

The biological effects of emamectin benzoate (SLICE[®]) on spot prawn (*Pandalus platyceros*)

by

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B.Sc., University of Victoria, 2007

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Supervisory Committee

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Abstract

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British Columbia salmon aquaculture operations use the chemotherapeutant emamectin benzoate (EMB trade name SLICE[®]), a synthesized avermectin compound, delivered through feed to decrease sea lice (*Lepeophtheirus salmonis*) parasite abundance on production fish. Avermectins bind to ion channels in crustaceans and disrupts nerve impulse transmission. Detectable amounts of EMB can accumulate in the depositional area around farms during SLICE[®] treatment periods, thus presenting potential for exposure to populations of proximate non-target species. The distribution of spot prawn (*Pandalus platyceros*), an economically important crustacean, overlaps with areas of intensive salmon farm activity. The primary objective of this research was to determine if EMB exposure had a measurable biological effect on spot prawns in the field and in the laboratory. The field component was conducted in the Broughton Archipelago, BC, to determine if emamectin benzoate residues could be detected near actively treating salmon farms, and whether farm proximity affected spot prawn size distribution. Three laboratory experiments tested the mortality, molting and behavioural response of spot prawns to SLICE[®] feed pellet exposure and acute exposure to EMB through sediment over ten, 30 and 45-day durations.

Measurable amounts of EMB was detected in the marine sediment near five farm sites during the field survey and was found to persist between treatment periods. Male and transitional stage spot prawns captured near farm sites attained a greater size and had better body condition compared to reference sites, indicating prawns may benefit from direct or indirect farm food subsidies. However, at several farm sites the size distribution of prawns changed over the sampling period, a trend not observed at reference sites, demonstrating that farm activity may alter prawn population dynamics. Laboratory results indicated that only prawns that had been starved prior to exposure would initially

consume SLICE[®] pellets, but feeding rates declined with subsequent exposures. Depressed consumption rates was not a residual effect of EMB, but rather an aversion to the SLICE[®] pellet diet as prawns resumed feeding when offered a preferred diet. Sediment EMB exposures to doses 808 $\mu\text{g kg}^{-1}$ and greater increased prawn mortality, largely due to the inability of molting individuals to successfully complete ecdysis. Exposed individuals accumulated EMB in their abdomen tissue with levels increasing with exposure dose. Prawns exposed to EMB through sediment at concentrations 1419 and 3330 $\mu\text{g kg}^{-1}$ displayed a significant reduction in olfactory detection and orientation behaviours to food stimuli.

This research highlights that spot prawns may avoid SLICE[®] pellets for preferential food sources, and that only short term EMB exposure 50 to 200 magnitude greater than levels present in the marine environment elicited a measurable response in spot prawn mortality rates, molting success and behaviour. However, preliminary trends in the field survey data indicate that there may be population differences occurring in spot prawns inhabiting areas near treating salmon farms that are not observed in reference populations. These results signify the inherent pitfalls in current management policy that base decisions on short-term acute toxicity laboratory exposure results that may not be indicative of the response of marine populations near active salmon farms to long-term chronic EMB exposure.

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Chapter 1. General Introduction

1.0 Fisheries and aquaculture

Seafood is one of the world's most valuable and globalized commodities. An exponentially increasing human population (United Nations 2011) and rising per capita seafood consumption is increasing demand for fishery products. From 1998 to 2008 there has been a 50 percent global increase in seafood exports and in 2011 seafood exports were valued over US \$125 billion (FAO 2010; 2012). Today over seven billion people rely on fish to provide more than 15 percent of their animal protein intake, and in poorer coastal areas this can rise to 90 percent (FAO 2010; 2012). Global capture fisheries have not been able to keep pace with this rising worldwide demand as the viability of marine ecosystems and fisheries have become undermined due to over-exploitation, habitat degradation, pollution, ocean acidification, hypoxia, invasive species, climate change, and disease (Lotze et al. 2006; Dulvy, Sadovy, and Reynolds 2003; Feely et al. 2004; Chan et al. 2008; Fabry et al. 2008; Grantham et al. 2004; Diaz 2001).

Unprecedented growth and industrialization of global fisheries after the 1950s initially brought large catch returns, but since the 1970s, collapses of fisheries have become evident (Pauly, Christensen, and Gu enette 2002) with many productive areas and fisheries being depleted (Myers and Worm 2003; Lotze et al. 2006). Globally there has been a significant reduction in large ocean fish predators since pre-industrial times (Christensen et al. 2003; Myers and Worm 2003) with little hope for recovery if current exploitation continues (Hutchings 2000; Myers and Worm 2005). The loss of apex oceanic predators has resulted in trophic cascades in marine food webs with potential for ecosystem degradation (Myers et al. 2007). Consequently fisheries have expanded to less accessible areas (Myers and Worm 2003) and have switched from harvesting high trophic level species to target less valuable lower trophic levels (Pauly et al. 1998; Essington, Beaudreau, and Wiedenmann 2006). In some cases large time lags (decades to centuries) have been observed between overfishing of a stock and changes in ecological communities as other species have compensated and filled missing niches, until those species are in turn overfished (Jackson et al. 2001; FAO 2011). From the late 1980's till

early 2000's reported world fisheries landings declined by 0.7 million tonnes per year (Pauly, Christensen, and Guénette 2002) and have remained stable over the last decade (FAO 2010; 2011; 2012). Fifty-seven percent of global fishery stocks are designated as fully exploited while 30 percent are over exploited (FAO 2011). As the majority of present global fisheries have reached their production capacity and constraints on marine resources are amplified by increasing worldwide demands for seafood, there has been a substantial growth and development of the aquaculture industry to bridge the gap in the growing demand (Naylor et al. 2000).

Globally, aquaculture is the fastest growing food producing industry with half of the seafood consumed worldwide being farmed products (FAO 2010). Global aquaculture production (excluding aquatic plants) has risen from one million tonnes to 60 million tonnes from the early 1950s to 2010 and is now worth US \$119 billion, with the Asia-Pacific region dominating global production (FAO 2010; 2012). The majority of aquaculture production (excluding aquatic plants) is freshwater fishes (56.4 percent) and molluscs (23.6 percent) while crustaceans, diadromous/marine fishes and other aquatic organisms comprise the balance (9.6%, 9.1%, and 1.4% respectively) (FAO 2012). Aquaculture has been conducted on a rural subsistence scale for thousands of years, though in recent decades industrial scale farming of high-value species has become prevalent (Naylor et al. 2000). Commercial-scale intensive aquaculture involves high stocking density of monocultures to provide products to global and regional markets. Intensive aquaculture typically rely on capture fisheries to provide feed, and currently seven out of the ten largest capture fisheries are not destined for direct human consumption (FAO 2008). High-value carnivorous salmon aquaculture requires feed from these capture fisheries.

1.1 The rise of salmon farming

Salmon farming began in Norway during the 1960's. Production expanded to Japan, Chile, Scotland, Ireland, New Zealand, Australia, the Faroe Islands, the US, and Canada during the 1980's; currently Norway and Chile are the leading producers followed by the UK and Canada (MOE 2010b). British Columbia is the largest producer of cultured salmon in Canada (MOE 2010b) and the industry is regulated federally by Fisheries and Oceans Canada. In 2010, total BC salmon production (both wild caught and farmed) was

101,800 tonnes with a landed value of \$568.9 million (MAL 2011). Cultured salmon represents 77 percent of quantity and 88 percent of landed value of BC salmon production (MAL 2011). Atlantic salmon (*Salmo salar*) is the most commonly cultured salmon species in Canada (94 percent), however three species of Pacific salmon are also cultured in low abundance (six percent cumulatively): coho (*Oncorhynchus kisutch*), sockeye (*O. nerka*) and chinook (*O. tshawytscha*) (MOE 2010b). The majority of cultured salmon produced in BC is exported, with over 80 percent sold to the US (MAL 2011).

The salmon aquaculture industry in BC has seen significant expansion over the last several decades. In 1984 there were less than five fish farms on the BC coast (Ellis 1996). Currently there are 10 companies that own 147 open net cage farms and farm applications in BC; 90 percent are owned by three multinational Norwegian companies: Mainstream (Cermaq), Marine Harvest, and Grieg Seafood (MAL 2011b; Living Oceans Society 2011).

Salmon are cultured in high density within open net systems that are permeable to the surrounding environment. Producing salmon this way externalizes costs associated with water filtration and oxygenation as well as waste dispersal, which is done by surrounding waters. This type of system can allow any nutrient and chemical inputs as well as pathogens to disperse freely to the marine environment. A typical single farm in BC operates between six and 24 cages with an average of 35,000-50,000 fish per cage (DFO 2011a). Production at such high density requires large inputs of feed and chemicals.

1.2 Salmon aquaculture feed and waste deposition

Open-net systems allow uneaten feed and cultured fish fecal material to distribute to the surrounding environment. Video detection systems have helped to significantly reduce feed pellet waste at salmon farms (Parsonage and Petrell 2003) and currently between 1 – 17% of delivered feed goes uneaten and falls to the ocean floor (Brooks and Mahnken 2003; Chamberlain et al. 2007; Strain and Hargrave 2005). In 2005 between 1,097 – 18,863 tonnes of uneaten feed was lost directly to the marine environment in BC (Cubitt et al. 2008). In addition to uneaten feed waste, up to 33% of feed eaten by cultured salmon is expelled as feces (Weston 1986). The combination of uneaten feed and waste

products that are deposited in marine sediment in the vicinity of salmon farms can represent up to 19 percent of the total organic matter in the original feed (Stucchi et al. 2005).

Tides, currents, the size and orientation of the farm, production fish condition, marine sediment composition, and depth and bathymetry characteristics of a site can all affect the amount of waste deposition that occurs in the benthic habitat (Kalantzi and Karakassis 2006; Cubitt, Butterworth, and McKinley 2008). Deposition to the benthos does not extend much beyond 150 meters as particulate waste matter falls vertically and concentrates directly underneath farms (Weston 1990; Sutherland, Martin, and Levings 2001; Schendel et al. 2004; Chamberlain et al. 2007). However, farm origin particles have been found in the water column up to 300 meters away (Schendel et al. 2004) due to dispersion via strong currents (Sutherland, Martin, and Levings 2001; Cromey et al. 2002).

The accumulation of uneaten feed and feces below net-pens has resulted in nutrient enrichment, enhanced bacterial numbers, increased sediment oxygen consumption, alterations in biochemical sediment properties including sediment texture, the production and release of methane and hydrogen sulphide, and shifts in benthic infaunal communities (Sutherland, Martin, and Levings 2001). In addition to nutrient inputs, uneaten feed and salmon waste can be contaminated with drugs, antibiotics, and heavy metals (zinc, copper and cadmium) that accumulate in sediment and organisms in proximity to salmon farms (Brooks and Mahnken 2003; Smith, Yeats, and Milligan 2005; Yeats et al. 2005; Debruyn et al. 2006; Dean, Shimmield, and Black 2007; Samuelsen et al. 1992; Capone et al. 1996; Yeats 2002). According to the Fisheries and Oceans Canada regulations, all operational farm sites are required to monitor the benthic environment at their peak production, using sediment grabs at soft bottom sites and underwater video at hard bottom sites (DFO 2013). At soft bottom sites the levels of sulphide in the sediment is measured and at hard bottom sites the cover of bacterial mats (*Beggiatoa* spp.) and presence of opportunistic polychaete complexes is assessed. When thresholds levels of sulphide ($> 1300 \mu\text{mol}$ at 30 m from farm and $> 700 \mu\text{mol}$ at 125 m from farm) or bacterial mats ($> 10\%$ cover) and polychaetes (complexes found in two-thirds or more of surveyed area) are exceeded, fallowing must occur to remediate the benthic environment

until further monitoring establishes that satisfactory recovery below threshold limits has been observed. Remediation of sites can take several months to years; heavily impacted sites could take four to seven years to recover (Brooks et al. 2003; Brooks, Stierns, and Backman 2004). Periods of fallowing may be standard operating procedure by some aquaculture operations with rotation of production and fallowing between sites.

1.3 Disease outbreaks in salmon aquaculture

Disease outbreaks are common in cultured salmon operations and when there is no barrier between farmed and wild populations pathogens can spread freely between farm and wild hosts. The high host density in salmon farms creates the capacity to amplify pathogens and thus initiate novel epidemiological dynamics. Many novel host-parasite relationships have been introduced with open net pen culturing of fish and include pathogenic species from Isopoda, Copepoda, Cestoda, Mycrosporidia and Myxozoa (Kent 2000). Disinfectants, antibiotics and other drug compounds are used to manage fungal, ectoparasitic, protozoal and bacterial disease outbreaks on salmon farms (Burka et al. 1997). Treatment protocols and drugs available are strictly regulated and must be prescribed by licensed veterinarians (Cubitt, Butterworth, and McKinley 2008; BurrIDGE et al. 2010).

1.3.1 Sea lice parasites

Sea lice are globally distributed marine copepods from the family Caligidae that are parasitic on the epidermis of fish hosts. *Lepeophtheirus salmonis* and *Caligus elongatus* are common sea lice species in the northern hemisphere and *C. teres* and *C. rogercresseyi* in the southern hemisphere. *L. salmonis*, a salmonid-specific species of sea lice found in the northeast Pacific Ocean, exists naturally at low ambient levels and can infect all salmonids including Pacific (*Onchorynchus* spp.) and Atlantic (*Salmo salar*) salmon (Kabata 1979; Wooten et al. 1982). *L. salmonis* develop through three free-living pelagic stages, of which the final stage must infect a host (Costello 2006). *L. salmonis* parasitize fish hosts through attached chalimus stages and mobile pre-adult and adult stages (Heuch and Nordhagen 2000; Kabata 1979; Costello 2006). Sea lice feed on host epidermis, mucus, and blood (Brandal, Egidius, and Romslo 1976; Kabata 1974) and can cause lesions in the skin epidermis of hosts compromising osmoregulation, and increase stress

and vulnerability to secondary infections (Pike and Wadsworth 1999; Bowers et al. 2000; Mustafa et al. 2000; Sackville et al. 2011). Further, fecundity, growth, and survival of salmon can be comprised by sea lice infection (Nolan, Reilly, and Bonga 1999; Pike and Wadsworth 1999; Morton and Routledge 2005; Krkosek et al. 2006; Wootten et al. 1982; Grimnes and Jakobsen 1996).

Caligidae copepod species are responsible for the majority of disease outbreaks in cultured salmon (Johnson et al. 2004). Johnson et al. (2004) report the global annual cost of the treatment and management of sea lice infestations on farms and product value lost in production due to mortality, reduced growth rate, and carcass downgrading exceeds US \$100 million.

Cultured salmon in the Northern hemisphere are grown in coastal areas sympatric with wild salmon populations, so there is the potential for significant cross infestation. Initially production fish are infected from sea lice on wild salmon populations migrating past salmon farms to spawning grounds. Where fish hosts are aggregated in high densities, such as with salmon farms, localized populations of *L. salmonis* can exceed ambient levels (Wootten et al. 1982). Mechanisms regulating the transmission and abundance of parasites can become undermined when wildlife populations encounter spill over of parasite reservoirs residing within farm animal populations (Daszak, Cunningham, and Hyatt 2000). Studies in Ireland, Scotland, Norway and Canada have documented a spatial association between wild fish populations infected with sea lice and salmon farms (MacKenzie, Longshaw, and Begg 1998; Bjørn and Finstad 2001; Heuch and Mo 2001; Bjørn 2002; Butler 2002; Morton et al. 2004; Krkosek, Lewis, and Volpe 2005; Krkosek et al. 2006; Tully and Whelan 1993).

Permanent presence of adult salmon hosts in coastal farms results in a sustained source of sea lice in coastal environments that can have significant consequences for fish that may not encounter this parasite naturally. Juvenile salmon do not encounter parasites which are associated with adult salmon populations during the early months of their marine phase as the seaward migration of juvenile Pacific wild salmon precedes the return of wild adult salmon (Krkosek et al. 2006; Krkosek, Gottesfeld, et al. 2007b; Groot and Margolis 1991; Quinn and Myers 2004). Salmon farms enable juvenile Pacific salmon temporal and spatial sympatry with farmed adult salmon and their associated sea

lice parasite loads (Krkosek et al. 2006; Krkosek, Gottesfeld, et al. 2007b). First reports of sea lice on juvenile salmon in BC were from the Broughton Archipelago in 2001 (Morton and Williams 2003). Since then, high loads of sea lice have been documented on juvenile pink, chum, and sockeye salmon in regions of BC with high concentrations of salmon farms (Morton et al. 2004; Krkosek, Lewis, and Volpe 2005; Price, Morton, and Reynolds 2010; Price et al. 2011). The consequences of *L. salmonis* infections can be more severe for juvenile salmon because sea lice are relatively large in comparison to host size (Holmes and Zobar 1990), and small fry lack protective scales. Krkosek et al. (2007a) predicted an extirpation of wild pink salmon from the Broughton Archipelago in four salmon generations due to the impacts from salmon farms, and specifically sea lice.

Scientific and public pressure in BC has prompted management to address the problem of sea lice transmission from farmed to wild populations, particularly during the juvenile salmon outmigration period. Beginning in 2003 BC regulatory authorities required salmon aquaculture operations to monitor sea lice on production fish as a pre-emptive measure to avoid large infestations. Facility operators report sea lice abundance on production fish to Fisheries and Oceans Canada on a monthly basis as part of their license conditions (DFO 2012c). If levels surpass the regulatory threshold of three motile sea lice per fish management procedures must be initiated (DFO 2012c; Saksida et al. 2007; PSF 2009). However, there have been recommendations to treat when more than three percent of near-farm migrating juvenile pink and chum salmon (which weigh less than one gram) have one or more sea lice (PSF 2009). Good husbandry practices on farms are also effective in preventing sea lice outbreaks and include: low stocking density, reducing infection to newly introduced juveniles by treatment of fish prior to restocking and year-class separation, fallowing, and improved water circulation including routine defouling of nets (Johnson et al. 2004). Between-cohort fallowing, in which all production fish are removed for a period of time, seems especially effective to reduce sea lice transmission to wild salmon populations (Morton, Routledge, and Williams 2005; Morton et al. 2011). New advances in sea lice control have included vaccines (Raynard et al. 2002), cleaner fish (Deady, Varian, and Fives 1995; Treasurer 1993), and modifying salmon behaviour (Dempster et al. 2011), but further development is required before adoption by industry. Once an outbreak of sea lice occurs within a farm, fish husbandry

methods are no longer as effective. To provide immediate control of infections, industry relies heavily on the use of chemotheraputants that can be applied topically as bath treatments or as an in-feed preparation to reduce sea lice parasite loads on production fish (Rae 1979).

1.3.2 Sea lice anti-parasitic treatments

There has been a diverse array of treatments used for sea lice in salmon aquaculture that are typically applied under veterinarian prescription. The classes of chemotheraputants used as sea lice treatments include: organophosphates, pyrethroids, chitin synthesis inhibitors, hydrogen peroxide, and avermectins. Effectiveness of these compounds vary as some are only successful in reducing the adult lice phase, leaving juvenile stages unaffected (Burka et al. 1997). Avermectins and chitin synthesis inhibitors are administered as in-feed preparations while the rest are delivered in a topical bath treatment. Ultimately all chemical compounds used are released to the environment during and after treatment. All anti-sea lice compounds lack specificity; therefore there is concern with applications affecting non-target organisms.

In most countries that have salmon farming industries sea lice have developed resistance to chemicals used and outbreaks cannot be managed until alternatives are found. The development of resistance is heavily dependent on the frequency of chemical application on a farm and within a management area. In the terrestrial environment the sustainability of chemicals as an integral part of pest management is degrading as hundreds of insect pest species have become resistant to one or more chemical classes of pesticides (Denholm et al. 2002). Reduced sensitivity of sea lice to chemical treatment has been reported for various compounds (Treasurer, Grant, and Davis 2000; Denholm 2002; Sevatdal and Horsberg 2003; Fallang et al. 2004; Sevatdal, Copley, et al. 2005a).

