	-0.2607 to 0.3718	No	ns	0.9776
Row 2					
0.5 vs. 0	-0.04445	-0.3607 to 0.2718	No	ns	0.9901
5 vs. 0	0.1111	-0.2052 to 0.4273	No	ns	0.7930
50 vs. 0	0.1667	-0.1496 to 0.4829	No	ns	0.4907
200 vs. 0	0.3333	0.01710 to 0.6496	Yes	*	0.0357
Row 3					
0.5 vs. 0	-0.01112	-0.3274 to 0.3051	No	ns	0.9999
5 vs. 0	0.1333	-0.1829 to 0.4496	No	ns	0.6746
50 vs. 0	-0.1445	-0.4607 to 0.1718	No	ns	0.6125
200 vs. 0	0.1556	-0.1607 to 0.4718	No	ns	0.5508
Row 4					
0.5 vs. 0	0.07778	-0.2385 to 0.3940	No	ns	0.9286
5 vs. 0	0.2667	-0.04958 to 0.5829	No	ns	0.1221
50 vs. 0	0.04442	-0.2718 to 0.3607	No	ns	0.9901
200 vs. 0	0.4333	0.1171 to 0.7496	Yes	**	0.0039
Row 5					
0.5 vs. 0	0.1222	-0.1940 to 0.4385	No	ns	0.7353
5 vs. 0	-0.1334	-0.4496 to 0.1829	No	ns	0.6745

50 vs. 0	0.03332	-0.2829 to 0.3496	No	ns	0.9967
200 vs. 0	0.3556	0.03931 to 0.6718	Yes	*	0.0226
Row 6					
0.5 vs. 0	-0.02778	-0.3440 to 0.2885	No	ns	0.9983
5 vs. 0	0.1000	-0.2162 to 0.4162	No	ns	0.8453
50 vs. 0	0.4333	0.1171 to 0.7496	Yes	**	0.0039
200 vs. 0	0.4111	0.09487 to 0.7274	Yes	**	0.0065
Row 7					
0.5 vs. 0	0.000	-0.3162 to 0.3162	No	ns	>0.9999
5 vs. 0	0.03332	-0.2829 to 0.3496	No	ns	0.9967
50 vs. 0	0.3667	0.05043 to 0.6829	Yes	*	0.0178
200 vs. 0	0.5222	0.2060 to 0.8385	Yes	***	0.0004

Naïve Combo Burrowing

Table Analyzed	Combo Burrowing				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	21.12	<0.0001	****	Yes	
Day	13.81	<0.0001	****	Yes	
concentration	56.25	<0.0001	****	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	2.503	24	0.1043	F (24, 70) = 6.984	P<0.0001
Day	1.636	6	0.2726	F (6, 70) = 18.26	P<0.0001
concentration	6.663	4	1.666	F (4, 70) = 111.6	P<0.0001
Residual	1.045	70	0.01493		
Data summary					
Number of columns (concentration)	5				
Number of rows (Day)	7				
Number of values	105				

Within each row, compare columns (simple effects within rows)					
Number of families	7				
Number of comparisons per family	4				
Alpha	0.05				
Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant ?	Summary	Adjusted P Value
Row 1					
0.5 vs. 0	0.05556	-0.1937 to 0.3049	No	ns	0.9486
5 vs. 0	0.000	-0.2493 to 0.2493	No	ns	>0.9999
50 vs. 0	0.000	-0.2493 to 0.2493	No	ns	>0.9999
200 vs. 0	0.1111	-0.1382 to 0.3604	No	ns	0.6322
Row 2					
0.5 vs. 0	0.000	-0.2493 to 0.2493	No	ns	>0.9999
5 vs. 0	0.000	-0.2493 to 0.2493	No	ns	>0.9999
50 vs. 0	0.05556	-0.1937 to 0.3049	No	ns	0.9486
200 vs. 0	0.3333	0.08403 to 0.5826	Yes	**	0.0050
Row 3					
0.5 vs. 0	-0.1110	-0.3603 to 0.1383	No	ns	0.6329
5 vs. 0	-0.1666	-0.4159 to 0.08274	No	ns	0.2840
50 vs. 0	-0.05544	-0.3047 to 0.1939	No	ns	0.9488
200 vs. 0	0.4779	0.2286 to 0.7272	Yes	****	<0.0001
Row 4					
0.5 vs. 0	-0.1111	-0.3604 to 0.1382	No	ns	0.6321
5 vs. 0	-0.2222	-0.4715 to 0.02707	No	ns	0.0941
50 vs. 0	0.1111	-0.1382 to 0.3604	No	ns	0.6322
200 vs. 0	0.7222	0.4729 to 0.9715	Yes	****	<0.0001
Row 5					
0.5 vs. 0	0.1667	-0.08264 to 0.4160	No	ns	0.2835
5 vs. 0	0.05556	-0.1937 to 0.3049	No	ns	0.9486