Organophosphates

Organophosphate compounds are cholinesterase inhibitors that hinder neuromuscular transmission (Baillie and Wright 1985). Organophosphates are applied as bath treatments and are only effective on adult sea lice stages (Roth et al. 1996). Four organophosphate compounds have been developed for sea lice treatment: malathion, trichlorfon, dichlorvos, and azamethiphos (Haya et al. 2005). Azamethiphos, the active ingredient in

the formulation Salmosan[®], is currently the only organophosphate compound used in the aquaculture industry as the other compounds had small therapeutic indexes (i.e. narrow margins of safety for production fish when used at doses to treat sea lice) or resistance of sea lice was observed (Horsberg and Hoey 1989; Jones, Sommerville, and Wootten 1992; Tully and McFadden 2000; Haya et al. 2005). Azamethiphos is registered for use in Norway, Scotland and Chile. Variable sensitivity of sea lice to azamethiphos has already been observed (Roth et al. 1996) and resistance is confirmed in insect pests (Levot and Hughes 1989). Organophosphate compounds are not likely to accumulate in sediment and tissue but remain in an aqueous phase, due to water solubility and low adsorption coefficient (Roth et al. 1993).

Pyrethroids

Pyrethroid compounds interact with sodium ion channels, which depolarize nerve endings resulting in interference with nerve membrane function (Miller and Adams 1982). Pyrethroids are applied as topical bath treatment and are effective on all attached stages of sea lice including adults (Burrige et al. 2010). Pyrethroids are highly toxic to crustaceans but have high degradability and are rapidly metabolized (Haya et al. 2005; Kahn 1983; Davis 1985). These compounds also bind quickly to particles and have high absorption into sediment. Pyrethroid compounds commonly used in the aquaculture industry include cypermethrin (Excis[®] and Betamax[®]) and deltamethrin (AlphaMax[®] and Pharmaq[®]). Cypermethrin is currently used in Scotland and Norway while deltamethrin is used in Norway, Chile and on an emergency basis in eastern Canada. Cypermethrin can persist for weeks in sediment (Kahn 1983) and has been found in low concentrations in the water collected around treating farms at least 100 meters away (Pahl and Opitz 1999; Hunter and Fraser 1995; SEPA 1998). Reduced sensitivity of sea lice has been observed with the use of deltamethrin in Norway (Sevatdal and Horsberg 2003).

Hydrogen peroxide

Bath treatments of hydrogen peroxide have been used for sea lice treatment for several decades and are often resorted to when sea lice populations are resistant to other prescribed chemicals. Hydrogen peroxide causes mechanical paralysis in sea lice due to

bubble formation in the haemolymph and gut resulting in positive buoyancy, lifting lice to the water surface (Bruno 1994). In addition hydrogen peroxide causes inactivation of enzymes and DNA replication, and peroxidation of lipid and cellular organelle membranes (Cotran et al. 1989). Hydrogen peroxide is not effective on juvenile lice stages and has inconsistent efficacy on pre-adult and adult stages (Treasurer, Wadsworth, and Grant 2000; Mitchell and Collins 1997). In the 1990s hydrogen peroxide was used in Faroe Islands, Norway, Scotland and Canada (Treasurer and Grant 1997), and has recently been used in Scotland and Chile (SEPA 2009; Bravo 2010). Environmental concern with the use of hydrogen peroxide is low as it is miscible in water and rapidly degrades to water and oxygen products (Bruno 1994; Richard et al. 2007; Miller, Rose, and Waite 2009). There is some evidence that sea lice have developed resistance against hydrogen peroxide in Scotland (Treasurer, Wadsworth, and Grant 2000).

Chitin synthesis inhibitors

The mode of action by which chitin synthesis inhibitors work is unclear (Savitz, Wright, and Smucker 1994), however, they seem to prevent the synthesis of chitin, an important component of the exoskeleton of insects and crustaceans. These compounds have the potential to be highly toxic to molting species (SEPA 1999b; Fischer and Hall 1992). Chitin synthesis inhibitors are most effective on larval, juvenile and pre-adult stages but there is reduced efficacy with adult lice. These compounds are extremely effective in breaking infection cycles as they target younger lice stages, but treatment must occur before adults are present. There are two chitin synthesis inhibitor products used for sea lice treatment: teflubenzuron (Calicide[®]) and diflubenzuron (Lepsidon[®]). Teflubenzuron was used in Scotland in 2007 and eastern Canada in 2009 and diflubenzuron was used in Chile in 2008 (Burrige et al. 2010). The compounds have low water solubility and will bind to sediment and organic particles. Teflubenzuron can be found in marine sediment in proximity to treating farms 645 days after treatment (Haya et al. 2005; SEPA 1999b).

Avermectins

Avermectin compounds attach to specific high-affinity binding sites in arthropods and open glutamate-gated chloride channels, which increases membrane permeability to

chloride ions, inhibits nerve impulse transmission, and causes hyperpolarization of nerve and muscle tissue (McKellar and Benchaous 1996; Roy et al. 2000; Wolstenholme and Rogers 2005). This causes paralysis in sea lice and termination of all activities, including feeding. In mammals, avermectin compounds increase the release of the inhibitory neurotransmitter γ -amino-butyric acid (GABA). Two products have been formulated for sea lice treatment as an in-feed medication: ivermectin and emamectin benzoate (EMB, trade name SLICE[®]), however ivermectin is no longer used in the industry due to its toxicity to production fish (Palmer et al. 1987; O'Halloran et al. 1992). EMB was originally synthesized as a pesticide for lepidopteron control in agriculture in the US and Japan (Lasota and Dybas 1991), and aside from its aquaculture uses has been prescribed in the forestry industry in North America to treat ash wood for the emerald ash borer (*Agrilus planipennis*) (Poland et al. 2011).

1.4 Emamectin benzoate (SLICE[®])

EMB is a semi-synthetic derivative of abamectin, synthesized from fermentation products of the bacteria, *Streptomyces avermitilis* (Merck Animal Health 2009). EMB is a mixture of two avermectin homologues (90% 4'-epimethy-amino-4'-deoxyavermectin B_{1a} benzoate and 10% 4'-epimethy-amino-4'-deoxyavermectin B_{1b} benzoate) (SEPA 1999). SLICE[®] premix contains EMB (0.2%), butylated hydroxyanisole (0.01%), propylene glycol (2.5%), maltodextrin (47.40%), and cornstarch. While EMB is the main active ingredient, butylated hydroxyanisole and propylene glycol have anti-microbial activities (Mayor et al. 2009) but are reported to have negligible risk to the environment (SEPA 1999). EMB is lipophilic, has low water solubility, and a high adsorption octanol–water partition coefficient for organic particulates ($\log K_{ow} = 5$) so will tightly bind to marine sediment (SEPA 1999). EMB has several metabolites, including the 8,9-Z isomer, N-formylated, N-methylformylated emamectins (Bright and Dionne 2005), and the most significant being the N-demethylated metabolite (Chukwudebe et al. 1996; Kim-Kang et al. 2004).

Once production fish ingest SLICE[®] coated feed pellets, EMB is absorbed in the gut and distributed to fish plasma, mucus, skin and muscle (Sevatdal, Magnusson, et al. 2005b; Whyte et al. 2011). Concentrations of EMB is highest in fish mucus and lowest in the skeletal muscle (Sevatdal, Magnusson, et al. 2005b). The amount of EMB taken up by

individual salmon is influenced by site, season, and the disease status of the fish (Berg and Horsberg 2009). Sea lice that feed on the blood and tissue of treated salmon will uptake EMB. EMB is extremely effective, causing 98 percent disengagement of all juvenile and adult stages on Atlantic salmon (Stone et al. 2000b). Efficacy of SLICE[®] treatments in reducing juvenile lice stages on salmon has been recorded up to 69 days after treatment, after which effectiveness declines (Stone et al. 2000a). The optimum treatment concentration is 50 µg EMB per kilogram of production fish per day for seven days (Stone et al. 1999). The concentration of EMB in SLICE[®] pellets ranges from one to 25 µg g⁻¹, however, the most common dosage is 10 µg g⁻¹. Emamectin benzoate is currently used in Norway, Scotland, Chile and Canada. Resistance of sea lice to EMB has been observed in Scotland (Lees et al. 2008), Chile (Bravo et al. 2008) and eastern Canada (Igboeli et al. 2012; AVC-CAHS 2009; Westcott et al. 2010). No published literature has documented resistance of sea lice to EMB in BC.

1.4.1 Canadian usage

SLICE[®] has been used in Canada since 1999 and is the only sea lice treatment applied in BC. For ten years SLICE[®] was used on an emergency case-by-case basis under Health Canada's Emergency Drug Release program, until Health Canada's Veterinary Drug Directorate approved the chemotherapeutant in 2009 (Intervet 2009). Canada Food Inspection Agency monitors therapeutant drug residues once cultured salmon are harvested using a Quality Management Program (DFO 2012a). In Canada, production fish can receive up to three treatments per year and five treatments maximum throughout the entire grow out cycle. In BC, the average number of treatments per production cycle is 1.2. Treatment of aquaculture pens is usually done in the fall or winter to reduce sea lice populations before the spring wild juvenile salmon out migration period. In BC there have been attempts in some regions to enact coordinated management of sea lice on salmon farms along juvenile salmon migration routes for a more integrative pest management approach. Individual salmon farms alternate annually with SLICE[®] treatments and fallowing; a treatment regime that accommodates the 18-month grow out period required to produce farmed salmon. This type of treatment regime may be beneficial in removing sea lice effectively along salmon migration routes for the duration of the treatment, but if all areas are treated simultaneously benthic habitat and non-target

populations may also be exposed on a larger scale. Information regarding EMB treatment applications by the salmon aquaculture industry in BC is not available in the public domain due to proprietary concerns. This lack of transparency unfortunately makes it difficult to assess the in-field risk to non-target organisms.

1.4.2 Emamectin benzoate deposition in the marine environment

During the seven-day treatment period, production fish excrete EMB and its desmethyl metabolite in feces to the marine environment; EMB excretion is largely complete by one day post-treatment (Kim-Kang et al. 2004). EMB residues in tissue and skin decline slowly in Atlantic salmon and metabolism of EMB is limited. During and after SLICE[®] treatments detectable amounts of EMB and its desmethyl metabolite accumulate in the benthic environment proximate to salmon farms as a result of uneaten SLICE[®] pellets and salmon waste products (Telfer et al. 2006; SEPA 2004a; DFO 2012b). The accumulation and persistence of EMB in the sediment is dependent upon the farm's frequency and extent of SLICE[®] use, the type of sediment and its physical-chemical properties, the sediment microorganism community, and the hydrodynamic characteristics (DFO 2012b; Hurt et al 2006; Hand and Fleming 2007). EMB residues in marine sediment near treating farms has been assessed in Norway, Scotland, France, the US and Canada (Table 1). The majority of EMB accumulates within 60 meters of treating farms but can be detected up to 150 meters away (Telfer et al. 2006; DFO 2012b). Typical concentrations found near cages are 0.5 – 35 $\mu\text{g kg}^{-1}$ and are highest several weeks after treatment. Four weeks post-treatment, EMB residues decline either through dilution or degradation to metabolites (DFO 2012b), and the suggested half-life of EMB in marine sediment is 165 – 250 days (McHenry 1999; SEPA 2004b). EMB can still be detected in sediment 1.5 years post-treatment (DFO 2012b). The combination of an annual treatment regime and the prolonged persistence of EMB residues in marine sediment may result in elevated environmental levels and chronic exposure to non-target organisms.

EMB is subject to photolysis and degrades in the water column at depths that light can penetrate (Mushtaq, Chukwudebe, and Wrzesinski 1998). In BC EMB has been detected in the water column around treating salmon farms due to the development of a more sensitive analysis methodology (Ikonomou and Surridge 2011). EMB levels in

subsurface water around a treating salmon farm ranged from 0.006 to 0.635 ng/L and dissipated quickly in the water column with no residue found after four to five weeks (DFO 2012b).

1.4.3 Emamectin benzoate in marine organisms

Since EMB disseminates into marine environments and is toxic to arthropods and nematodes, it is a concern with regard to non-target organisms in the vicinity of treating salmon farms. Non-target organisms may be exposed to EMB through consumption of uneaten medicated pellets, incidental ingestion of EMB contaminated sedimentary particles, direct exposure with contaminated sediment, and through gill respiration from EMB that has leached into the water overlaying and within the sediment. Adsorption of molecules through the gill membrane in fish is much more restrictive than through the gastrointestinal tract (Wood and Part 1997; Trischitta et al. 1999; de Wolf et al. 2007), making it unlikely that this is a major uptake pathway. Since EMB tightly binds to organic material and will remain in the sediment, marine invertebrates associated with the benthos will be most at risk. EMB residues have been detected in crustaceans (*Pagurus* spp., *Carcinus maenas*, and *Munida rugosa*), echinoderms (*Asterias rubens*), molluscs (*Buccinum undatum*), and fish (*Scyliorhinus canicula*, *Myoxocephalus scorpius*, and *Conger conger*) near treating salmon farms in Scotland (Telfer et al. 2006). EMB was detected in blue mussels (*Mytilus edulis*) deployed 100 meters from farms one week post-treatment, and at ten meters one month post-treatment, indicating mussels may be accumulating higher levels of EMB closer to farms and were depurating EMB with time (Telfer et al. 2006).

1.4.4 Emamectin benzoate effects on non-target species

It is difficult to obtain ecotoxicological information regarding chemotherapeutic treatments as they are often in confidential reports (Crane et al. 2006) and comparison between studies is complicated by different experimental methods, reporting procedures, exposure routes, and test organisms (Mayor et al. 2008).

Though EMB is a lipophilic compound ($\log K_{ow} = 5$), the large molecular weight (~1000 g/mol) and size of the EMB molecule, as well as polar characteristics, indicate EMB is not likely lipophilic *in vivo* and will not bioconcentrate in organisms (Van Den

Berg et al. 1995; Nendza and Hermens 1995). EMB does not bioaccumulate in blue gill sunfish (*Lepomis macrochirus*) (Chukwudebe et al. 1996) or rats (Mushtaq et al. 1996; Mushtaq, Allen, and Crouch 1997).

In laboratory conditions EMB is toxic to both parasitic sea lice and free-living copepods (Willis et al. 2003), though there has been no observed effect of SLICE[®] treatments on zooplankton abundance and diversity when sampled over 31 months near treating farms in Scotland (Willis et al. 2005). One Scottish field study determined that while nutrient inputs had the largest effect on benthic community diversity, differences due to SLICE[®] treatments were observed at several sampling locations, with a decrease in diversity four months post-treatment, returning to pre-treatment levels one year after treatment (Telfer et al. 2006).

There is significant research on laboratory EMB exposures to marine and freshwater organisms through water, sediment and food (Table 2). The most sensitive organism is the mysid shrimp (*Americamysis bahia*) with 96 hour LC₅₀ (lethal concentration dose where 50 percent of exposed individuals die) of 0.04 µg L⁻¹ (Conner et al. 1994).

A considerable amount of EMB exposure research has been conducted in eastern Canada on the American lobster (*Homarus americanus*), a decapod crustacean. Some of the major findings include premature molting and the loss of eggs with the cast in ovigerous lobsters force-fed an EMB dose between 0.22 – 0.39 µg g⁻¹ EMB (Waddy et al. 2002). Molting, or ecdysis, is an essential physiologic process in crustaceans, in which exoskeletons, predominantly composed of chitin, are cast off and regenerated to allow for development, growth, and reproduction (Chang 1993; Waddy, Merritt, et al. 2007b). Waddy et al. (2002) hypothesize that EMB disrupts the neuroendocrine control of molting glands, causing an acceleration of molting in crustaceans. American lobsters administered a single dose of 0.5 µg g⁻¹ had lower rates of premature molting than lobsters given a succession of lower doses at two week intervals amounting to the same cumulative exposure (Waddy et al. 2010). The impact of low-dose chronic exposure may have greater effects than acute exposure as higher mortality was observed during ecdysis in lobsters delivered four to eight multiple small doses compared to lobsters delivered one large dose. EMB pellets are acutely toxic to adult and juvenile lobsters at high

concentrations, however, the seven-day LC₅₀ of adults was 644 µg g⁻¹, 25 times higher than the maximum EMB concentration in commercially prepared medicated feed (Burridge et al. 2004).

The maximum observed *in situ* concentrations of EMB in marine sediment and water is below levels that cause direct mortality to documented marine organisms in acute laboratory exposures. However, ecotoxicological research conducted regarding the sub lethal impacts of EMB or low-dose chronic exposures on non-target species is very limited and in particular there is little information on the impact on Pacific Northwest crustacean species. In British Columbia reside several commercially important crustacean species that have populations overlapping with areas of intense salmon farming. One of the most valuable species is the spot prawn, *Pandalus platyceros*, which supports a coast-wide fishery.

Table 1: Concentration of emamectin benzoate (EMB) and its desmethyl metabolite in marine sediment at salmon farm sites in Canada, the US, Scotland, France and Norway. Near field is ≤ 25 m, Far field is ≥ 100 m. LOD = level of detection.