50 vs. 0	0.5556	0.3063 to 0.8048	Yes	****	<0.0001
200 vs. 0	0.8778	0.6285 to 1.127	Yes	****	<0.0001
Row 6					
0.5 vs. 0	0.05556	-0.1937 to 0.3049	No	ns	0.9486
5 vs. 0	0.000	-0.2493 to 0.2493	No	ns	>0.9999
50 vs. 0	0.5000	0.2507 to 0.7493	Yes	****	<0.0001
200 vs. 0	0.8223	0.5730 to 1.072	Yes	****	<0.0001
Row 7					
0.5 vs. 0	0.000	-0.2493 to 0.2493	No	ns	>0.9999
5 vs. 0	0.05556	-0.1937 to 0.3049	No	ns	0.9486
50 vs. 0	0.8889	0.6396 to 1.138	Yes	****	<0.0001
200 vs. 0	1.000	0.7507 to 1.249	Yes	****	<0.0001

Chronic Combo Avoidance

Table Analyzed	Combo 5 ug/kg Avoidance
Two-way ANOVA	Ordinary
Alpha	0.05

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	22.66	0.0103	*	Yes
Day	2.829	0.3131	ns	No
concentration	41.58	<0.0001	****	Yes

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	1.631	30	0.05437	F (30, 84) = 1.926	P=0.0103
Day	0.2037	6	0.03394	F (6, 84) = 1.203	P=0.3131
concentration	2.993	5	0.5986	F (5, 84) = 21.21	P<0.0001

Residual	2.371	84	0.02822

Within each row, compare columns (simple effects within rows)					
Number of families	7				
Number of comparisons per family	5				
Alpha	0.05				
Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Row 1					
0 vs. CC	0.1111	-0.2403 to 0.4625	No	ns	0.8849
0.5 vs. CC	0.1111	-0.2403 to 0.4625	No	ns	0.8849
5 vs. CC	0.2667	-0.08475 to 0.6181	No	ns	0.1976
50 vs. CC	0.1000	-0.2514 to 0.4514	No	ns	0.9211
200 vs. CC	-0.02222	-0.3737 to 0.3292	No	ns	0.9997
Row 2					
0 vs. CC	0.1556	-0.1959 to 0.5070	No	ns	0.6816
0.5 vs. CC	0.1000	-0.2514 to 0.4514	No	ns	0.9212
5 vs. CC	0.1667	-0.1848 to 0.5181	No	ns	0.6235
50 vs. CC	0.2778	-0.07363 to 0.6292	No	ns	0.1683
200 vs. CC	0.02223	-0.3292 to 0.3737	No	ns	0.9997
Row 3					
0 vs. CC	0.2333	-0.1181 to 0.5848	No	ns	0.3084
0.5 vs. CC	0.1778	-0.1737 to 0.5292	No	ns	0.5653
5 vs. CC	0.1111	-0.2403 to 0.4625	No	ns	0.8849
50 vs. CC	0.2889	-0.06252 to 0.6403	No	ns	0.1424
200 vs. CC	0.08891	-0.2625 to 0.4403	No	ns	0.9497
Row 4					
0 vs. CC	0.1000	-0.2514 to 0.4514	No	ns	0.9211
0.5 vs. CC	0.2778	-0.07362 to 0.6292	No	ns	0.1682

5 vs. CC	0.2556	-0.09585 to 0.6070	No	ns	0.2306
50 vs. CC	0.2778	-0.07363 to 0.6292	No	ns	0.1683
200 vs. CC	0.1000	-0.2514 to 0.4514	No	ns	0.9211
Row 5					
0 vs. CC	0.1667	-0.1847 to 0.5181	No	ns	0.6233
0.5 vs. CC	0.1111	-0.2403 to 0.4626	No	ns	0.8849
5 vs. CC	0.3389	-0.01252 to 0.6903	No	ns	0.0626
50 vs. CC	0.3667	0.01527 to 0.7181	Yes	*	0.0377
200 vs. CC	-0.2111	-0.5625 to 0.1403	No	ns	0.4016
Row 6					
0 vs. CC	0.1111	-0.2403 to 0.4626	No	ns	0.8849
0.5 vs. CC	0.04447	-0.3070 to 0.3959	No	ns	0.9974
5 vs. CC	0.4445	0.09304 to 0.7959	Yes	**	0.0077
50 vs. CC	0.5889	0.2375 to 0.9403	Yes	***	0.0002
200 vs. CC	-0.2778	-0.6292 to 0.07367	No	ns	0.1683
Row 7					
0 vs. CC	0.1000	-0.2514 to 0.4514	No	ns	0.9212
0.5 vs. CC	0.3666	0.01514 to 0.7180	Yes	*	0.0378
5 vs. CC	0.6111	0.2597 to 0.9626	Yes	***	0.0001
50 vs. CC	0.5278	0.1764 to 0.8792	Yes	**	0.0011
200 vs. CC	-0.2778	-0.6292 to 0.07366	No	ns	0.1683

Chronic Combo Burrowing

Table Analyzed	Combo 5 ug.kg Burrowing				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	5.280	0.2704	ns	No	
Day	3.104	0.0040	**	Yes	

Concentration	79.12	<0.0001	****	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.7576	30	0.02525	F (30, 84) = 1.183	P=0.2704
Day	0.4454	6	0.07424	F (6, 84) = 3.479	P=0.0040
Concentration	11.35	5	2.271	F (5, 84) = 106.4	P<0.0001
Residual	1.793	84	0.02134		
Within each row, compare columns (simple effects within rows)					
Number of families	7				
Number of comparisons per family	5				
Alpha	0.05				
Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Row 1					
0 vs. CC	0.1889	-0.1167 to 0.4945	No	ns	0.3745
0.5 vs. CC	0.1333	-0.1723 to 0.4389	No	ns	0.6931
5 vs. CC	0.2667	-0.03892 to 0.5723	No	ns	0.1077
50 vs. CC	0.4333	0.1277 to 0.7389	Yes	**	0.0023
200 vs. CC	0.7556	0.4500 to 1.061	Yes	****	<0.0001
Row 2					
0 vs. CC	0.07778	-0.2278 to 0.3834	No	ns	0.9485
0.5 vs. CC	0.1444	-0.1611 to 0.4500	No	ns	0.6263
5 vs. CC	0.2667	-0.03892 to 0.5723	No	ns	0.1077
50 vs. CC	0.3889	0.08329 to 0.6945	Yes	**	0.0073
200 vs. CC	0.7556	0.4500 to 1.061	Yes	****	<0.0001
Row 3					
0 vs. CC	0.1333	-0.1723 to 0.4389	No	ns	0.6931
0.5 vs. CC	0.2000	-0.1056 to 0.5056	No	ns	0.3214
5 vs. CC	0.1889	-0.1167 to 0.4945	No	ns	0.3745
50 vs. CC	0.3111	0.005514 to 0.6167	Yes	*	0.0445