Country	Distance from net pen edge	Dates	Wet weight concentration ($\mu\text{g kg}^{-1}$) (highest mean value from any location at any time during study)	
			Emamectin benzoate	Desmethyl metbolite
Canada (BC) ¹	Near field	2009	Sediment: 35.0 Water: 0.635 ng/L (50 m from farm)	Water: <LOD
	Far field		Sediment: 0.12 Water: 0.006 ng/L	Water: <LOD
Canada (Eastern)	Near field ²	1999	0.762	0.365
	Near field ²	2000	5.29	1.3
	Near field ²	2001	2.56	0.65
	NA ³	2002	<LOD	
Scotland	Near field ⁴	1997	2.73	0.71
	Far field ⁴		0.62	< 0.25
	Near field ⁵	2003	27.9	NA
	Near field ⁶	2004	4.60	NA
	Near field ⁷	2006	5.38	< LOD
US (Maine) ²	Near field	2001	0.89	0.13
	Near field	2003-2004	3.47	< 0.5
France ²	Near field	2001	1.15	
	Far field		0.95	
Norway ²	Near field	2002	5.65	
	Far field		< 0.5	

¹Ikonomou 2012, ²Endris per comm. 2011, ³Parker and Mallory 2003, ⁴Telfer et al. 2006, ⁵Thomas 2004, ⁶Thomas 2005, ⁷Thomas 2007

* All measurements are for sediment unless otherwise stated

Table 2. Data from laboratory EMB acute and chronic exposures to marine and freshwater invertebrates and fish species

Scientific name	Common name	Endpoint	Effect measurement	Media type	Duration	Concentration	Reference
Crustacea							
<i>Daphnia magna</i>	Water flea	EC50	Immobilization	Freshwater	48 h	1.0 µg L ⁻¹	Homes and Swigert 1993
		EC50	Immobilization	Freshwater	48 h	3.8 µg L ⁻¹	Blankinship et al 2002a
		NOEC	Mortality	Freshwater	21 d	0.088 µg L ⁻¹	Zelinka et al. 1994b
		LOEC/ NOEC	Reproduction	Freshwater	21 d	0.16 / 0.088 µg L ⁻¹	McHenery and Mackie 1999
		LC50	Mortality	Freshwater	21 d	0.128 µg L ⁻¹	McHenery and Mackie 1999
<i>Americamysis bahia</i>	Mysid shrimp	LC50	Mortality	Seawater	96 h	0.04 µg L ⁻¹	Conner et al. 1994
		NOEC	Growth	Seawater	28 d	0.0087 µg L ⁻¹	Blankinship et al 2002b
		LC50/NOEC	Mortality	Seawater	96 h	0.043 / 0.018 µg L ⁻¹	McHenery and Mackie 1999
<i>Acartia clausi</i>	Copepod	LOEC/ NOEC	Egg production	Seawater	7 d	0.159 / 0.05 µg L ⁻¹	Willis and Ling 2003
		EC50	Immobilization	Seawater	48 h	0.29 µg L ⁻¹	Willis and Ling 2003
<i>Pseudocalanus elongatus</i>	Copepod	EC50	Immobilization	Seawater	48 h	0.45 µg L ⁻¹	Willis and Ling 2003
<i>Temora longicornis</i>	Copepod	EC50	Immobilization	Seawater	48 h	2.81 µg L ⁻¹	Willis and Ling 2003
<i>Oithona similis</i>	Copepod	EC50	Immobilization	Seawater	48 h	231 µg L ⁻¹	Willis and Ling 2003
		LC50/NOEC	Mortality	Seawater	10 d	6.32 / 3.20 µg L ⁻¹	McHenery and Mackie 1999
			Mortality	Sediment	10 d	193.1 / 114.6 µg kg ⁻¹	McHenery and Mackie 1999
<i>Corophium volutator</i>	Amphipod	LC50	Mortality	Sediment	10 d	153 µg kg ⁻¹	Mayor et al. 2008
<i>Eohaustorius estuarius</i>		Amphipod	LC50	Mortality	Sediment	10 d	185 µg kg ⁻¹
<i>Artemia salina</i>	Brine shrimp	IC100	Immobilization	Seawater	6 h	1730 µg L ⁻¹	Blizzard et al. 1989; Mrozik et al. 1995
<i>Nephrops norvegicus</i>	Dublin	LC50	Mortality	Feed	96 h	> 68200 µg kg ⁻¹	McHenery and Mackie 1999
	Bay prawn	LC50	Mortality	Feed	192 h	> 68200 µg kg ⁻¹	Aufderheide 1999c
		LC50/NOEC	Mortality	Seawater	96 h	983 / 814 µg L ⁻¹	McHenery and Mackie 1999
		LC50/NOEC	Mortality	Seawater	192 h	572 / 440 µg L ⁻¹	McHenery and Mackie 1999
<i>Crangon crangon</i>	Bay shrimp	LC50/NOEC	Mortality	Seawater	96 h	224 µg L ⁻¹	Aufderheide 1999a
		LC50	Mortality	Seawater	192 h	166 µg L ⁻¹	Aufderheide 1999a

<i>Homarus americanus</i>	American lobster	LC50	Mortality	Feed	192 h	> 69300 $\mu\text{g kg}^{-1}$	Aufderheide 1999b
		LC50	Mortality	Feed	7 d	>589 $\mu\text{g g}^{-1}$ (juvenile) 644 $\mu\text{g g}^{-1}$ (adult)	Burridge et al. 2004
		NOEL*	Molting	Feed		0.12 $\mu\text{g g}^{-1}$ bw	Waddy et al. 2007a
Mollusca							
<i>Mytilus galloprovincialis</i>	Mediterranean mussel	EC50	Development	Seawater	48 h	314 $\mu\text{g L}^{-1}$	Aufderheide 2002
		LC50	Mortality	Seawater	48 h	> 713 $\mu\text{g L}^{-1}$	Aufderheide 2002
<i>Crassostrea virginica</i>	Eastern oyster	EC50/NOEC	Shell deposition	Seawater	96 h	530 / 260 $\mu\text{g L}^{-1}$	Zelinka et al. 1994a
		LC50/NOEC	Mortality	Seawater	96 h	665 / 260 $\mu\text{g L}^{-1}$	Zelinka et al. 1994a
Annelida							
Infaunal polychaetes in sediment core		NOEC	Mortality	Sediment	21 d	460 $\mu\text{g kg}^{-1}$	Black et al. 2000
<i>Arenicola marina</i>	Lugworm	LC50/NOEC	Mortality	Sediment	10 d	111 / 56 $\mu\text{g kg}^{-1}$	McHenery and Mackie 1999
<i>Hediste diversicolor</i>	Ragworm	LC50	Mortality	Sediment	10 d	1368 56 $\mu\text{g kg}^{-1}$	Mayor et al. 2008
Fish							
<i>Lepomis macrochirus</i>	Bluegill sunfish	LC50/NOEC	Mortality	Freshwater	96 h	180 / 87 $\mu\text{g L}^{-1}$	Homes and Swigert 1993b
<i>Pimephales promelas</i>	Fathead minnow	LC50/NOEC	Mortality	Freshwater	96 h	194 / 156 $\mu\text{g L}^{-1}$	Drottar and Swigert 1995
		NOEC	Mortality	Freshwater	32 d	28 $\mu\text{g L}^{-1}$	Drottar 1995
		NOEC	Hatching success	Freshwater	32 d	54 $\mu\text{g L}^{-1}$	Drottar 1995
		NOEC	Time to hatch	Freshwater	32 d	54 $\mu\text{g L}^{-1}$	Drottar 1995
		NOEC	Growth	Freshwater	32 d	12 $\mu\text{g L}^{-1}$	Drottar 1995
<i>Oncorhynchus mykiss</i>	Rainbow trout	LC50/NOEC	Mortality	Freshwater	96 h	174 / 48.7 $\mu\text{g L}^{-1}$	Holmes et al. 1993
<i>Cyprinodon variegatus</i>	Sheephead minnow	LC50/NOEC	Mortality	Freshwater	96 h	1340 / 860 $\mu\text{g L}^{-1}$	Martin and Swigert 1994
<i>Cyprinus carpio</i>	Common carp	LC50	Mortality	Freshwater	96 h	260-444 $\mu\text{g L}^{-1}$	Wallace 2001b

*Study examined chronic effects after given single dose

1.5 Spot prawns

Spot prawns are the largest of eight commercially important shrimp species in BC. This species belongs to the family Pandalidae, which are geographically distributed from California to Alaska and the Sea of Japan to the Korea Strait (Watson 1994). Spot prawns can be found inhabiting areas from the intertidal to 487 meters deep and are often associated with habitats of smaller gravel and cobble rock types and mixed sediment (Schlining 1999).

Pandalid shrimp are protandric hermaphrodites, so first mature as males and transition into females in the latter part of life. Larvae hatch from eggs attached to the underside of adult females during March or April and will enter a pelagic life stage with movements dictated by tides and currents for the next three months until settling in the benthic environment (Figure 1; Boutillier and Bond 1999). Juveniles rear in kelp beds for the first summer and then leave the nursery habitat at 16 – 20 mm length (Marliave and Roth 1995). Juveniles then migrate to deeper adult grounds and mature as males by the second autumn. Most male prawns enter the transitional phase after two years and by four years most transitional stages will have become females (Butler 1980). The spot prawn spawning period occurs from August to October and mated females will carry 2000 – 4000 eggs for five months (DFO 2011; Butler 1967; Hynes 1930). Once females become ovigerous they will not molt again. After releasing hatched larvae in spring over a period of ten days spent females die, typically at an age of four to five years.

Once on adult grounds spot prawns have limited migration ranges. Unpublished tagging studies report that mature spot prawns remain within two miles of release locations over several months (Boutillier and Bond 2000). Significant difference in growth rates and parasite loads is observed in adult populations only separated by tens of kilometres, further supporting limited migration (Bower, Meyer, and Boutillier 1996; Bower and Boutillier 1990). The population distribution of spot prawns is very patchy, making them vulnerable to serial depletion and local overfishing (Orensanz et al. 1998).

Spot prawns are generalist opportunistic feeders that consume other small shrimp, amphipods, small molluscs, worms, euphausiids, limpets, annelids, sponge, plankton, and dead animal material (Barr 1973; Butler 1980; Mormorunni 2001). Adults are benthic nocturnal foragers that exhibit daily diel vertical migration of 100s of meters to feed in

the shallows during the night (Chew et al. 1974). Adult spot prawns are an important food resource for fish species associated with the benthos, such as rockfish, as well as octopus (Bergstrom 2000), while larval pandalids are prey for other pelagic and planktonic marine organisms (Parsons 2005).

Several factors have been identified to be important in the success of spot prawn recruitment and reproduction: variation in ocean conditions, critical benthic habitat, intertidal areas, changes in suspended organic material, food supply, change in protective cover, obstruction in migratory pathways, and level of harvest (ADFG 1985).

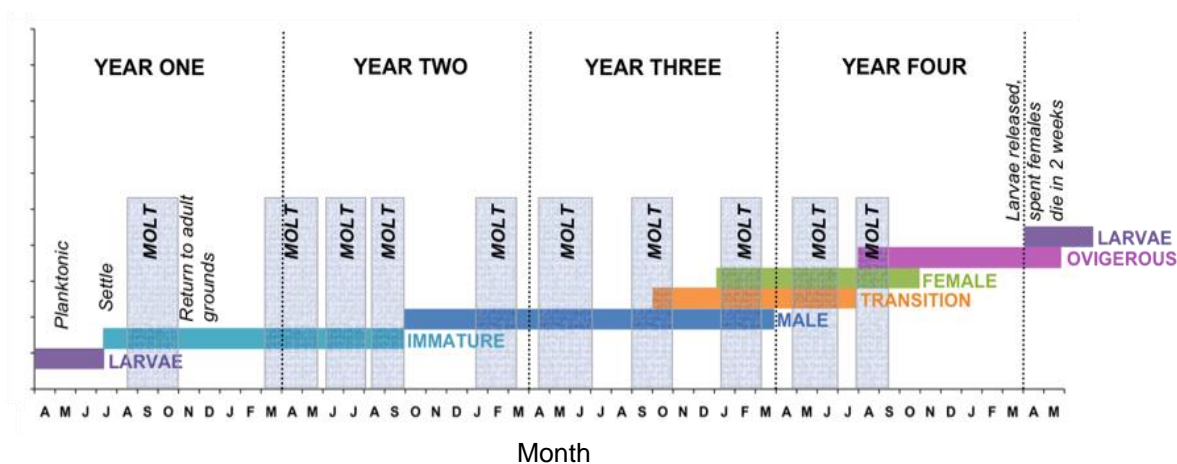


Figure 1: Spot prawn (*Pandalus platyceros*) lifecycle

1.5.1 The spot prawn fishery

The global harvest of shrimp species (wild caught and produced via aquaculture) has been characterized as unsustainable due to environmental impacts including bottom trawling, bycatch, habitat destruction, heavy reliance on chemical inputs, as well as the social degradation of coastal communities (de Groot 1984; Boyd and Clay 1998; Naylor et al. 1998; Morgan and Chuenpagdee 2003; Alverson et al. 1994). In contrast, the spot prawn fishery in the Northeast Pacific Ocean is lauded as a sustainable fishery (Roberts 2008) due to its well-managed low-impact fishing method using traps or 'pots', which minimizes habitat destruction as well as bycatch compared to trawl fisheries. The fishery is primarily community-based and fishermen are able to contribute to the management of the fishery leading to more long-term sustainable harvest.

The BC spot prawn fishery began in Howe Sound circa 1914 but did not become a significant fishery until the mid 1970s (DFO 2011). During the 1980's the number of commercial prawn vessels increased six-fold. In 1990 there was a limit set on the number of available commercial licences and there are currently 252 licences (of which 52 are communal commercial licences for First Nations). In 2000, 84 percent of BC commercial prawn fishermen lived outside of metropolitan areas and over half of commercial licence holders participate exclusively in the spot prawn fishery and so are dependent upon the fishery for their livelihood (Mormorunni 2001). Today approximately 60 percent of commercial landings are from coastal waters on the inside of Vancouver Island between 40 to 100 meters depth (DFO 2011).

To manage growth and recruitment overfishing in the fishery, British Columbia implemented size limitations in 1988 and a fixed escapement system using a spawner index. This index was developed in 1979 to insure enough females are left in the population to meet the reproductive requirements of the stock. The fishery targets prawns in the final two years of their life (no ovigerous females are retained) and the current minimum size limit is 33 mm carapace length (length from the posterior of the eye orbit to the posterior mid-dorsal margin of the carapace) (DFO 2011). The prawn commercial fishery season in BC commences in May to protect ovigerous females and the egg-hatching period, and allows for increased growth prior to harvest. The fishing season is brief and intensive, usually lasting only 60 days, so harvesting during summer molting periods is generally avoided as well. Further prawn fishery management includes seasonal and in season area closures, gear limits, trap mesh size requirements, daily fishing time restrictions, and a daily single haul limit (DFO 2011). In addition all commercial prawn fishers are required to maintain an accurate log of daily harvest operations. Information that must be recorded includes the latitude and longitude, time of haul, and catch in weight (DFO 2011).

Approximately 2,400 tonnes of spot prawn are harvested annually in BC (MOE 2010a). In 2010, spot prawns had a commercial landing value of \$20.5 million (DFO 2011), making spot prawns BC's seventh most valuable commercial fishery overall, but the second most valuable per kilogram after geoduck, *Panopea generosa* (MAL 2010; MAL 2011). The majority of commercial catch is exported to the Japanese market (~ 80

– 90 percent), so the fishery relies heavily on the Asian economy. In recent years there has been a surge within the fishery to locate and sell to local consumers. Efforts have included green labelling, marketing, direct sales to local grocery stores, and spot prawn festivals in Vancouver, Cowichan Bay and Victoria BC.

1.5.2 Risk of SLICE[®] treatments to spot prawns

There is significant overlap between salmon farm tenures and spot prawn commercial fisheries in BC, particularly in the areas surrounding Vancouver Island where 60 percent of landings occur (Figure 2). Commercial harvest regularly occurs near salmon farms, and there is local perception by some fishermen that prawns aggregate near these sites, though no published studies have quantified this. The catch abundance of a closely related species, pink shrimp (*Pandalus borealis*), is highly correlated with the amount of particulate organic carbon in the environment (Ramseier et al. 2000). Waste products from salmon farms represent a significant contribution of nutrients to the benthic environment, so it is possible that spot prawns may be attracted to these sites. Aggregations of American lobsters near salmon farms have been observed in Atlantic Canada (Iwama 1991; Findlay, Watling, and Mayer 1995). The trophic subsidy from uneaten fish feed pellets is the suggested attractant to farm sites (Hargrave 2003). Wild fish species are found to aggregate around fish farms because of the infrastructure and nutrient subsidies to the environment (Carss 1990; Tuya et al. 2006; Sudirman et al. 2009; Arechavala-Lopez et al. 2010). If farms do attract spot prawns with nutrient subsidies, EMB exposure to populations may become amplified.

Toxicity of EMB may also be augmented if its application period overlaps with important spot prawn phenology. Molting and reproduction in crustaceans both require a significant amount of energy investment as well as the production of hormones and pheromones (Chang et al. 2001; Dunham 1978; Hardege and Terschak 2011; Raethke, MacDiarmid, and Montgomery 2004). Crustaceans can be heavily impacted by chemicals and heavy metals during this time due to their sensitive endocrine system (Rodriguez et al. 2007). Currently in BC, salmon farms are encouraged to treat with SLICE[®] during the fall and winter, which is when the spot prawn breeding season is underway and some life stages are molting (Butler 1964). The overlap of SLICE[®] treatments with these sensitive time periods or life stages may make spot prawn populations more vulnerable.

In BC, EMB has been detected in the muscle tissue of wild-caught spot prawns up to 150 meters from a treating farm, 100 days post-treatment when sampling ceased (DFO 2012b). In an eight-day laboratory sediment EMB exposure with spot prawns, higher rates of mortality (15 – 20 percent) were observed in prawns exposed to concentrations between 100 – 800 $\mu\text{g kg}^{-1}$, relative to controls, but no difference in mortality was observed in the two highest exposure concentrations, 1200 and 4800 $\mu\text{g kg}^{-1}$ (Veldhoen et al. 2012). It was postulated that high doses of EMB may induce early compensatory mechanisms in spot prawn compared to lower doses. No effect of EMB concentration on molting frequency was observed. EMB was detected in spot prawn tail muscle tissues in individuals exposed to all nominal concentrations. Eight-day exposures at the lowest EMB dose (100 $\mu\text{g kg}^{-1}$) altered mRNA abundance patterns within prawn muscle tissue relating to protein encoding for translation, transcription regulation, and apoptosis relative to control groups (Veldhoen et al. 2012).

It is important to understand the biological effect of EMB exposure on spot prawn survival and molting so the risk of exposure levels observed in the environment can be framed in an ecologically relevant context. The uptake mechanism of EMB by prawns is unknown; either passively through sediment and water, or directly through consumption of medicated feed or contaminated material. Studies with American lobsters have documented an aversion of individuals to SLICE[®] medicated pellets after an initial exposure (Burridge et al. 2004). American lobsters offered a choice between both medicated and unmedicated pellets and a natural food source (crabs and sea urchins) would reject pellets after the first exposure, preferring natural food sources (Waddy, Mercer, et al. 2007a). An unpublished study exposing medicated pellets to spot prawns and Dungeness crab (*Metacarcinus magister*) for seven days found that both species consumed low quantities of SLICE[®] pellets but would resume feeding when offered natural food sources (van Aggelen, Linssen, and Endris 2003). These studies indicate that marine decapods have an aversion to SLICE[®] pellets, but it is important to form more conclusive observations about whether this same effect extends to spot prawns. To have a better understanding regarding the impact of EMB exposure on non-target spot prawn both in-field assessments and controlled laboratory exposures need to be conducted.

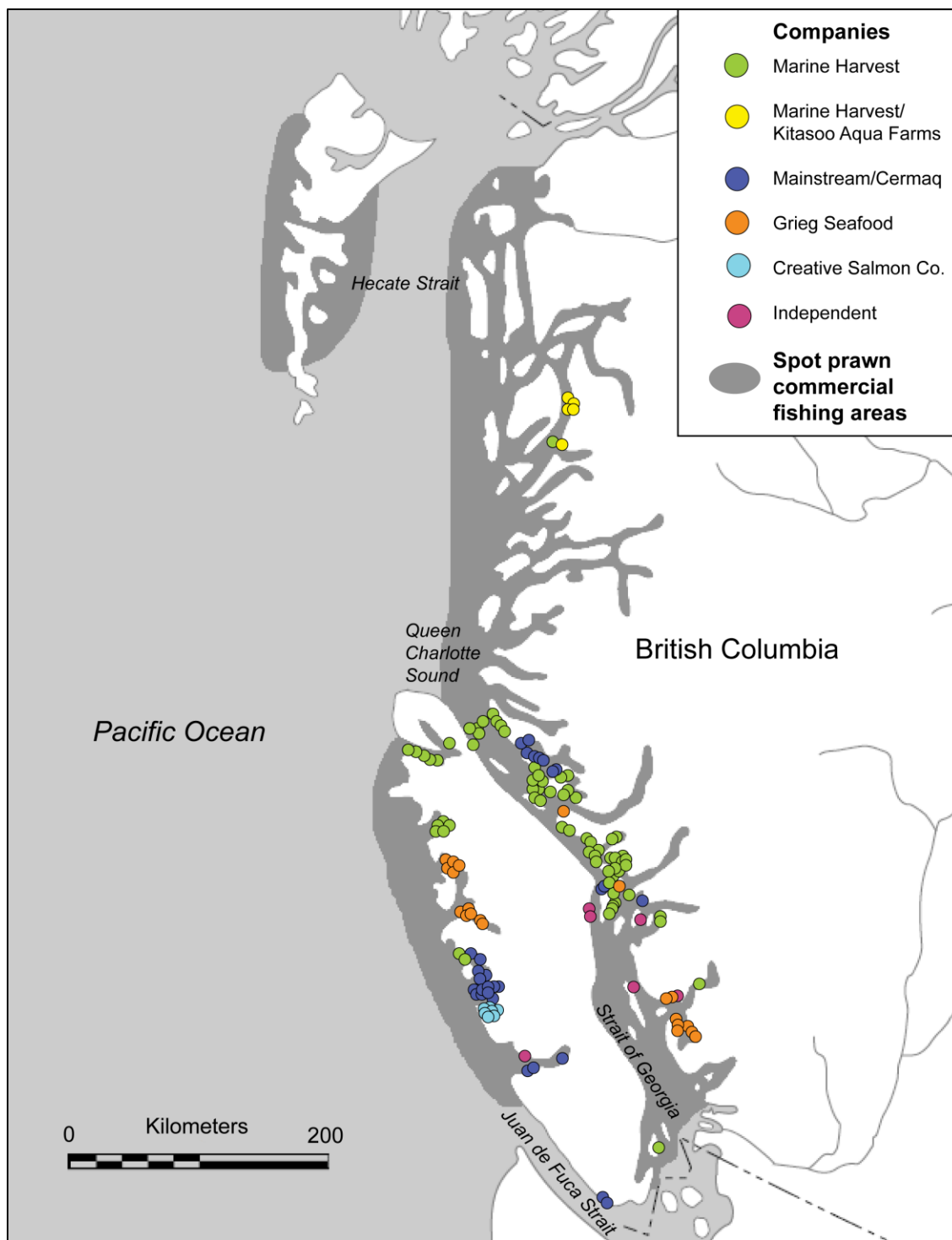


Figure 2: Open-net salmon farms in British Columbia and spot prawn commercial fishing areas

1.6 Thesis outline

In this thesis five questions are addressed, providing baseline data on the biological effects of EMB exposure on spot prawns:

- (1) Can EMB be detected in marine sediments at farm sites in the Broughton Archipelago following SLICE[®] exposure?
- (2) Does the catch abundance, size and sex distribution of spot prawns in proximity to salmon farms change after a SLICE[®] treatment in comparison to reference populations?
- (3) Will spot prawns consume SLICE[®] pellets and/or exhibit food aversion learning with EMB exposure?
- (4) Does mortality, molting occurrence, and EMB muscle tissue concentration of spot prawns increase with increasing EMB exposure concentration and duration?
- (5) Does EMB exposure affect a prawn's ability to detect and orientate towards food stimuli?