200 vs. CC	0.9444	0.6389 to 1.250	Yes	****	<0.0001
Row 4					
0 vs. CC	0.06667	-0.2389 to 0.3723	No	ns	0.9724
0.5 vs. CC	0.01111	-0.2945 to 0.3167	No	ns	0.9999
5 vs. CC	0.1500	-0.1556 to 0.4556	No	ns	0.5928
50 vs. CC	0.3222	0.01664 to 0.6278	Yes	*	0.0351
200 vs. CC	0.9444	0.6389 to 1.250	Yes	****	<0.0001
Row 5					
0 vs. CC	0.000	-0.3056 to 0.3056	No	ns	>0.9999
0.5 vs. CC	0.1444	-0.1611 to 0.4500	No	ns	0.6263
5 vs. CC	0.3833	0.07775 to 0.6889	Yes	**	0.0083
50 vs. CC	0.6056	0.3000 to 0.9111	Yes	****	<0.0001
200 vs. CC	0.9444	0.6389 to 1.250	Yes	****	<0.0001
Row 6					
0 vs. CC	0.1889	-0.1167 to 0.4945	No	ns	0.3745
0.5 vs. CC	0.2667	-0.03892 to 0.5723	No	ns	0.1077
5 vs. CC	0.3611	0.05551 to 0.6667	Yes	*	0.0144
50 vs. CC	0.5611	0.2555 to 0.8667	Yes	****	<0.0001
200 vs. CC	1.000	0.6944 to 1.306	Yes	****	<0.0001
Row 7					
0 vs. CC	0.1333	-0.1723 to 0.4389	No	ns	0.6931
0.5 vs. CC	0.1444	-0.1611 to 0.4500	No	ns	0.6263
5 vs. CC	0.3611	0.05552 to 0.6667	Yes	*	0.0144
50 vs. CC	0.8333	0.5278 to 1.139	Yes	****	<0.0001
200 vs. CC	0.9444	0.6389 to 1.250	Yes	****	<0.0001

Appendix II. Oxygen Consumption Statistical Analyses

Amphipod

Emamectin Benzoate

Table Analyzed	EMB				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	12.78	0.1201	ns	No	
Day	10.95	0.0022	**	Yes	
Concentration	36.01	<0.0001	****	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	110302	12	9192	F (12, 60) = 1.586	P=0.1201
Day	94511	3	31504	F (3, 60) = 5.437	P=0.0022
Concentration	310916	4	77729	F (4, 60) = 13.42	P<0.0001
Residual	347627	60	5794		

Within each row, compare columns (simple effects within rows)					
Number of families	4				
Number of comparisons per family	4				
Alpha	0.05				
Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Row 1					
0 vs. EMB 0.1	135.0	-0.06459 to 270.0	No	ns	0.0501
0 vs. EMB 0.5	83.43	-51.59 to 218.4	No	ns	0.3479
0 vs. EMB 1	68.70	-66.31 to 203.7	No	ns	0.5190
0 vs. EMB 5	103.1	-31.96 to 238.1	No	ns	0.1823
Row 2					
0 vs. EMB 0.1	17.18	-117.8 to 152.2	No	ns	0.9932
0 vs. EMB 0.5	116.6	-18.47 to 251.6	No	ns	0.1093
0 vs. EMB 1	94.47	-40.55 to 229.5	No	ns	0.2455

0 vs. EMB 5	114.1	-20.92 to 249.1	No	ns	0.1204
Row 3					
0 vs. EMB 0.1	138.6	3.616 to 273.7	Yes	*	0.0424
0 vs. EMB 0.5	229.4	94.40 to 364.4	Yes	***	0.0003
0 vs. EMB 1	223.3	88.27 to 358.3	Yes	***	0.0004
0 vs. EMB 5	171.8	36.74 to 306.8	Yes	**	0.0082
Row 4					
0 vs. EMB 0.1	116.6	-18.47 to 251.6	No	ns	0.1093
0 vs. EMB 0.5	195.1	60.05 to 330.1	Yes	**	0.0023
0 vs. EMB 1	257.6	122.6 to 392.7	Yes	****	<0.0001
0 vs. EMB 5	260.1	125.1 to 395.1	Yes	****	<0.0001

Ivermectin

Table Analyzed	IVM				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	10.67	0.0963	ns	No	
Row Factor	22.82	<0.0001	****	Yes	
Column Factor	34.59	<0.0001	****	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	100666	12	8389	F (12, 60) = 1.672	P=0.0963
Row Factor	215295	3	71765	F (3, 60) = 14.30	P<0.0001
Column Factor	326312	4	81578	F (4, 60) = 16.26	P<0.0001
Residual	301033	60	5017		