These questions were addressed through a field survey and several laboratory experiments. Chapter 2 describes a preliminary field survey undertaken in 2009 to investigate the EMB concentration in marine sediments and the catch abundance, size and sex distribution of spot prawns near four treating farms and four reference sites in the Broughton Archipelago, British Columbia, at different times before and after SLICE[®] treatment. In Chapter 3, the feeding response and acute toxicity of spot prawns to SLICE[®] pellets was investigated. In Chapters 4 and 5, experimental data are presented on the effects of EMB exposure through prepared sediment to spot prawn survival, molting ability, and food detection behavioral responses. In the final chapter the results of all chapters are discussed as well as the level of risk SLICE[®] treatments pose to commercially important spot prawn populations.

Chapter 2. Emamectin benzoate detection in marine sediment and spot prawn catch abundance and size distribution at active salmon farms in the Broughton Archipelago, BC

2.0 Introduction

The accumulation and persistence of emamectin benzoate (EMB) and its desmethyl metabolite in the marine sediment near farms treating with the chemotherapeutant SLICE[®] is dependent upon the farm's production capacity, the frequency and extent of SLICE[®] usage, the type of sediment and its physical-chemical properties, the sediment microorganism community, and the hydrodynamic (tides and currents) characteristics (DFO 2012b; Hurt et al 2006; Hand and Fleming 2007). GIS models are able to approximate the spread of waste from farms, which would include EMB residues tightly bound to particles, however, site specific monitoring is essential in understanding deposition fate as these models are not completely accurate when compared to in-field observations (Chamberlain et al. 2007). The majority of EMB accumulates in sediment directly underneath farms to 60 meters away, but can be detected up to 150 meters (Telfer et al. 2006; DFO 2012b). A larger EMB footprint could be possible as farm origin particles have been found in the water column up to 300 meters away (Schendel et al. 2004). Typical concentrations found near cages in Canada, the US, and Europe are within 0.5 – 35 $\mu\text{g kg}^{-1}$ and are highest several weeks after treatment. Four weeks post-treatment EMB residues decline through dilution and degradation to metabolites, however, EMB can still be detected in sediment 1.5 years post-treatment (DFO 2012b). Since EMB tightly binds to marine sediment, marine invertebrates associated with the benthos will be most at risk. Published reports of EMB concentrations in the marine environment near BC salmon farms has been limited and further investigation is warranted.

Considerable research has been conducted on the effects of nutrient inputs from salmon farms on community diversity and distribution. It is well established that benthic species diversity increases with distance from salmon farms (Telfer et al. 2006) up to a

distance of 300 meters in some sites (Neofitou, Vafidis, and Klaoudatos 2010), which is correlated with total organic carbon in sediment (Hyland et al. 2005). Macrobenthic communities near farms are often dominated by high abundances of opportunistic polychaetes (*Capitella* spp.), which are commonly found in polluted sediments (Brown, Gowen, and McLusky 1987; Weston 1990; Telfer et al. 2006; Neofitou, Vafidis, and Klaoudatos 2010). Only one published study has examined the effects of SLICE[®] treatments on the macrobenthic invertebrate community. Observations at several sampling locations show a decrease in biodiversity four months post-treatment, returning to pre-treatment levels within a year (Telfer et al. 2006). Salmon farms can attract mobile invertebrates, such as American lobsters (*Homarus americanus*) (Iwama 1991; Findlay, Watling, and Mayer 1995; Hargrave 2003), and pelagic wild fish species (Carss 1990; Tuya et al. 2006; Sudirman et al. 2009; Arechavala-Lopez et al. 2010) with nutrient subsidies from uneaten pellets. Commercial harvest of spot prawns regularly occurs near salmon farms, and there is some speculation that prawns aggregate near these sites, however no published studies have quantified this. The catch abundance of a closely related species, pink shrimp (*Pandalus borealis*), is highly correlated with the amount of particulate organic carbon in the environment (Ramseier et al. 2000). Waste products from salmon farms represent a significant contribution of nutrients to the benthic environment, so it is possible that spot prawns may be attracted to farm sites, putting them more at risk to SLICE[®] treatment.

In BC, EMB has been detected in the muscle tissue of wild-caught spot prawns up to 150 meters from a treating farm, 100 days post-treatment (DFO 2012b). The effects of SLICE[®] on spot prawn populations are likely to be localized in space, as the EMB footprint under salmon farms is restricted to several hundred meters. SLICE[®] effects on spot prawns are also likely to be temporally localized as spot prawns have a larval planktonic period so recruitment into the adult benthic population is not dependent on the local spawning stock. However, once in the adult population spot prawns have limited migration (Bower, Meyer, and Boutillier 1996; Boutillier and Bond 2000; Bower and Boutillier 1990) so it may be possible to observe the effects of SLICE[®] on adult size and sex distribution before and after a SLICE treatment.

This chapter reports the concentrations of EMB in the marine sediment near five treating salmon farms in the Broughton Archipelago, BC, and a preliminary pilot survey conducted to determine the catch abundance and size distributions of spot prawns caught in the vicinity of treating salmon farms.

2.1 Methods and materials

2.1.1 Site selection

Five farm sites and four reference sites were sampled for marine sediment and spot prawns in the Broughton Archipelago, BC, from January to April 2009 (Table 3; Figure 3). Two farm sites, F1 and F2, are located in Sutlej Channel and were sampled one week before, during, and one week after SLICE[®] treatment. Farm sites F3 and F4 are located in Knight Inlet and were sampled ten days and two months after SLICE[®] treatment. F5 is located in Fife Sound and was sampled two months after treatment for sediment only. As cooperation from the aquaculture industry was not possible at the time of sampling this resulted in some of the mismatch between survey work and the SLICE[®] treatment schedules on farms. Production fish age and stocking density were different across the five sites. Reference sites were chosen based on similar physical features which included similar water depths, currents and tidal flows to farm sites. Reference site R1 is an old farm tenure site that has been decommissioned since 2001 (Marty et al. 2010).

Table 3: Salmon farm and reference site information on sites sampled in the Broughton Archipelago in winter 2009. ¹Data obtained from Marty et al. (2010).

Site	2009 SLICE [®] dates	Prior SLICE [®] dates ¹	Depth sampled (m)	# production fish and year class ¹
F1	Feb 10-16	Jan 2006	88	~278,000 (1 st year class)
F2	Feb 10-16	Feb 2008	92	~280,000 (1 st year class)
F3	Jan 12-19	Jan 2007	79	~520,000 (2 nd year class)
F4	Jan 12-19	Jan 2007	77	~570,000 (2 nd year class)
F5	-	Nov 2008	51	~ 678,000 (2 nd year class)
R1	-	-	85	-
R2	-	-	85	-
R3	-	-	73	-
R4	-	-	97	-

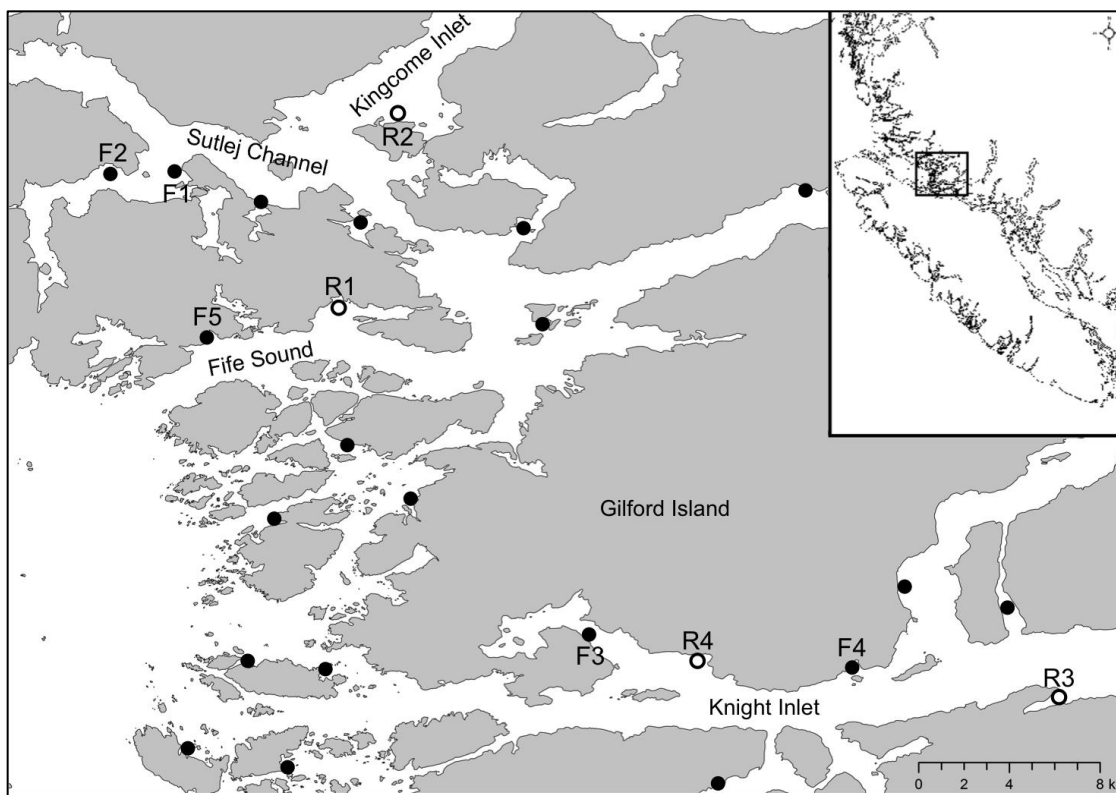


Figure 3: Study area sites in the Broughton Archipelago, British Columbia. F1 to F5 are farm sites (dark circles) and R1 to R4 are reference sites (open circles).

2.1.2 Sample collection

Four 70 cm diameter commercial prawn traps (2 x 3 cm size mesh), baited with commercial prawn bait, were set for three hours at each site between the hours of 8 am and 5 pm to collect spot prawns. Sediment samples were obtained using a petite ponar. Sediment was sub-sampled through the top flaps of the grabber to obtain sediment from the top five to ten centimetres. At farm sites traps were deployed as close to farms as possible and were reset at the same coordinates on subsequent visits. The four traps at each site were placed on one line with 15 m line between each trap. Prawns and other species were enumerated from each trap and then 25 prawns were randomly selected to retain. In some cases two hauls were required to obtain a sufficient number of prawn samples. Prawns retained were frozen to -20°C and later in the lab were weighed, the carapace length was measured and the sex was determined as either male, transitional, female or ovigerous female. To weigh ovigerous females the eggs were removed and weighed separately. A condition factor (Fulton's K) was calculated to assess body

condition of prawns.

$$\text{Condition Factor} = \text{Mass} / \text{Carapace Length}^{2.6} * 100$$

Fulton's K is commonly used to evaluate fish condition (Bolger and Connolly 1989; Lambert and Dutil 1997; Grant and Brown 1999) but has been used to assess condition for the blue king crab (*Paralithodes platypus*) and golden king crab (*Lithodes aequispinus*) (Hawkes, Meyers, and Shirley 1986) as well as the pink shrimp (*Pandalus borealis*) (Brillon, Lambert, and Dodson 2005).

Comparisons between spot prawn carapace length, mass, and body condition at sites were carried out in two ways: (1) spot prawns were classified by gender as either male or transitional stage and compared over the entire 2009 sampling period from January to April, and (2) spot prawn size and condition differences were compared temporally in reference to SLICE[®] treatment dates using a non gender-biased approach. These two approaches were necessary as low sample sizes prohibited one large overall analysis of different genders temporally.

2.1.3 Sediment analysis

Sediment samples were analyzed for EMB and its desmethyl metabolite at the Institute of Ocean Sciences, Sidney BC, according to the methodology of Ikonomou and SurrIDGE (2011). Final sample extracts were analyzed by high performance liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS). Instrumentation used included a Dionex P680 (Dionex, Sunnyvale, CA) HPLC system and Sciex API 5000 triple quadrupole mass spectrometer using multiple reaction monitoring (MRM) mode acquiring positive ions. HPLC parameters are in Table 4 and MRM mass spectrometer parameters in Table 5. One analysis method modification is the TurboIonSpray[®] optimized conditions used were: ionspray voltage: +5500V, curtain gas 20 au (arbitrary units), nebulizer gas flow 30 au, turbo-ion gas flow 20 au, collision gas 4 au, and turbo-ion gas temperature 500°C.

To provide an additional level of detection confirmation a second MRM transition was observed and chromatographic retention times were compared to authentic standards for further identification. Acquisition, quantitation, and data processing of all MRM signals were achieved using Analyst software (1.4.0). A nine-point calibration curve was

generated for each analyte and used for quantitation. Instrument linearity was demonstrated (r^2 were >0.99) over a concentration range of 0.020 - 25 ng/mL. Results were acceptable if percent recovery of performance standards were within 40 to 110 % of the expected value. Method quality assurance and control included two duplicates, one matrix spike, one lab blank, and bracketing verification standards in each sample batch of 20 samples. The level of detection (LOD) and level of quantification (LOQ) of EMB in sediments is respectively 0.053 and 0.177 $\mu\text{g kg}^{-1}$ and for the desmethyl metabolite, LOD and LOQ in sediment is 0.080 and 0.266 $\mu\text{g kg}^{-1}$. In prawn tissue samples the EMB LOD and LOQ is 0.041 and 0.090 $\mu\text{g kg}^{-1}$, and for the desmethyl metabolite is 0.058 and 0.142 $\mu\text{g kg}^{-1}$.

Table 4: High performance liquid chromatography (HPLC) parameters

HPLC Column:	Reverse phase C ₁₈ column (Xtera C ₁₈ -MS, 4.6 x 30 mm, 5 μ particle size, Waters) and guard column (Opti-Guard® C ₁₈ , 1mm, chromatographic specialties)
Mobile phase:	A: methanol B: 10 mM ammonium acetate in HPLC water
Gradient Program:	Time 0-2: 70% A, 30% B Time 2-2.1: 90% A, 10% B Time 11.8-11.9: 70% A, 30% B
Column Temp:	35°C \pm 0.1°C
Flow Rate:	0.3 mL / minute
Injection Volume:	10 μ L
Run Time:	15 minutes

Table 5: MRM mass spectrometer parameters

Analyte	Precursor ion (m/z) Qualifying ion (q)	Precursor Ion Identity	Precursor - product transition (m/z)	Retention Time (min)	Collision energy (V)	Declustering potential (V)
EMB	886.4	[M+H] ⁺	886.4→158.2	5.8	48	50
	886.4(q)	[M+H] ⁺	886.4→126.4			
Desmethyl metabolite	872.6	[M+H] ⁺	872.6→144.6	5.7	35	50
	872.6 (q)	[M+H] ⁺	872.6→112.4			
Terbutometon	226.5	[M+H] ⁺	226.5→170.0	3.2	25	20

2.1.4 Statistical analysis

All analyses and figures were completed using statistical computing software *R* (R Development Core Team 2011). Differences were considered to be significant if $\alpha < 0.05$. Model selection was undertaken using Akaike information criteria (AIC) and a likelihood ratio test to determine the best-fit models.

Catch per unit effort data of prawns captured from sites in Suttle Channel and Knight Inlet were fit separately using generalized linear mixed effects models (GLMM) fit by maximum likelihood with poisson distribution and repeated measures. Similar models were fit with binomial distribution to compare the male:transitional prawn sex ratio at the different sites and times. Study site was included as a random effect to account for potential correlation among individuals within sites, uneven sample sizes among sites, and repeated observations as sampling was conducted several times relative to the farm treatment schedule (Pinheiro and Bates 2000).

To compare carapace length, body mass and condition of prawns collected at the different sites, first an ANOVA was used to compare male and transitional prawns collected over the entire study duration at the different sites. While sample sizes of males and transitionals were unequal at the eight sites, equal variance and normality assumptions were met so an ANOVA was used. Females were excluded from this analysis due to low sample size (four caught total at six of the eight sites). A linear mixed effects model fit by maximum likelihood with repeated measures was then used to compare the length and mass of spot prawns at farm and reference sites sampled at different times. Sites in Knight Inlet and Suttle Channel were analyzed with separate linear mixed effects models due to different treatment schedules, sampling schedules, and because they are geographically separated in different inlets. These models did not differentiate between sex due to low sample sizes.

2.2 Results

2.2.1 EMB sediment concentrations

EMB was detected at the parts-per-billion (ppb) level at all salmon farm sites during all sampling occasions (Table 6). Laboratory percent recoveries of EMB were between 70 and 90% and thus it was not necessary to correct the data according to recovery levels. At

farm sites F1 and F2, EMB was detected in the samples obtained before the 2009 SLICE® treatment indicating EMB had persisted in the sediment since the previous SLICE® treatments; three years prior for site F1 and one year prior for site F2 (Marty, Saksida, and Quinn 2010). The desmethyl metabolite was only detected in the sediment at farm sites F1 and F2. EMB was detected at very low levels in the sediment at site F3, 525 m away from the farm edge both ten days and 2 months after treatment. No EMB or desmethyl metabolite residues were found at the sediment obtained at the reference locations.

2.2.2 Prawn abundance

The most common species captured in traps were spot prawns and coonstripe shrimp (*Pandalus hypsinotus*) (Table 7). Other species captured in low abundance included squat lobster (*Munida quadrispina*), tanner crab (*Chionoecetes bairdi*), great sculpin (*Myoxocephalus polyacanthocephalus*), walleye pollock (*Theragra chalcogramma*), spotted ratfish (*Hydrolagus colliei*), and unidentified hermit crabs (*Pagurus* spp.).

There was no difference in average prawn catch at farm and reference sites in Suttlej Channel (Figure 4; GLMM, $\chi^2 = 0.6695$, $df = 1$, $p = 0.413$). At both farm and reference sites more prawns were caught per trap in late January and early February before farms had applied SLICE® treatment than when the sites were sampled later on in February after SLICE® treatment (Figure 4; GLMM, $\chi^2 = 101.65$, $df = 2$, $p < 0.001$).