Within each row, compare columns (simple effects within rows)

Number of families	4
Number of comparisons per family	4
Alpha	0.05

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Row 1					
0 vs. IVM 0.01	88.33	-37.31 to 214.0	No	ns	0.2418
0 vs. IVM 0.05	52.75	-72.89 to 178.4	No	ns	0.6751

0 vs. IVM 0.1	101.8	-23.82 to 227.5	No	ns	0.1441
0 vs. IVM 0.5	101.8	-23.82 to 227.5	No	ns	0.1441
Row 2					
0 vs. IVM 0.01	62.57	-63.07 to 188.2	No	ns	0.5376
0 vs. IVM 0.05	23.31	-102.3 to 149.0	No	ns	0.9723
0 vs. IVM 0.1	135.0	9.309 to 260.6	Yes	*	0.0315
0 vs. IVM 0.5	88.33	-37.31 to 214.0	No	ns	0.2418
Row 3					
0 vs. IVM 0.01	79.75	-45.90 to 205.4	No	ns	0.3250
0 vs. IVM 0.05	85.88	-39.76 to 211.5	No	ns	0.2638
0 vs. IVM 0.1	213.5	87.83 to 339.1	Yes	***	0.0003
0 vs. IVM 0.5	197.5	71.88 to 323.2	Yes	***	0.0008
Row 4					
0 vs. IVM 0.01	241.7	116.0 to 367.3	Yes	****	<0.0001
0 vs. IVM 0.05	247.8	122.2 to 373.5	Yes	****	<0.0001
0 vs. IVM 0.1	262.5	136.9 to 388.2	Yes	****	<0.0001
0 vs. IVM 0.5	292.0	166.3 to 417.6	Yes	****	<0.0001

Combination Exposure

Table Analyzed	Combo				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	6.812	0.6023	ns	No	
Row Factor	1.280	0.5939	ns	No	
Column Factor	51.73	<0.0001	****	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	65083	12	5424	F (12, 60) = 0.8477	P=0.6023
Row Factor	12232	3	4077	F (3, 60) = 0.6373	P=0.5939
Column Factor	494304	4	123576	F (4, 60) = 19.32	P<0.0001
Residual	383871	60	6398		

Within each row, compare columns (simple effects within rows)					
Number of families	4				
Number of comparisons per family	4				
Alpha	0.05				
Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Row 1					
0 vs. Combo 0.01/0.1	190.2	48.28 to 332.0	Yes	**	0.0050
0 vs. Combo 0.05/0.5	147.2	5.340 to 289.1	Yes	*	0.0397
0 vs. Combo 0.1/1	147.2	5.340 to 289.1	Yes	*	0.0397
0 vs. Combo 0.5/5	250.3	108.4 to 392.2	Yes	***	0.0002
Row 2					
0 vs. Combo 0.01/0.1	121.5	-20.42 to 263.3	No	ns	0.1136
0 vs. Combo 0.05/0.5	112.9	-29.01 to 254.8	No	ns	0.1555
0 vs. Combo 0.1/1	155.8	13.93 to 297.7	Yes	*	0.0270
0 vs. Combo 0.5/5	165.6	23.74 to 307.5	Yes	*	0.0171
Row 3					
0 vs. Combo 0.01/0.1	225.7	83.86 to 367.6	Yes	***	0.0007
0 vs. Combo 0.05/0.5	235.6	93.67 to 377.4	Yes	***	0.0004
0 vs. Combo 0.1/1	235.6	93.67 to 377.4	Yes	***	0.0004
0 vs. Combo 0.5/5	201.2	59.32 to 343.1	Yes	**	0.0028
Row 4					
0 vs. Combo 0.01/0.1	144.8	2.886 to 286.7	Yes	*	0.0442
0 vs. Combo 0.05/0.5	202.4	60.55 to 344.3	Yes	**	0.0026
0 vs. Combo 0.1/1	214.7	72.82 to 356.6	Yes	**	0.0013
0 vs. Combo 0.5/5	290.8	148.9 to 432.6	Yes	****	<0.0001