At farm sites in Knight Inlet the average catch of prawns in traps was greater two months after treatment (April) than ten days after treatment (January), while the average catch at reference sites did not change over the time period (Figure 5; GLMM, $\chi^2 = 43.846$, $df = 1$, $p < 0.001$).

2.2.3 Sex ratio

In Suttlej Channel there was no difference in the proportion of males to transitional stages over the sampling period, except at the time sampled during the treatment period when a greater ratio of males were captured at reference sites compared to farm sites (GLMM, $\chi^2 = 9.427$, $df = 2$, $p = 0.009$). In Knight Inlet at both farm and reference sites the proportion of males to transitional stages caught was greater ten days after treatment compared to two months after treatment (GLMM, $\chi^2 = 80.117$, $df = 1$, $p < 0.001$).

Table 6: Collection time, location and EMB and AB sediment concentrations from salmon farm and reference sites sampled in the Broughton Archipelago in 2009.

Site	2009 Sampling	Sampling time relative to treatment schedule	Distance/ direction from farm	Sediment		
				Observation	[EMB] ($\mu\text{g kg}^{-1}$)	[desmethyl methbolite] ($\mu\text{g kg}^{-1}$)
F1	Jan 28	13 days pre-2009 treatment (3 years post-treatment)	80m north	Fine grained	0.3031	< LOD
	Feb 16	Day 7 of treatment			0.8533	0.109
	Feb 25	9 days post treatment			0.4645	< LOD
F2	Jan 28	13 days pre-2009 treatment (1 year post-treatment)	130m east	Shell with fine grain	0.2020	< LOD
	Feb 4	6 days pre-treatment			0.1893	0.081
	Feb 16	Day 7 of treatment			0.1019	< LOD
	Feb 25	9 days post treatment			0.4158	0.088
	Mar 4	16 days post treatment			0.0984	< LOD
F3	Jan 30	12 days post treatment	525m east	Pebble and sand	0.0800	< LOD
	April 2	74 days post treatment			0.0570	< LOD
F4	Jan 31	13 days post treatment	20m east	Sand	0.0973	< LOD
	April 1	73 days post treatment			0.0726	< LOD
F5	Jan 26	2 months post treatment	50m west	Sand and shell	0.1290	<LOD
R1	Jan 28	-	>5000m	Shell and rocks	< LOD	< LOD
	Feb 15				< LOD	< LOD
	Feb 28				< LOD	< LOD
R2	Jan 27	-	>5000m	Clay and sand	< LOD	< LOD
	Feb 15				< LOD	< LOD
	Feb 26				< LOD	< LOD
R3	Jan 31	-	>5000m	Woody debris and sand	< LOD	< LOD
	April 1				< LOD	< LOD
R4	Jan 30	-	>5000m	Sand	< LOD	< LOD
	April 2				< LOD	< LOD

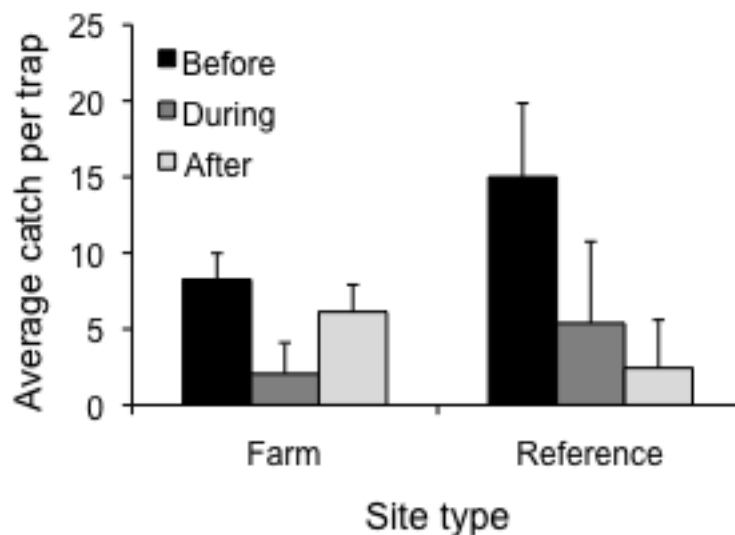


Figure 4: Average spot prawn catch per trap at farm and reference sites in Sutlej Channel before, during and after SLICE® treatments. Error bars are standard error. There was no difference in average prawn catch at farm and reference sites. At all sites more prawns were caught earlier on in season.

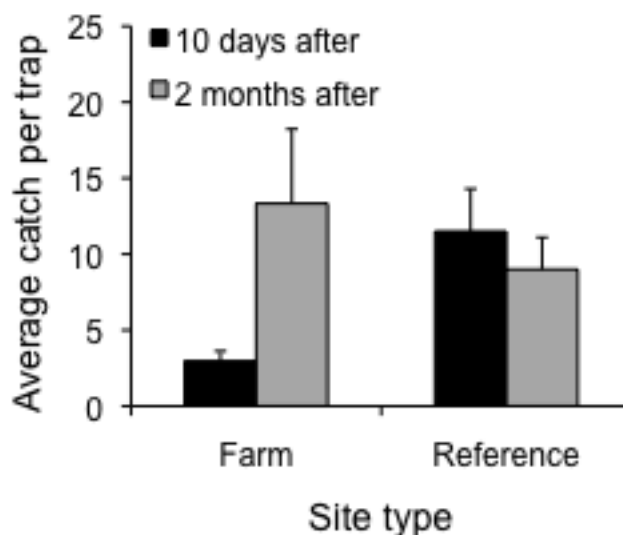


Figure 5: Average spot prawn catch per trap at farm and reference sites in Knight Inlet ten days and two months after SLICE treatments. Error bars are standard error. At farm sites average catch was greater two months after treatment (April) than ten days after treatment (January), while the average catch at reference sites did not change over the time period.

Table 7: Average catch of spot prawns and coonstripe shrimp at four farm sites treating with SLICE[®] and four reference sites in the Broughton Archipelago in 2009

Site	Treatment date	2009 sampling dates	# traps	Avg. catch per trap (\pm SE)	
				Spot prawn	Coonstripe shrimp
F1	Feb 10-16 2009	Jan 27	4	11 (2)	0 (0)
		Feb 16	12	2 (1)	< 1 (< 1)
		Feb 25	8	9 (3)	0.8 (< 1)
F2	Feb 10-16 2009	Feb 4	4	6 (2)	0 (0)
		Feb 16	8	2 (1)	0 (0.0)
		Feb 25	8	3 (2)	0 (0)
F3	Jan 12-19 2009	Jan 30	16	1 (1)	1 (< 1)
		Apr 2	8	2 (1)	< 1 (< 1)
F4	Jan 12-19 2009	Jan 31	12	5 (1)	0 (0)
		Apr 1	4	34 (6)	0 (0)
R1	-	Jan 27	4	21 (9)	0 (0)
		Feb 15	4	8 (5)	0 (0)
		Feb 28	4	18 (7)	0 (0)
R2	-	Jan 27	4	9 (3)	5 (1)
		Feb 15	4	3 (2)	3 (1)
		Feb 26	8	2 (1)	2 (1)
R3	-	Jan 25	4	15 (4)	1 (1)
		Apr 1	4	11 (1)	2 (1)
R4	-	Jan 29	4	8 (4)	1 (< 1)
		Apr 2	4	8 (4)	1 (1)

2.2.4 Gender biased size distribution and condition

On average male and transitional prawns collected near farm sites were significantly larger (carapace lengths and mass) than the same sex stages at reference sites over the entire study period from January to April 2009 (Figure 6; Males, N = 238 – carapace length ANOVA: $F_{1, 236} = 68.78$, $p < 0.001$; mass ANOVA: $F_{1, 236} = 49.46$, $p < 0.001$; Transitionals, N = 186 – carapace length ANOVA: $F_{1, 184} = 82.79$, $p < 0.001$; mass ANOVA: $F_{1, 184} = 60.62$, $p < 0.001$). However, the difference observed between male

prawns at farm and reference sites is attributed to the larger size of males at two of the farms sites, F1 and F2.

The carapace lengths and mass of male prawns collected at both F1 and F2 were significantly greater than male prawns collected from all other sites, but were not different from one another (Carapace length: ANOVA with pair wise comparisons using a bonferroni correction: $F_{7, 230} = 22.21$, $p < 0.001$; Mass: ANOVA with pair wise comparisons using a bonferroni correction: $F_{7, 230} = 22.77$, $p < 0.001$). The size of male prawns at farm sites F3 and F4 was not significantly different from male prawns collected at reference sites ($p > 0.05$), except prawns from F4 had significantly larger carapace lengths than prawns from R1 ($p < 0.05$), but not different masses.

Transitional stage prawns collected from all four farm sites were larger than transitional prawns from reference sites with the largest sizes at sites F2 and F1 (Carapace length: ANOVA with pair wise comparisons using a bonferroni correction: $F_{7,178} = 17.11$, $p < 0.001$; Mass: ANOVA with pair wise comparisons using a bonferroni correction: $F_{7,178} = 23.25$, $p < 0.001$).

Transitional stages had better condition at farm sites than reference sites (Table 8; ANOVA: $F_{1, 184} = 4.205$, $p = 0.042$), while there was no difference for male stages (ANOVA: $F_{1, 236} = 1.675$, $p = 0.197$). Farm site F4 had the lowest condition males while reference site R3 had the highest condition male and transitional stages overall.

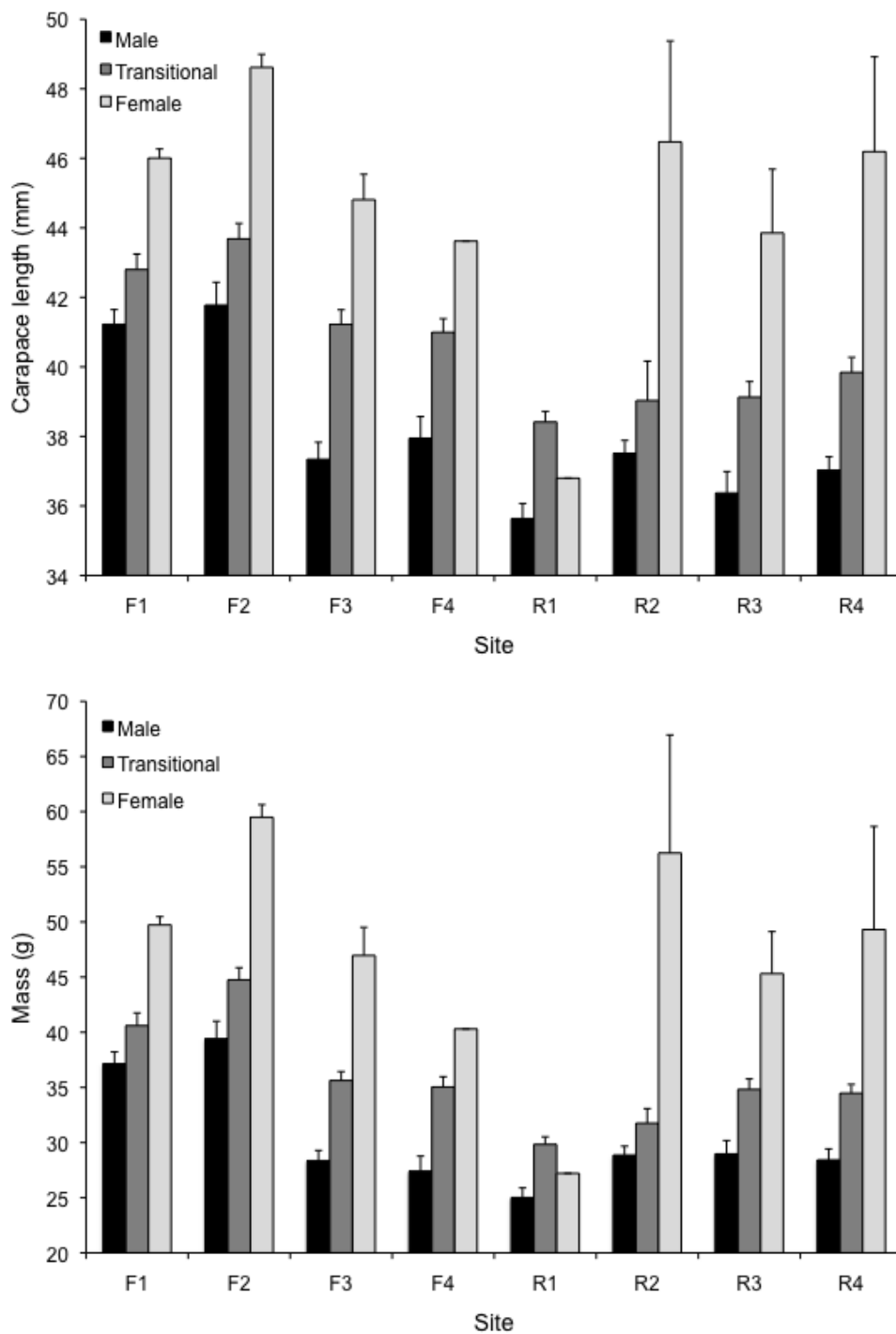


Figure 6: Average carapace length and mass of male, transitional, and female spot prawns collected in 2009 from the Broughton Archipelago at four farms sites (F1 –F4) treating with SLICE[®] and four reference sites (R1 – R4). Error bars are standard error. On average male and transitional prawns collected near farm sites were significantly larger (carapace lengths and mass) than the same sex stages at reference sites over the entire study period from January to April 2009.

Table 8: Condition factor, number of brooding females and the total mass of eggs of spot prawns collected from January – April 2009 in the Broughton Archipelago.

Site	Male		Transitional		Female			
	N	Condition factor \pm SE	N	Condition factor \pm SE	N	Condition factor \pm SE	No. brooding	Brood mass (g) \pm SE
F1	41	0.23 \pm 0.002	33	0.23 \pm 0.002	2	0.24 \pm <0.001	2	8.00 \pm 1.24
F2	23	0.24 \pm 0.002	24	0.24 \pm 0.002	34	0.24 \pm 0.001	34	9.32 \pm 0.33
F3	25	0.23 \pm 0.003	14	0.23 \pm 0.006	3	0.24 \pm 0.006	3	8.15 \pm 0.16
F4	22	0.21 \pm 0.003	27	0.22 \pm 0.002	1	0.22	0	NA
R1	47	0.23 \pm 0.002	27	0.23 \pm 0.002	1	0.23	0	NA
R2	41	0.23 \pm 0.003	12	0.23 \pm 0.012	3	0.25 \pm 0.007	3	6.06 \pm 2.69
R3	16	0.25 \pm 0.007	26	0.25 \pm 0.005	8	0.24 \pm 0.008	5	8.06 \pm 0.41
R4	23	0.24 \pm 0.005	23	0.24 \pm 0.004	4	0.22 \pm 0.013	2	9.46 \pm 2.00

2.2.5 Size distribution at different sampling times in Sutlej Channel

There was no difference in the average carapace length between spot prawns collected before and during SLICE[®] treatments in Sutlej Channel (Likelihood Ratio Test, $\chi^2 = 2.6750$, $p = 0.102$) so they were pooled and compared to prawns collected one week after treatment. Prawns collected at both farm (F1 and F2) and reference sites (R1 and R2) one week after SLICE[®] treatment had larger carapace lengths than prawns collected before and during treatment (Figure 7; Linear mixed effect model, $\chi^2 = 8.5113$, $df = 1$, $p = 0.014$). There was no difference between the mass of prawns collected during the different sampling times (Figure 7; Linear mixed effect model, $\chi^2 = 4.8209$, $df = 2$, $p = 0.090$). Prawns sampled at farms were significantly larger and heavier than prawns collected at reference sites (Figure 7; Length: Linear mixed effect model, $\chi^2 = 8.2901$, $df = 1$, $p = 0.004$; Mass: Linear mixed effect model, $\chi^2 = 6.4809$, $df = 1$, $p = 0.011$), but there was no difference in the condition of prawns collected at farm and reference sites (Table 9; Linear mixed effect model, $\chi^2 = 2.578$, $df = 1$, $p = 0.108$) or at different times ($p = 0.949$).

2.2.6 Size distribution at different sampling times in Knight Inlet

At farm sites F3 and F4, prawns collected two months after treatment had larger carapace lengths compared to prawns collected ten days after treatment (Figure 7; Linear mixed effect model, $\chi^2 = 9.940$, $df = 1$, $p = 0.002$). The size of prawns caught at reference sites R3 and R4 did not change over the two collection periods. Comparing mass of prawns yielded the same results as carapace length. The condition of spot prawns collected at reference sites in Knight Inlet was higher than prawns collected at farm sites (Table 9; Linear mixed effect model, $\chi^2 = 4.706$, $df = 1$, $p = 0.030$) and did not depend on time captured ($p = 0.655$).

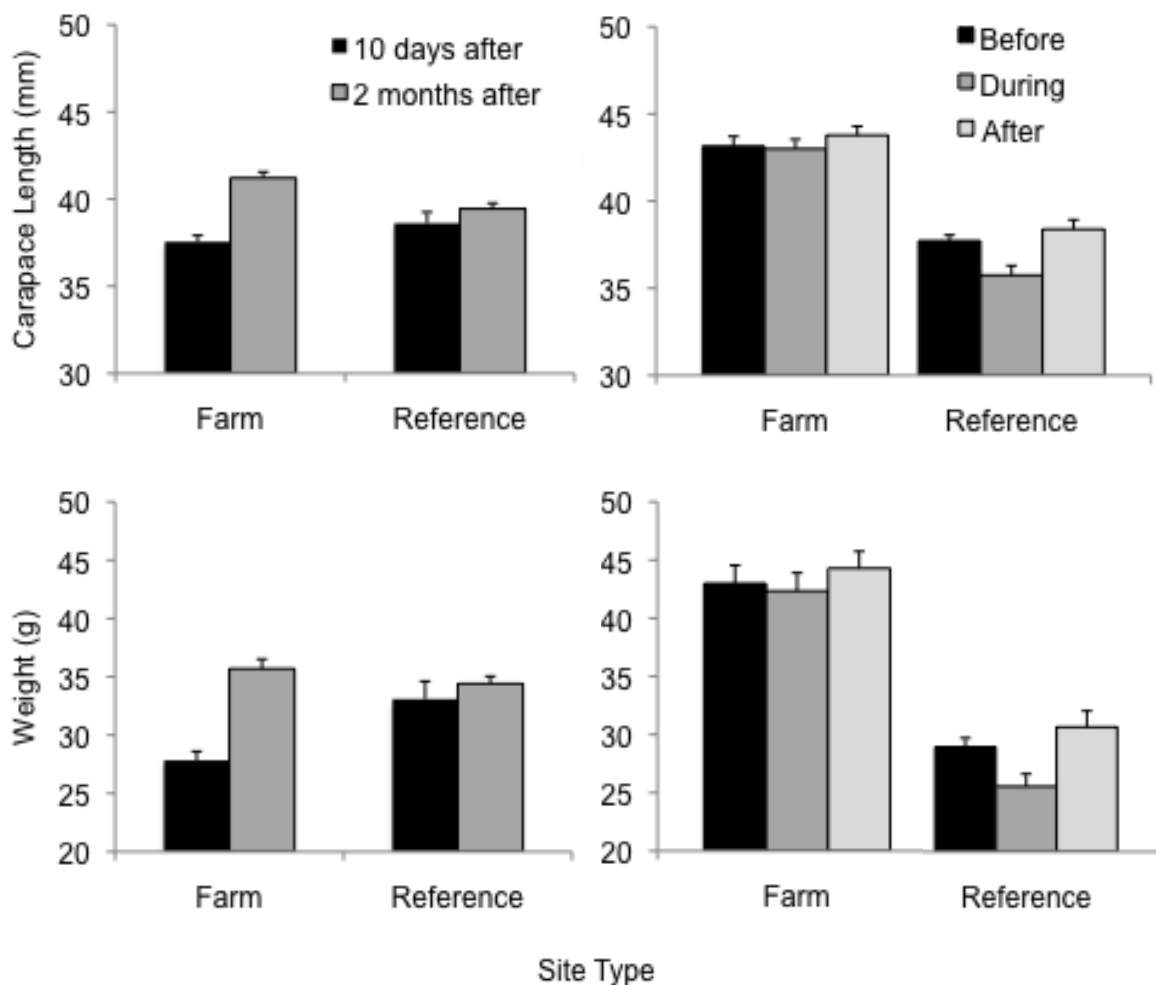


Figure 7: Average carapace length and mass of spot prawns collected from salmon farm sites and reference sites in Knight Inlet (left panels) and Sutlej Channel (right panels) in the Broughton Archipelago in 2009. Error bars are standard error. Knight Inlet: At farm sites, prawns collected two months after treatment were larger than prawns collected ten days after treatment, while the size of prawns caught at reference sites did not change over the survey period. Sutlej Channel: Prawns sampled at farms were significantly larger than prawns collected at reference sites. Prawns collected at both farm and reference sites one week after SLICE[®] treatment were larger than prawns collected before and during treatment.