Polychaetes

Emamectin Benzoate

Table Analyzed	Emb
Two-way ANOVA	Ordinary
Alpha	0.05

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	22.34	0.0584	ns	No
Row Factor	6.021	0.1167	ns	No
Column Factor	33.25	<0.0001	****	Yes

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	82490	12	6874	F (12, 40) = 1.939	P=0.0584
Row Factor	22234	3	7411	F (3, 40) = 2.091	P=0.1167
Column Factor	122763	4	30691	F (4, 40) = 8.659	P<0.0001
Residual	141779	40	3544		

Within each row, compare columns (simple effects within rows)

Number of families	4
Number of comparisons per family	4
Alpha	0.05

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Row 1					
0 vs. EMB 0.1	-47.08	-170.7 to 76.53	No	ns	0.7341
0 vs. EMB 0.5	1.122	-122.5 to 124.7	No	ns	>0.9999
0 vs. EMB 1	-35.35	-159.0 to 88.27	No	ns	0.8785
0 vs. EMB 5	-51.24	-174.9 to 72.38	No	ns	0.6756
Row 2					
0 vs. EMB 0.1	3.293	-120.3 to 126.9	No	ns	>0.9999
0 vs. EMB 0.5	23.90	-99.72 to 147.5	No	ns	0.9661
0 vs. EMB 1	-93.52	-217.1 to 30.10	No	ns	0.1845

0 vs. EMB 5	-201.4	-325.0 to -77.74	Yes	***	0.0007
Row 3					
0 vs. EMB 0.1	-57.17	-180.8 to 66.45	No	ns	0.5906
0 vs. EMB 0.5	-36.50	-160.1 to 87.12	No	ns	0.8663
0 vs. EMB 1	-56.57	-180.2 to 67.05	No	ns	0.5992
0 vs. EMB 5	-85.15	-208.8 to 38.47	No	ns	0.2522
Row 4					
0 vs. EMB 0.1	24.39	-99.23 to 148.0	No	ns	0.9636
0 vs. EMB 0.5	-51.11	-174.7 to 72.50	No	ns	0.6775
0 vs. EMB 1	-170.0	-293.6 to -46.40	Yes	**	0.0043
0 vs. EMB 5	-118.9	-242.5 to 4.705	No	ns	0.0623

Ivermectin

Table Analyzed	Ivm
Two-way ANOVA	Ordinary
Alpha	0.05

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	13.62	0.1173	ns	No
day	28.20	<0.0001	****	Yes
concentration	30.62	<0.0001	****	Yes

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	37183	12	3099	F (12, 40) = 1.647	P=0.1173
day	76970	3	25657	F (3, 40) = 13.64	P<0.0001
concentration	83580	4	20895	F (4, 40) = 11.11	P<0.0001
Residual	75257	40	1881		

Within each row, compare columns (simple effects within rows)	
Number of families	4
Number of comparisons per family	4
Alpha	0.05

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Row 1					
0 vs. IVM 0.1	-1.925	-91.99 to 88.14	No	ns	>0.9999
0 vs. IVM 0.5	26.92	-63.14 to 117.0	No	ns	0.8614
0 vs. IVM 1	42.79	-47.28 to 132.9	No	ns	0.5683
0 vs. IVM 5	-21.65	-111.7 to 68.41	No	ns	0.9292
Row 2					
0 vs. IVM 0.1	0.9990	-89.07 to 91.06	No	ns	>0.9999
0 vs. IVM 0.5	4.910	-85.15 to 94.97	No	ns	0.9998
0 vs. IVM 1	-14.82	-104.9 to 75.24	No	ns	0.9808
0 vs. IVM 5	-144.5	-234.5 to -54.40	Yes	***	0.0008
Row 3					
0 vs. IVM 0.1	-51.56	-141.6 to 38.51	No	ns	0.4068
0 vs. IVM 0.5	-82.10	-172.2 to 7.961	No	ns	0.0828
0 vs. IVM 1	-64.03	-154.1 to 26.04	No	ns	0.2284
0 vs. IVM 5	-134.5	-224.6 to -44.48	Yes	**	0.0018

Row 4					
0 vs. IVM 0.1	-71.06	-161.1 to 19.00	No	ns	0.1575
0 vs. IVM 0.5	-83.92	-174.0 to 6.146	No	ns	0.0741
0 vs. IVM 1	-102.3	-192.3 to -12.19	Yes	*	0.0217
0 vs. IVM 5	-148.8	-238.9 to -58.74	Yes	***	0.0006

Combination (Ivermectin and Emamectin benzoate) – day 7, 14 and 21

Table Analyzed	Combo day 21
Two-way ANOVA	Ordinary
Alpha	0.05

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	19.29	0.1816	ns	No
Day	0.3830	0.8845	ns	No
concentration	33.70	0.0021	**	Yes

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	73440	8	9180	F (8, 30) = 1.551	P=0.1816
Day	1458	2	729.0	F (2, 30) = 0.1232	P=0.8845
concentration	128270	4	32067	F (4, 30) = 5.420	P=0.0021
Residual	177509	30	5917		

Within each row, compare columns (simple effects within rows)	
Number of families	3
Number of comparisons per family	4
Alpha	0.05

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value

Row 1					
0 vs. Combo 0.1	-84.29	-246.2 to 77.65	No	ns	0.4814
0 vs. Combo 0.5	-86.21	-248.1 to 75.72	No	ns	0.4618
0 vs. Combo 1	-88.42	-250.4 to 73.51	No	ns	0.4397
0 vs. Combo 5	-117.8	-279.7 to 44.15	No	ns	0.2073
Row 2					
0 vs. Combo 0.1	3.512	-158.4 to 165.4	No	ns	>0.9999
0 vs. Combo 0.5	-38.46	-200.4 to 123.5	No	ns	0.9285
0 vs. Combo 1	-34.16	-196.1 to 127.8	No	ns	0.9516
0 vs. Combo 5	-243.7	-405.7 to -81.81	Yes	**	0.0020
Row 3					
0 vs. Combo 0.1	-4.332	-166.3 to 157.6	No	ns	>0.9999
0 vs. Combo 0.5	-91.69	-253.6 to 70.25	No	ns	0.4082
0 vs. Combo 1	-148.2	-310.1 to 13.75	No	ns	0.0804
0 vs. Combo 5	-103.2	-265.1 to 58.77	No	ns	0.3084

Combination (Ivermectin and Emamectin benzoate) – No 5 µg/kg – day 7-28

Table Analyzed	Combo no 5
Two-way ANOVA	Ordinary
Alpha	0.05

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	13.56	0.4051	ns	No
Row Factor	2.999	0.5502	ns	No
Column Factor	38.71	0.0002	***	Yes

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	30023	9	3336	F (9, 32) = 1.078	P=0.4051
Row Factor	6640	3	2213	F (3, 32) = 0.7151	P=0.5502
Column Factor	85719	3	28573	F (3, 32) = 9.232	P=0.0002
Residual	99037	32	3095		

Within each row, compare columns (simple effects within rows)					
Number of families	4				
Number of comparisons per family	3				
Alpha	0.05				
Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Row 1					
0 vs. Combo 0.1	-84.29	-196.3 to 27.72	No	ns	0.1746
0 vs. Combo 0.5	-86.21	-198.2 to 25.79	No	ns	0.1613
0 vs. Combo 1	-88.42	-200.4 to 23.58	No	ns	0.1470
Row 2					
0 vs. Combo 0.1	3.512	-108.5 to 115.5	No	ns	0.9996
0 vs. Combo 0.5	-38.46	-150.5 to 73.55	No	ns	0.7335
0 vs. Combo 1	-34.16	-146.2 to 77.84	No	ns	0.7939
Row 3					
0 vs. Combo 0.1	-4.332	-116.3 to 107.7	No	ns	0.9994
0 vs. Combo 0.5	-91.69	-203.7 to 20.32	No	ns	0.1279
0 vs. Combo 1	-148.2	-260.2 to -36.18	Yes	**	0.0073
Row 4					
0 vs. Combo 0.1	-34.53	-146.5 to 77.47	No	ns	0.7889
0 vs. Combo 0.5	-98.15	-210.2 to 13.85	No	ns	0.0962
0 vs. Combo 1	-165.8	-277.8 to -53.82	Yes	**	0.0026