Table 9: Condition factor and gender of spot prawns captured at farm and reference sites in the Broughton Archipelago, BC.

Site	Dates	No. captured at site	Condition factor (SE)	No. caught per gender		
				Male	Transitional	Female
F1	Jan 27 th	25	0.23 (0.002)	17	8	0
	Feb 16 th	26	0.23 (0.002)	11	15	0
	Feb 25 th	25	0.23 (0.002)	13	10	2
F2	Feb 4 th	22	0.24 (0.002)	6	5	11
	Feb 16 th	15	0.24 (0.002)	4	4	7
	Feb 25 th	24	0.24 (0.002)	9	5	10
F3	Jan 30 th	19	0.23 (0.004)	19	0	0
	April 2 nd	23	0.23 (0.004)	6	14	3
F4	Jan 31 st	25	0.21 (0.003)	19	6	0
	April 1 st	25	0.22 (0.002)	3	21	1
R1	Jan 27 th	25	0.23 (0.003)	17	8	0
	Feb 15 th	25	0.22 (0.002)	21	4	0
	Feb 28 th	25	0.23 (0.002)	9	15	1
R2	Jan 27 th	25	0.23 (0.002)	17	7	1
	Feb 15 th	13	0.25 (0.010)	11	2	0
	Feb 26 th	18	0.23 (0.006)	13	3	2
R3	Jan 25 th	25	0.25 (0.006)	12	6	7
	April 1 st	25	0.26 (0.005)	4	20	1
R4	Jan 29 th	25	0.24 (0.005)	19	3	3
	April 2 nd	25	0.23 (0.004)	4	20	1

2.3 Discussion

This study had demonstrated that EMB and its desmethyl metabolite can be detected in marine sediment in the vicinity of treating net pens, at distances up to 500 m away. The highest concentration of EMB residues detected was 0.853 $\mu\text{g kg}^{-1}$, 80 m away from farm site F1 during the treatment period. Levels of EMB detected at each farm site did not vary much over the sampling period and is likely because the majority of sampling locations were located more than 60 m from farms, outside of the main depositional footprint. The EMB concentrations found in the sediment are consistent with levels observed in the depositional area near salmon farms in BC (DFO 2012b), Atlantic Canada (Endris per. comm. 2011; Parker and Mallory 2003), Scotland (Telfer et al. 2006; SPAH 2004; Thomas 2004; 2005; 2007), United States, France and Norway (Endris per.

comm. 2011). The levels of EMB found in the vicinity of treating farms in the Broughton Archipelago was well below the standard allowable limits ($0.056 - 0.115 \text{ mg kg}^{-1}$) that can be present in marine sediment, which are derived from acute toxicity tests with sensitive sediment dwelling species *Corophium volutator* and *Arenicola marina* (McHenery 1999). However, in this study it was not possible to sample directly underneath net pens where the highest concentrations of EMB have been observed to occur.

Persistence of EMB in marine sediment has been measured for up to 175 days (Bright and Dionne 2005) and 1.5 years in the vicinity of treating salmon farms in BC (DFO 2012b). This survey observed EMB to persist in the sediment at one salmon farm (F1) up to three years after the last treatment, and at another farm (F2), one year after the last treatment. The maximum persistence time of EMB in marine sediment *in situ* still has not been determined as field studies measuring levels around salmon farms usually conclude after several hundred days, when EMB is still detected in the sediment.

Prawn catch abundance in traps in Sutlej Channel had a similar declining pattern with time at both farm and reference sites, so no effect of farm activity could be detected on catch abundance. However, in Knight Inlet while no difference in average prawn catch was observed at reference sites over time, more prawns were caught per unit effort two months after SLICE[®] treatment at farm sites compared to ten days after. Lower catch abundances could be attributed to migration, mortality, and/or the patchy distribution of spot prawns (Orensanz et al. 1998). Without more longitudinal and spatial assessment it is difficult to make correlations between SLICE[®] treatment schedules, catch abundances, and prawn populations in the vicinity of salmon farms.

When comparing prawn size distribution over the entire sampling period from January to April using a gender biased approach, it was observed that male and transitional stage prawns captured at farm sites were generally larger than the same gender stages at reference sites. These greater sizes attained at farm sites may be attributed to direct food subsidies from farms in the form of uneaten pellets or indirectly from prawn prey items attracted to farm sites. Between one and 17 percent of delivered feed goes uneaten and falls to the ocean floor (Brooks and Mahnken 2003; Chamberlain et al. 2007; Strain and Hargrave 2005). Cubitt et al (2008) calculated that in 2005

between 1,097 – 18,863 tonnes of uneaten feed was lost directly to the marine environment in BC. Depending on individual farm production status this could result in a tonne to potentially over a hundred tonnes of feed deposited per year at a single farm, representing a significant source of food for pelagic and benthic species inhabiting the area. An increase in weight, total length, and body condition of wild fish species captured near aquaculture sites compared to reference sites has been observed in the Mediterranean (Fernandez-Jover et al. 2007; Arechavala-Lopez et al. 2010) and Norway (Skog et al. 2003). Better body condition can significantly increase fish spawning and hatching success, as individuals can invest in greater fecundity and the production of high quality eggs and sperm (Izquierdo, Fernandez-Palacios, and Tacon 2001). The relationship between greater carapace length of females and the number of eggs produced is established in species closely related to spot prawns, *Pandalus borealis* (Shumway et al. 1985; Parsons and Tucker 1986; Brillon, Lambert, and Dodson 2005) and *Pandalus jordani* (Hannah, Jones, and Long 1995). While comparison of prawn females during the field survey was not possible due to the limited number captured, better condition was observed in transitional stages at farm sites compared to reference sites. In addition 42% of the prawns caught at farm site F2 were ovigerous female spot prawns, an anomaly that was not observed at any other site.

Using a non-gender biased temporal comparison it was determined that the average carapace length, mass, and condition of prawns collected from farm sites in Suttlej Channel was not different from reference sites. At all Suttlej Channel sites carapace length was greater in prawns collected in the final sampling period compared to the two earlier sampling periods, which may be an indication of regular growth of individuals in the population. Observations from sites sampled in Knight Inlet demonstrate that while the carapace length and mass of prawns collected at the two reference sites did not change over the two collection periods spaced 1.5 months apart, the size of prawns from the two farm sites was significantly larger two months after treatment compared to ten days after treatment. Over a period of several months there seems to be a shift in the population size distribution to larger prawns indicating that either small prawns become harder to catch or are absent from the population because of migration or mortality. Based on the limited sampling regime (temporally and spatially) it

is difficult to correlate these results with the effects of EMB, however, it is important to stipulate that changes in the population size distribution around farms seem to be occurring. It is important to note that the majority of immature males would be excluded from this survey as they would fall through the mesh upon trap retrieval, however, this bias would be consistent across each sampling event and site.

While salmon farms are a predominant industry in the Broughton Archipelago there is also an intensive commercial prawn fishery. Due to minimum size restrictions on prawns retained, the spot prawn fishery is likely to have a measurable influence on the size distribution of spot prawn in an area as prawns with a carapace length greater than 33 mm are retained and smaller size and ovigerous females released. The fishing pressure at each site will vary depending on the number of fishermen targeting those sites, their gear capacity, and the duration of fishing at the site over the season. Information regarding the annual fishing pressure at each site sampled during this survey is unknown and it is possible that it may be causing the size differences observed. This survey was not conducted during the active commercial fishery, which would commence at the beginning of May; however, further studies and fisheries and aquaculture management should consider the interactive effects between both commercial and recreational fishing and the aquaculture industry on spot prawn population size distribution and abundance.

Though the field component of this research was preliminary, there is some evidence that coastal aquaculture may influence spot prawn populations directly. Initial findings demonstrate that larger male and transitional stages reside in areas near salmon farms sites compared to reference sites more than five kilometres away. In addition, immediately following SLICE[®] treatment at farms sampled in Knight Inlet, the catch abundance was depressed and a smaller size distribution of prawns was observed compared to sampling two months later at the same sites, a trend not observed at reference sites. The scale of this influence or how far ranging remains unknown. Aquaculture operations have been shown to influence fish biomass and abundance positively up to several kilometers away (Machias et al. 2005; Arechavala-Lopez et al. 2010). It is important to determine whether spot prawns aggregate near salmon farms, if prawns are benefiting from food subsidies at aquaculture sites by attaining larger sizes,

and if fishermen are targeting areas in the vicinity of farms to benefit from the net migration of more prawns into the area.

Coordinated sea lice management at salmon farms in the Broughton Archipelago has resulted in multiple farms in close proximity applying SLICE[®] treatments at the same time every other year, which may be amplifying both the spatial and temporal footprint of EMB. How long EMB can persist in the marine sediment at salmon farms remains to be unknown, though this research suggests it may be much longer than previously measured. Accurately understanding EMB persistence in marine sediment is crucial information in assessing the sustainability of the current SLICE[®] treatment regime in BC and determining whether benthic habitat and non-target populations may be exposed on a larger scale.

Chapter 3. Response of spot prawns to emamectin benzoate medicated feed exposure and toxicity effects on mortality and molting

3.0 Introduction

During sea lice outbreaks, salmon farms in BC administer SLICE[®] treatments over a seven-day period in which a dose of 50 µg EMB per kilogram of fish per day is delivered through feed. The concentration of EMB in SLICE[®] treated pellets ranges between 1 to 25 µg g⁻¹, with the most common dosage being 10 µg g⁻¹. During SLICE[®] treatments uneaten medicated pellets can accumulate in the benthic environment proximate to salmon farms (SEPA 2004a; Telfer et al. 2006); between one and 17 percent of delivered feed goes uneaten and falls to the ocean floor (Brooks and Mahnken 2003; Chamberlain et al. 2007; Strain and Hargrave 2005). On Canada's Atlantic coast American lobsters and other invertebrates have been observed aggregating near salmon farms (Iwama 1991; Findlay, Watling, and Mayer 1995); a trophic subsidy from uneaten fish feed pellets is the suggested attractant to farm sites (Hargrave 2003). Spot prawns (*Pandalus platyceros*) on the Pacific coast that inhabit areas near salmon aquaculture operations may be attracted to these sites year round because of nutrient inputs. If spot prawns consume salmon farm feed pellets the EMB exposure risk of wild populations during treatment applications may increase. It is important to determine if spot prawns will avoid consuming medicated SLICE[®] pellets, and if their level of starvation will affect consumption.

Learned aversion behaviours to food have been documented in the hermit crab *Pagurus granosimanus* and crayfish *Procambarus clarkii*, when a novel food source offered in combination with lithium chloride injection that induced illness caused individuals in future offerings of the same food to avoid consuming it (Wight, Francis, and Eldridge 1990; Arzuffi, Salinas-Loera, and Racotta 2000). This ability to avoid toxic foods after an initial exposure would be beneficial to animals that are generalist feeders, such as most crustaceans.

Several studies on both marine and terrestrial animals demonstrate avoidance of EMB medicated pellets, especially after an initial exposure. American lobsters (*Homarus americanus*) avoided consuming SLICE[®] medicated pellets after an initial exposure, especially high EMB doses, but resumed feeding when switched to a diet of unmedicated pellets or shrimp (Burrige et al. 2004). Lobsters offered a choice between both medicated and unmedicated pellets and a natural food source (crabs and sea urchins) would begin to reject pellets after the first exposure, preferring natural food sources (Waddy, Mercer, et al. 2007a). Spot prawns and dungeness crab (*Metacarcinus magistar*) exposed to medicated SLICE[®] pellets for seven days consumed low quantities, 0.001 – 0.023 g (0.006 – 0.2% body weight) for prawns and 0.01 – 1.68 g (0.001 – 0.3% body weight) for crabs, but would resume feeding at higher levels when offered a preferred diet of squid and trout (van Aggelen et al. 2003). Common responses in insects exposed to EMB include cessation of feeding (Ioriatti et al. 2009) and salmon and trout receiving higher than recommended doses of EMB had a decrease in appetite (Roy et al. 2000). Mallard ducks (*Anas platyrhynchos*) and northern bobwhite quail (*Colinus virginianus*) exposed to EMB displayed signs of toxicity including reduction of food consumption, though these symptoms declined once EMB exposure ceased (Chukwudebe et al. 1998). While it may be possible that EMB exposure can cause a physiological response by reducing an individual's ability to feed, these studies suggest that either the effect of EMB is transient or there is a reduction in food consumption as a result of food preference and aversion to EMB.

This chapter reports the results of a 13-day acute toxicity test of EMB (LC₅₀) and aims to address if spot prawns exhibit food aversion learning to SLICE[®] pellets, if there is a dose response, and whether a period of starvation prior to EMB exposure alters the amount of SLICE[®] pellets consumed.

3.1 Materials and methods

Salmon pellets medicated with a range of EMB concentrations were provided to wild caught adult prawns seven times over a 13-day period to estimate consumption rates. There were two control groups. One group was offered unmedicated salmon pellets and the other was offered a preferred diet of squid. Squid was offered two times, on days 15

and 17, to all treatment groups following the 13-day period. To test if level of starvation affected EMB consumption rates, prawns were subjected to either a seven day starvation period or a seven day period where they were fed unmedicated salmon pellets prior to consumption testing. Consumption was estimated by subtracting the weight of uneaten pellets remaining after four hours from the total weight of food offered.

3.1.1 Specimen collection and testing facilities

Pandalus platyceros were collected with commercial traps from Patricia Bay in Saanich Inlet, Victoria BC on April 7th 2011. The collection site had no known history of EMB use. Traps were baited with commercial prawn bait and set for three hours before being hauled. Prawns were held on ship in aerated holding tanks for 15 minutes, transferred to a 500 L aerated tank on a truck and transported within one hour to the University of Victoria Outdoor Aquatics Facility. Prawns were held communally in a dark 1000 L holding tank with shelter (one prawn per litre water) and pre-conditioned to 4 mm size pellets, which they were fed every other day, for a nine-day acclimation period. Flow through seawater obtained from Cattle Point, BC, was filtered, UV-treated and maintained at a temperature of 10.5°C and 30 ppt salinity.

3.1.2 Feed pellet preparation

Premix 0.2% SLICE[®] was obtained from Intervet Schering-Plough Animal Health and pure emamectin benzoate standard (technical grade) was purchased from Sigma Aldrich. Five treatments were prepared which included a feed pellet control, a squid control, and 1, 10 and 100 $\mu\text{g g}^{-1}$ EMB feed pellets. Pellets were prepared according to Burrige et al. (2004) and Roy et al. (2000). Fish feed pellets (4 mm, Skretting Vitalis) were weighed into 1 L plastic bags and the appropriate amount of SLICE[®] premix (0.2%) was added, plus an additional 10 % of premix. Additional premix is required to obtain the desired concentrations of EMB (Burrige et al. 2004), compensating for material lost in the preparation and transfer process. The 1 and 10 $\mu\text{g g}^{-1}$ pellets were prepared by adding the appropriate amount of SLICE[®] premix and 100 $\mu\text{g g}^{-1}$ pellets were prepared by adding pure emamectin benzoate to the premix to reduce the amount of non-active material in the pellets. SLICE[®] was added in three aliquots with two minutes of agitating

and turning the sealed bag after each addition. After the addition of SLICE[®] the pellets were coated in warmed herring oil (35 – 40°C), mixed for a further ten minutes and left overnight at 4°C to allow the oil to absorb. The same procedure was followed for preparing the unmedicated control pellets except SLICE[®] premix was not added.

Prepared feed pellets were frozen until individual portions were measured into one dram glass vials (average portion 0.442 g ± 0.032 SD). Squid, purchased from a grocery store, were cut into pieces equivalent to the average pellet size, and individual portions weighed into one dram glass vials (average portion 0.448 g ± 0.045 SD). Portions were between 1.5 – 2% of the average prawn body weight. Squid was chosen as a preferred diet alternative as it could be cut into pieces the same shape and size as fish feed pellets that maintain shape when submerged in water. The only diet study on spot prawns is unpublished data from Berkeley (1929) where polychaete worms, fish scales, sponge spicules, amphipods and unidentified crustaceans were found in prawn stomach contents (Butler 1967). Spot prawns are both scavengers and predators so it is possible that squid or other molluscs are part of their diet.

3.1.3 Feed pellet analysis

For analysis feed pellets were shipped frozen to Eurofin Avtech Pharmservices and analyzed in triplicate. EMB was extracted from 20 g of ground medicated feed samples homogenized into 10 mL of water and 100 mL of methanol for 60 minutes in a centrifuge tube. Samples were then shaken on a wrist action shaker for 60 minutes and sonicated for 15 minutes at 45 – 50°C, followed by one repeated shaking and sonication and the addition of 40 mL of water. Contents were allowed to settle overnight. Next 25 mL of supernatant from each sample was pipetted into the filtration column/solid phase extraction (SPE) cartridges (Isolute 2 g C18 pre-conditioned with methanol and water) followed by 2 X 10mL methanol:water (70:30) and 10 mL THF:water (40:60). EMB was eluted from each cartridge into test tubes with two separate 5 mL aliquots of 2% ammonium hydroxide in ethanol. Samples were placed into a sample concentrator heated to 45 – 50°C, evaporated to dryness under a stream of nitrogen, reconstituted with 2 mL methanol, and vortexed for 30 seconds. For the 100 µg g⁻¹ sample the reconstitution volume was adjusted to 12 mL to account for the high sample concentration. Samples

were filtered through a 0.45 μ syringe filter and analyzed using a reverse-phase gradient high performance liquid chromatography (HPLC) system with a UV detector (Waters 2695 Separations Module autosampler and pump and Waters 2487 Dual λ Absorbance UV detector; Table 10).

Table 10: High performance liquid chromatography (HPLC) parameters

HPLC Column:	Zorbax RX-C8, 250 x 4.6 mm, 5 μ m
Mobile phase:	A: 60:40/0.1% Phosphoric Acid:Acetonitrile B: 10:90/0.1% Phosphoric Acid:Acetonitrile C: 50:50 Tetrahydrofuran:Acetonitrile
Gradient Program:	Time 0: 100% A, Flow 1.0mL/minute Time 20: 45% A: 55% B, Flow 1.0mL/minute Time 20.1: 100% C, Flow 1.0mL/minute Time 30: 100% Flow 1.0mL/minute Time 30.1: 100% A, Flow 1.0mL/minute
Column Temp:	50°C \pm 5°C
Flow Rate:	1.0 mL / minute
Detection:	Ultraviolet, 244 nm
Injection Volume:	20 μ L
Run Time:	45 minutes

3.1.4 Exposure to emamectin benzoate

Four hundred prawns were haphazardly chosen and split evenly between two 100 L holding tanks kept outside (with a small clear patch in the lid to maintain a seasonal photoperiod) with continuously flowing seawater maintained at 10.5°C and 30 ppt salinity (Figure 8 and Figure 9). Prawns were held in tanks communally with shelter. Prawns were in male and transitional stages and were on average 33.23 mm (\pm 0.12 SE) carapace length (length from the base of the eye socket to the dorsal posterior of the carapace). Prawns in the two 100 L tanks were randomly assigned either a seven-day starvation treatment or fed 4 mm unmedicated feed pellets until satiation every day for seven days. At the end of seven days, 150 prawns from each of the two treatments were transferred and housed in individual 18x18x9 cm polypropylene plastic containers, and allowed to acclimate for 24-hours before the EMB testing period began, during which

time they were starved. Containers had nine 1.25 cm diameter holes on all sides to allow water flow and a small stone to anchor the container. To prevent the passing of feed from one container to another, plastic vexar 0.5 cm² mesh was glued over the holes. Thirty starved prawns and 30 satiated prawns were assigned randomly to each of five food treatments: unmedicated pellet, squid, 1 $\mu\text{g g}^{-1}$ EMB pellet, 10 $\mu\text{g g}^{-1}$ EMB pellet, or 100 $\mu\text{g g}^{-1}$ EMB pellets. Individual plastic containers were stacked five high, within five 100-litre tanks with filtered, flowing seawater (10.5°C and 30 ppt). Treatments were arranged by randomized block design with 60 containers per tank. This resulted in six of each treatment in each of the five 100 L tanks.

Prawns were fed their respective treatments, seven times every other day (13-day period) for four hours. After the 13-day period all prawns were then fed a preferred diet of squid twice, with feedings separated by a day.

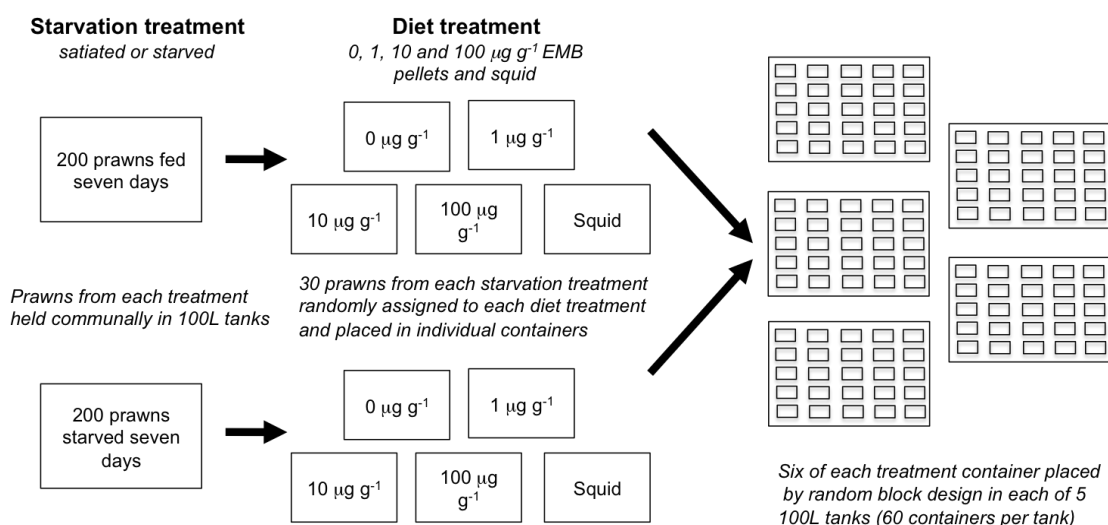


Figure 8: Randomized block design experimental layout for starvation and diet treatment of spot prawns.

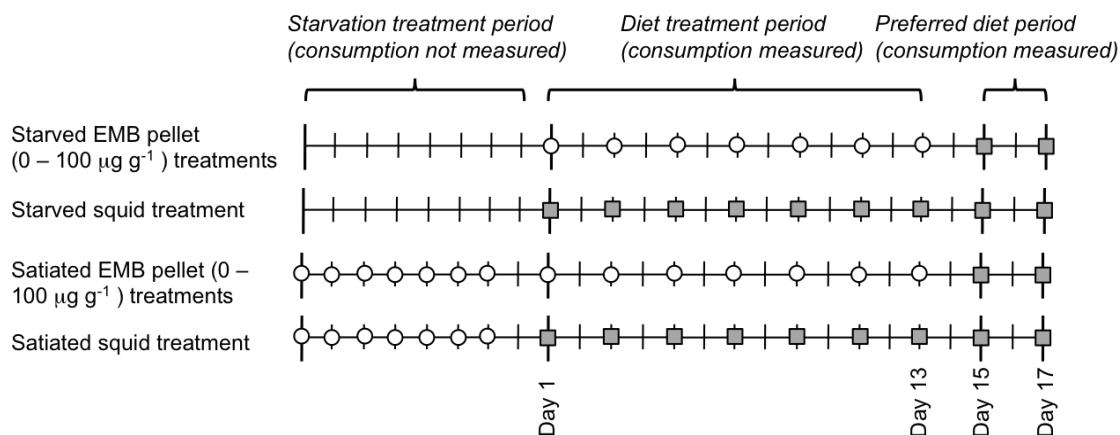


Figure 9: Experimental timeline for the pre-experiment starvation treatment period, diet treatment period and preferred diet period. ○ = 0 – 100 $\mu\text{g g}^{-1}$ pellet feeding, ■ = squid feeding, | = no feeding

At the end of each four-hour feeding period the remaining feed was retrieved with tweezers. All recovered food was dried at 60°C for 48 hours and weighed. A correction factor obtained by calibration trials was applied to the dry weights to account for water uptake in the experiment and removal of moisture from the drying oven. It is important to emphasize this is not a direct measure of food consumed, but rather the food recovered. It is possible that food consumption was overestimated if not all food was recovered because it was torn apart by the prawn or regurgitated. In addition to measuring food retrieved, prawns were observed during feeding periods for molting, morbidity, and mortality. Molting was an important observation as crustaceans exhibit depressed feeding rates in the days before, during and following ecdysis (Lloyd and Yonge 1947; Strong and Daborn 1980; Lipcius and Herrnkind 1982; O'Halloran and O'Dor 1988). In addition, exposure to emamectin benzoate was found to induce premature molting in American lobsters (Waddy et al. 2002; Waddy, Merritt, et al. 2007b) so the rate of molting was compared between different diet treatment groups.

At the conclusion of the experiment all prawns were weighed, their carapace length measured, sex determined and body condition assessed including shell rigidity (as a proxy for molting status), presence of black spotting, scarring, eye damage, and broken and missing appendages.

3.1.5 Statistical analysis

Data were analyzed using R (R Development Core Team 2011). Differences were considered to be significant if $\alpha < 0.05$. Model selection was undertaken using Akaike information criterion (AIC) and a likelihood ratio test to determine the best-fit models. Four prawns that died during the experimental period were excluded from all statistical analysis.

A linear mixed effects model fit by maximum likelihood with repeated measures was used to compare the response (daily food consumption in grams) by prawns over Day 1 to 13 of the experiment (lme4 package, Bates et al. 2011). Separate linear mixed effects models were used to analyze squid and pellet treatment groups as they had opposite severely skewed distributions. The response variable, food consumed for prawns fed unmedicated and medicated pellets, was natural log transformed to account for the right skew of the data, and to meet normality assumptions. Random effects were included in the full model to account for the spatial hierarchical structure of the data (i.e. one of each of ten treatments nested per block, six blocks nested in each of five 100-litre holding tanks) as prawns within the same 100-litre tank are not independent from one another. In addition the model included the random effect of the correlation between repeated measurements on each prawn as they are not independent observations. Daily food consumption in squid diet treatment groups was fit using a separate linear mixed effects model with the response variable square transformed to account for the left-skewedness of the data. The change in food consumption of treatment groups was examined when prawns were switched to a preferred squid diet on day 15 using a Friedman's test.

Daily feed and EMB (natural log transformed) ingestions were summed across the period of medicated feeding (day 1 to 13) of all treatment groups and compared using a Kruskal-Wallis and ANOVA model respectively. This analysis allowed comparison between food consumption of squid and pellet diet treatment groups.

Consumption of the preferred squid diet by all treatment groups on days 15 and 17 was compared with a Kruskal-Wallis test.

The percentage of molting in all treatment groups was compared with a generalized linear model with binomial distribution and log-log link. Molting in prawns of different carapace lengths was explored using a generalized additive model (GAM).

3.2 Results

3.2.1 Feed preparation and analysis

The difference between the target and measured concentrations of EMB prepared feed ranged from 3 to 5 % or 95 to 103 % of target (Table 11).

Table 11: Emamectin benzoate (EMB) concentrations in prepared 4 mm medicated fish feed pellets provided to spot prawns in feed exposure trials. LOD= Limit of detection.

Target EMB concentration ($\mu\text{g g}^{-1}$)	Measured EMB ($\mu\text{g g}^{-1}$) \pm SE	Mean % of target
0	<0.60 (LOD)	-
1	0.96 \pm 0.01	96
10	10.27 \pm 0.22	103
100	94.97 \pm 1.32	95

3.2.2 Daily food consumption of pellet treatment groups (Day 1 to 13)

There was no difference in daily consumption between the three medicated pellet treatment groups (Likelihood Ratio Test, $\chi^2 = 1.481$, $p = 0.477$) so they were pooled and compared to the control unmedicated pellet group. Prawns fed unmedicated pellets had greater daily food consumption than prawns offered medicated pellets (Figure 10; Linear mixed effects model, $df = 3$, $\chi^2 = 8.5579$, $p = 0.004$).

Food consumption remained static at low levels over time in satiated prawns fed pellets, while consumption rates of pellets by starved prawns declined with time (Figure 10; Linear mixed effects model, $df = 1$, $\chi^2 = 26.54$, $p < 0.001$). Starved prawns consumed the greatest amount of pellets on the first day of the study with unmedicated starved groups consuming 12 times more than satiated prawns fed the same diet, and medicated starved groups consuming six to seven times more than satiated prawns (Figure 10).

Consumption by all treatment groups fed unmedicated and medicated pellets increased significantly from Days 13 to 15 when they were switched from their assigned diet treatments to a preferred diet of squid (Figure 10; Friedman rank sum test; $\chi^2 = 236$, $df = 1$, $p < 0.001$).

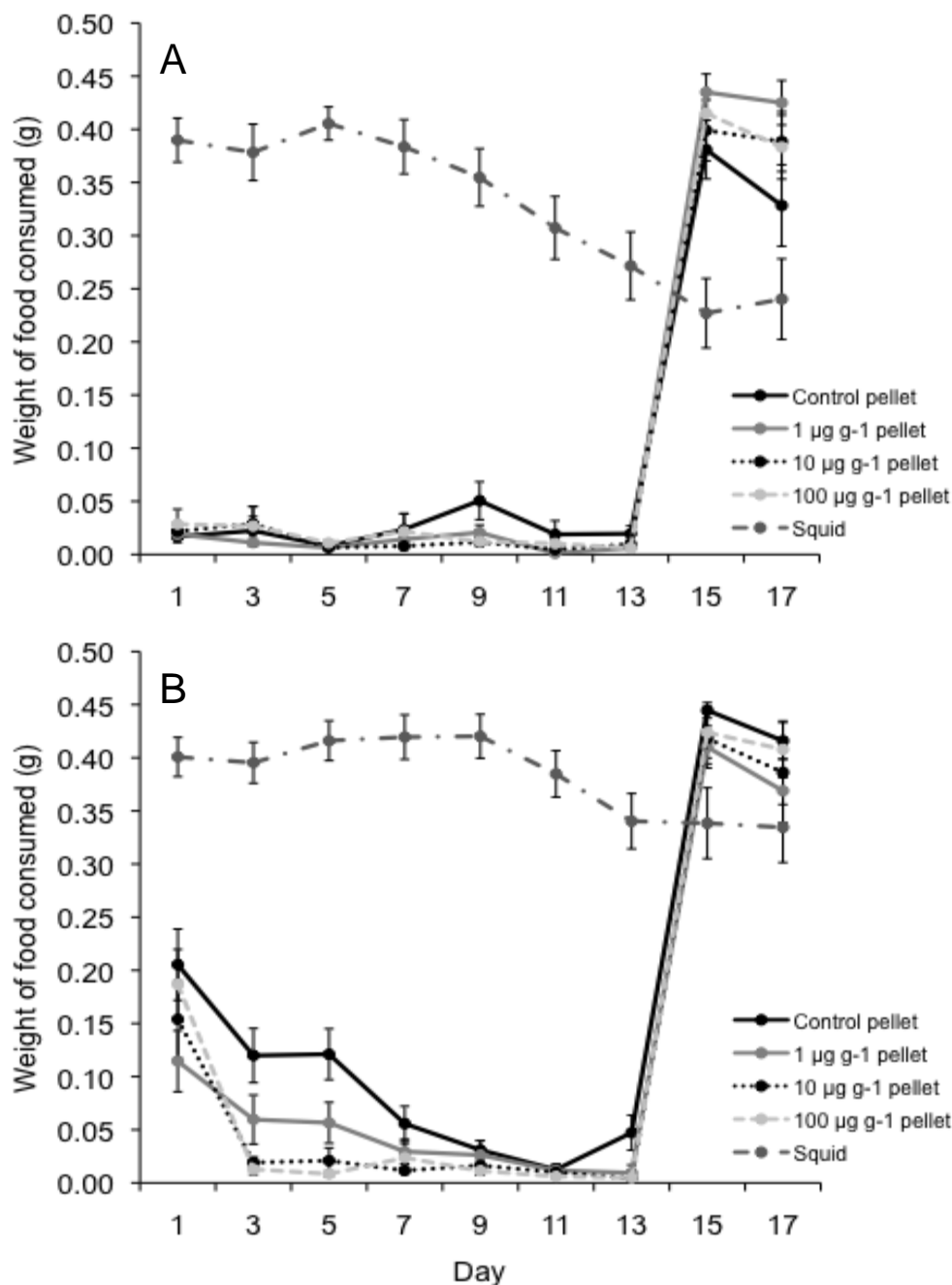


Figure 10: Daily food consumption by spot prawns over a 17-day period in each of two starvation treatments, (A) prawns fed to satiation for seven days and (B) prawns starved for seven days prior to testing period, and five food treatments (control pellet, 1, 10 and 100 µg g-1 EMB medicated pellets, and squid). Prawns in all groups were fed a diet of squid on days 15 and 17. Error bars are standard error. Prawns fed unmedicated pellets had greater daily consumption than prawns offered medicated pellets. Food consumption remained at low levels in satiated prawns fed pellets, while consumption rates in starved prawns declined with time. Consumption by all pellet treatment groups increased significantly when switched to a squid diet.

3.2.3 Daily food consumption of squid treatment groups (Day 1 to 13)

Consumption of squid decreased at a greater rate for satiated prawns compared to starved prawns as time progressed (Figure 10; Linear mixed effect model, $\chi^2 = 11.252$, $df = 1$, $p < 0.001$). Smaller prawns consumed less squid with time compared to larger prawns (Linear mixed effect model, $\chi^2 = 9.086$, $df = 1$, $p = 0.003$).

3.2.4 Overall cumulative food consumption (Days 1 to 13)

Both starved and satiated prawns given a squid diet consumed significantly more food overall summed across Days 1 to 13 than pellet diet groups (Figure 10; Kruskal-Wallis and pair wise comparisons using Wilcoxon rank sum test with a Bonferoni correction; $\chi^2 = 162.849$, $df = 9$, $p < 0.001$), but were not different from each other.

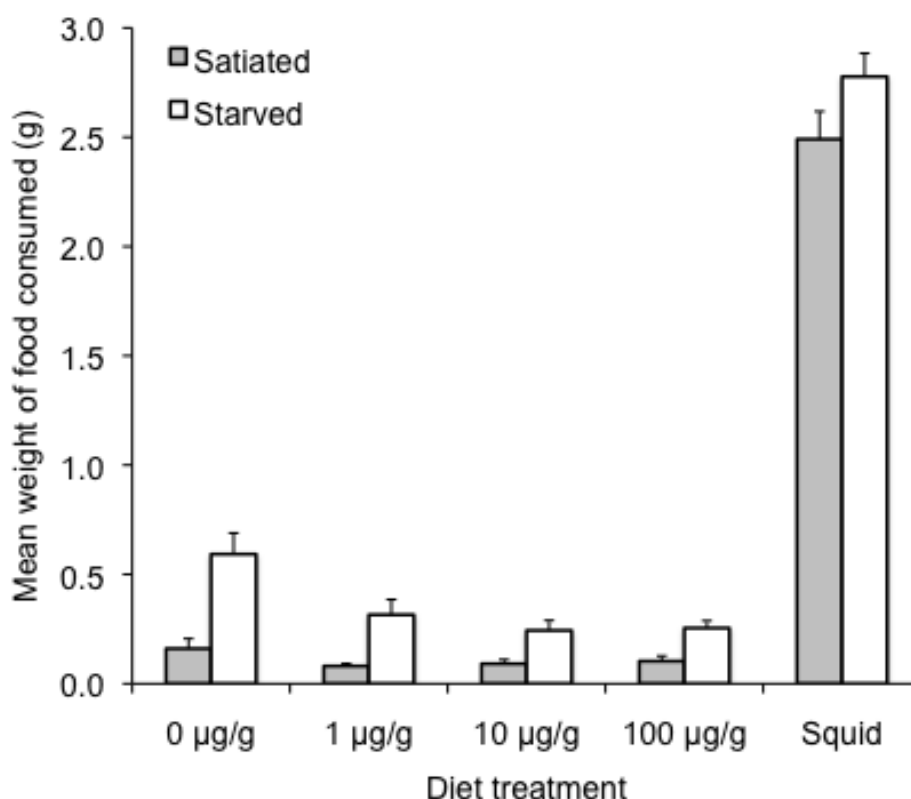


Figure 11: Cumulative weight of food consumed by spot prawn over days 1 to 13 in each of the starvation treatments (satiated and starved) and diet treatments (0, 1, 10 and 100 $\mu\text{g g}^{-1}$ EMB pellets and squid). Error bars are standard error. Prawns given a squid diet consumed significantly more food overall than pellet diet groups.

3.2.5 EMB consumption

Prawns exposed to $100 \mu\text{g g}^{-1}$ EMB pellets ingested significantly more EMB than prawns exposed to $10 \mu\text{g g}^{-1}$ (ANOVA, $F = 194.1$, $p < 0.001$) who in turn consumed significantly more EMB than prawns exposed to the $1 \mu\text{g g}^{-1}$ EMB pellets (Figure 12; ANOVA, $F = 167.32$, $p < 0.001$). Starved prawns consumed more EMB active ingredient than satiated prawns (ANOVA, $F = 33.66$, $p < 0.001$).

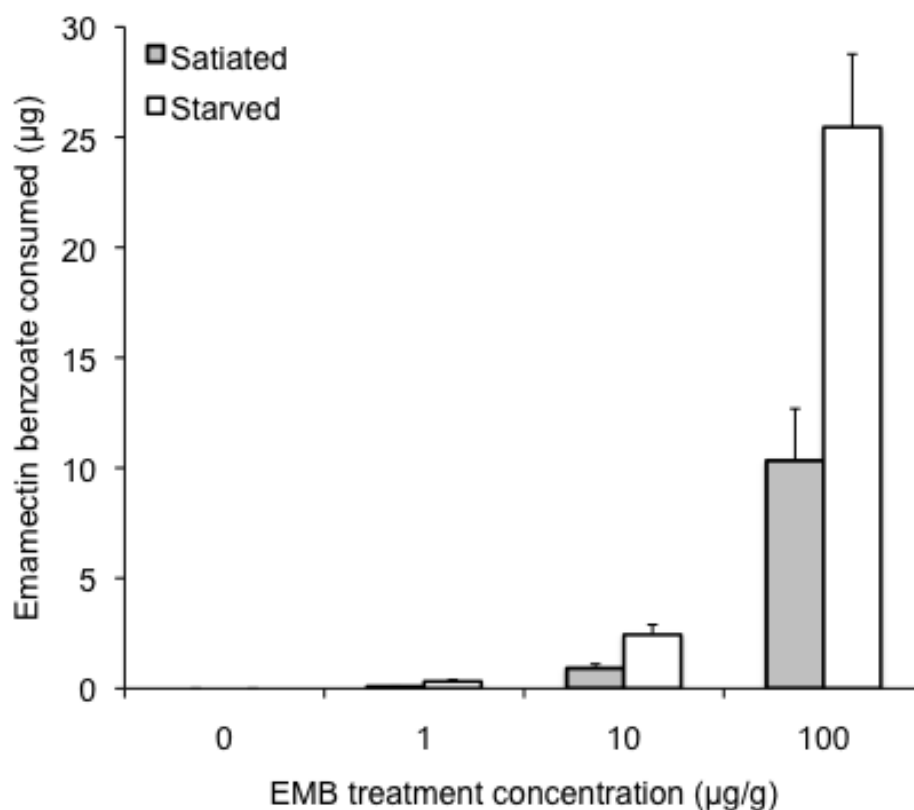


Figure 12: Relationship between the total amount of emamectin benzoate (EMB) active ingredient consumed by spot prawns over days 1 to 13 of the test exposure period and the EMB concentration of the feed the prawns were exposed to. Error bars are standard error. The amount of EMB consumed increased significantly with the EMB concentration of the pellet diet.

3.2.6 Preferred squid diet consumption (Day 15 and 17)

Consumption of a preferred squid diet totalled over Day 15 and 17 of the experimental period was compared in all diet treatments groups. There was no difference in consumption between all prawn groups fed a pellet diet (0 – 100 $\mu\text{g g}^{-1}$ EMB), but the two squid diet groups had significantly lower consumption of squid totalled over Day 15 and 17 (Figure 13; Kruskal-Wallis and pair wise comparisons using Wilcoxon rank sum test with a bonferoni correction; $\chi^2 = 27.1841$, $df = 9$, $p = 0.001$).

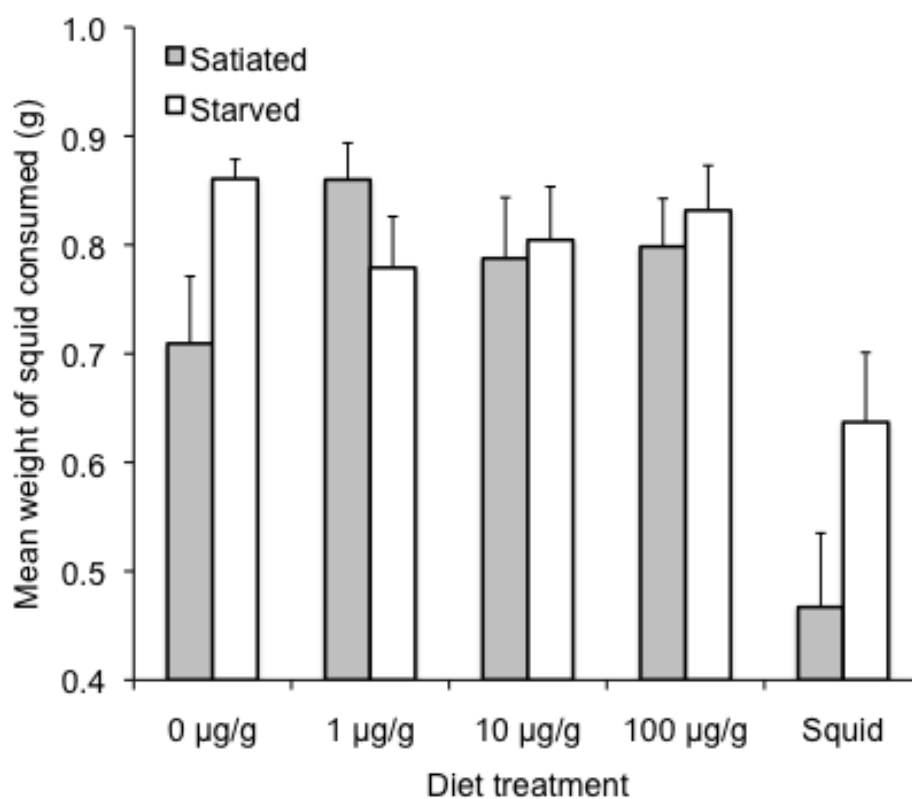


Figure 13: Summed weight of squid consumed by spot prawn over days 15 to 17 of the test exposure period in each of the starvation treatments (fed and starved) and pellet treatments (0, 1, 10 and 100 $\mu\text{g g}^{-1}$ EMB pellets and squid). Error bars are standard error. There was no difference in consumption of squid between all prawn groups fed a pellet diet.

3.2.7 Mortality and molting

Four prawns died during the experiment, one in each of the satiated unmedicated and 10 $\mu\text{g g}^{-1}$ EMB pellet groups and one in each of the starved 1 and 10 $\mu\text{g g}^{-1}$ EMB pellet groups (0 – 3.3% mortality per treatment).

Between 0 – 28 % of prawns in exposure groups molted during the experimental period (Figure 14). There was an increase in molting occurrence with increasing carapace length in spot prawns from 28 to 32 mm carapace length and then as prawn carapace length increased past 32 mm the occurrence of molting decreased (GAM, $p = 0.026$). Significantly fewer starved prawns molted than satiated prawns during the experimental period (Figure 14; GLM, $p = 0.005$). There was no significant effect of EMB pellet concentration on molting occurrence in prawns ($p > 0.05$).

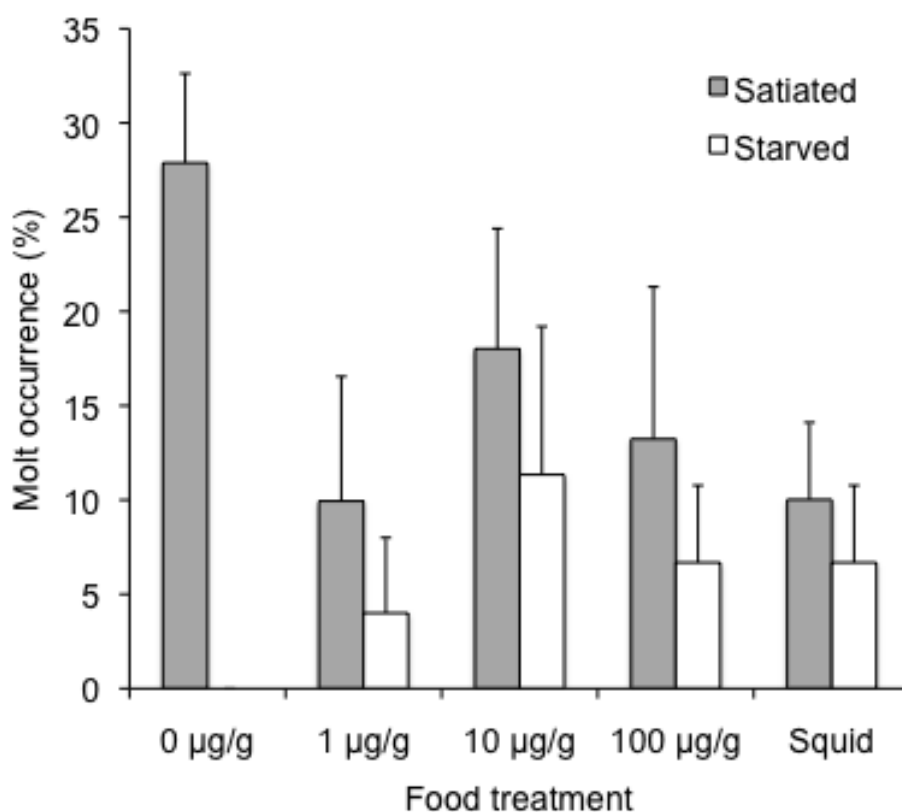


Figure 14: Molting occurrence of all spot prawn treatment groups during the experimental period day one to 17. Error bars are standard error. Significantly fewer starved prawns molted than satiated prawns during the experimental period.

3.3 Discussion

Starvation status of spot prawns prior to the feeding trial had the greatest influence on food consumption by all groups offered feed pellets, with satiated prawns maintaining lower consumption rates during the first 13 days compared to starved groups. This suggests that prawns will reject consuming both unmedicated and medicated pellets if they have had recent access to food.

Prawns offered unmedicated pellets consumed more food daily than prawns offered EMB medicated pellets. For starved prawns, consumption of both unmedicated and medicated pellets decreased with subsequent exposures, but at a greater rate for prawns fed medicated pellets. This trend suggests avoidance of EMB medicated pellets by prawns, even at levels as low as $1 \mu\text{g g}^{-1}$. Alternatively, EMB ingestion may be physiologically depressing prawn feeding behaviour. However, when prawns were switched from pellet diet treatments to a preferred squid diet after day 13, food consumption increased nine-fold. Further, there was no difference between the amount of preferred squid consumed by the different pellet treatment groups, even though prawns in the 10 and $100 \mu\text{g g}^{-1}$ groups consumed higher levels of EMB active ingredient. A return to feeding of the preferred squid diet after an exposure to medicated pellets indicates that ingestion of EMB for this duration either had no or very little transient physiological response in reducing feeding ability, and rather prawns were exhibiting food aversion learning.

Prawns did not exhibit a dose response to EMB as there was no difference in food consumption by treatment groups offered the three concentrations of medicated pellets. These results differ from Burridge et al. (2004) in which lobsters exhibited a strong dose response in decreasing pellet consumption with increasing EMB pellet concentration. A dose relationship may not have been apparent because this study tested much lower EMB pellet concentration ranges, $0 - 100 \mu\text{g g}^{-1}$ compared to the $0 - 1167 \mu\text{g g}^{-1}$ EMB pellet range that was tested by Burridge et al. (2004).

Cumulative consumption of medicated pellets by spot prawns across seven feedings over a 13-day period was on average 0.28 % (± 0.03 SE) of body weight for satiated prawns and 0.82 % (± 0.09 SE) for starved prawns in this experiment. These consumption values are considerably higher than the 0.01 – 0.2 % of body weight range

consumed over seven days in six-hour SLICE[®] pellet exposures with spot prawns that had been starved 24-hours (van Aggelen et al. 2003). This study had an increased period of starvation between feedings as prawns were fed every other day rather than every day as in van Aggelen et al. (2003), however, this does not explain the difference observed as the majority of food consumed was during the first day. van Aggelen et al. (2003) do not make explicit whether there was a difference in consumption as time progressed. A second explanation is that this study measured consumption based on dry weights of pellets rather than wet weights, which was deemed to be a more accurate measurement. There is no information as to what measurement was used by van Aggelen et al. (2003), and it was not possible to calibrate water uptake as pellets were left in water for six hours as opposed to four hours in this experiment, but if wet weights were used consumption could possibly be underestimated due to water uptake by pellets.

Starved and satiated prawns offered a squid diet treatment from day 1 to 13 consumed more of their food ration than all groups fed a pellet diet. Direct comparisons between groups fed squid versus pellets may not be relevant as the caloric value of squid (1.7 kJ per portion) is much less than feed pellets (though feed companies do not publish feed caloric values, the pellets used were minimum 24% fish oil equalling 4.4 kJ in each offered portion in oil alone), so it might be expected that squid groups would consume more. Cumulative consumption was similar between starved and satiated prawns offered a squid diet (they ingested most of the squid that was offered at each feeding), however, satiated prawns reduced their consumption as time progressed at a quicker rate than starved prawns. By days 15 and 17 prawns in the squid diet groups were consuming less than the pellet diet groups when all groups were fed a preferred squid diet. This may indicate that some upper limit of satiation was met in terms of how much food prawns can consume over time. These results, in addition to the increased food consumption observed when prawns fed pellets were switched to a squid diet on day 15, indicate that spot prawns do not find fish feed pellets (unmedicated or medicated) a particularly attractive food choice. Prawns may initially be attracted to salmon feed but rapidly become conditioned to reject it. It is not uncommon for crustaceans to become conditioned to the chemicals in formulated feeds, resulting in reduced future consumption of the feed (Kurmalay, Jones, and Yule 1990; Lee and Meyers 1996). Preference for

natural food sources over formulated feed pellets has also been found in spiny and homarid lobsters (Williams 2007).

Prawns exposed to the highest concentration of SLICE[®] pellets consumed the most EMB active ingredient during the 13-day exposure period. The amount of EMB consumed does not appear to be lethal to spot prawns in the time frame measured as only four prawns died during the experiment with no association to a particular treatment. Therefore the 14-day LC₅₀ of prawns must be higher than 100 µg g⁻¹, which is four times higher than the maximum 25 µg g⁻¹ concentration used in commercial SLICE[®] medicated fish feed. Different results may have been obtained if prawns had been force-fed or were held for a long period of time following exposure.

EMB concentration did not affect molting in prawns during the duration of the experiment, but a prawn's starvation condition before the experiment did. Prawns that had been fed to satiation prior to the experiment had a higher occurrence of molting than starved prawns. Since molting is a physically demanding process, having the necessary energy reserve would be important for successful ecdysis. Carapace length was also an important factor for molting, with the highest rate of molting occurring for prawns around 32 mm. When examining the size ranges of both male and transitional stages in this study, males were within 27.5 - 34.65 mm with an average 31.56 mm (± 0.12 SE) and transitional stages were within 30.20 - 38.50 mm with an average of 34.53 mm (± 0.12 SE) at the end of the experiment. Thus having the highest occurrence of molting occur around 32 mm is appropriate if prawns are morphing into the transitional phase.

Due to space and cost it was not possible for each prawn to have its' own water supply, thus resulting in 60 smaller containers in each of the five 100-litre tanks. Prawns held together in the 100 L tanks are not completely independent from one another due to chemical cues in the water. Though there were no differences in variance attributed to tanks and blocks, and it was accounted for in the model, it is important to note that individual prawns are not completely independent and that prawns may have been influenced by chemical cues from other prawns and diet treatments in the tanks. One possibility is that prawns fed the pellet diets may have detected chemical cues from the preferred squid diet and thus would not consume pellets.

Different results may have occurred if prawns had been fed for longer periods than four hours. A balance had to be reached to allow prawns enough time to consume food, but not so much that any remaining food, particularly pellets, could not be quantified if they began to disintegrate in the water. Also it was important to not have a difference between the ability to retain squid, with a form more resistant to dissolving, versus the pellets during food removal.

The time of year may also be another important factor in determining consumption rates of spot prawns of a particular food source. During some parts of the lifecycle spot prawns may require increased resources to sustain activities such as molting and reproduction.

Overall prawns seem to have a preference for squid over unmedicated and medicated pellets; even if prawns were starved or satiated they ate the majority of the squid that was offered during each feeding. Prawns exposed to SLICE[®] pellets in this experiment would only consume medicated pellets in initial exposures and if they had been previously starved. In areas near fish farms it may be unlikely that prawns will consume large quantities of uneaten SLICE[®] pellets that fall to the sea floor if they are able to recognize and reject EMB after initial exposures, especially if other preferred food sources are available. Though the migration capabilities of spot prawns is largely unknown, they are thought to not migrate to a large extent (DFO 1999) and preliminary tagging studies have shown that over several months mature spot prawns remain within two miles of their release location (Boutillier and Bond 2000). However, prawns do exhibit diurnal migration, migrating 100s of feet from deep to shallow waters at night (Chew et al. 1974) and have been observed to travel several meters in seconds during this research. If other food sources are available in nearby areas during periods when farms are treating with SLICE[®], spot prawns do not lack the ability in mobility to shift their distribution.

The low mortality rates observed in this study, even in prawns exposed to 100 $\mu\text{g g}^{-1}$ SLICE[®] pellets, in combination with low consumption rates measured in prawns exposed to medicated pellets, lends support that spot prawns are at low risk of direct mortality from acute exposures of SLICE[®] medicated pellets. However the effects of chronic exposures to SLICE[®] pellets, of concentrations routinely used by the industry, for

prawn populations inhabiting areas near salmon farms remains unknown. American lobsters delivered a single dose of $0.5 \mu\text{g g}^{-1}$ had lower rates of premature molting than lobsters given a succession of lower doses at two week intervals amounting to a similar cumulative exposure (Waddy et al. 2010). Higher mortality was observed during ecdysis in lobsters delivered four to eight multiple small doses compared to lobsters delivered one large dose. With long-term chronic exposure it is also important to consider not only direct mortality, but also possible sublethal effects including inability to feed or avoid predators, growth, and reproduction. There is the possibility that a population in the wild that resides near a fish farm may become habituated to eating fish feed pellets as an easier to obtain food resource. If this were to happen it is difficult to predict how prawns would respond to medicated pellets during SLICE[®] treatments if pellets were no longer a novel food source. Crayfish and hermit crabs only later rejected food sources that were novel to them (chicken and beef) when sickness was induced by lithium chloride shortly after feeding, but did not exhibit food aversion learning when the offered food source was part of their natural diet (Wight, Francis, and Eldridge 1990; Arzuffi, Salinas-Loera, and Racotta 2000).

If spot prawns are consuming fish feed they can be exposed to a variety of heavy metals and antibiotics. Rockfish populations in the Broughton Archipelago, BC, in proximity to salmon farms have been observed to have higher mercury content in their tissues, likely from the increased mercury content in commercial feed (Debruyn et al. 2006). This exposure to other contaminants on a regular basis may make prawns in vicinity to salmon farms more vulnerable to EMB exposure during SLICE[®] treatments. Future research should measure concentration of EMB in prawn tissue flesh after laboratory exposure to SLICE[®] pellets in addition to holding prawns for several months following exposure to observe long-term sub-lethal effects. Field surveys in combination with gut content analysis could be conducted to determine if spot prawns aggregate around salmon farms and if they consume fish feed pellets.

Chapter 4. Biological effects of emamectin benzoate acute sediment exposures on spot prawn mortality and molting ability

4.0 Introduction

During and after SLICE[®] treatments detectable amounts of EMB can accumulate in the benthic environment near salmon farms and in exposed non-target organisms (Telfer et al. 2006; SEPA 2004a; DFO 2012b). Concentrations of EMB found in sediments near treating farms in BC range between the limit of detection ($0.25 \mu\text{g kg}^{-1}$ for equipment used in most studies to date) to $35.0 \mu\text{g kg}^{-1}$ of wet sediment. Two potential routes of EMB uptake by prawns include directly through consumption of medicated feed and contaminated sediment particles, or passively through sediment and water, which will be the focus for this chapter.

Acute laboratory exposures of EMB through sediment have been conducted with the amphipods *Eohaustorius estuarius* (Kuo et al. 2010) and *Corophium volutator* (McHenery 1999; Mayor et al. 2008; Mayor et al. 2009), the polychaetes *Hediste diversicolor* (Mayor et al. 2008; Mayor et al. 2009) and *Arenicola marina* (McHenery 1999), and spot prawn (Veldhoen et al. 2012; DFO 2012b). To date no laboratory sediment EMB exposures have been conducted longer than ten days. Over an eight-day EMB exposure, Veldhoen et al. (2012) observed higher spot prawn mortality (15 – 20%) in individuals exposed to sediment containing mid-range EMB concentrations ($100 - 800 \mu\text{g EMB kg}^{-1}$ sediment) and reduced mortality in the two highest exposure concentrations, 1200 and $4800 \mu\text{g kg}^{-1}$, compared to controls. The authors suggest that high doses of EMB induce early compensatory mechanisms in spot prawn relative to low doses. EMB was detected in spot prawn abdominal muscle tissues in individuals exposed at all nominal concentrations and EMB exposures at all doses altered mRNA transcript levels within prawn muscle tissue relating to protein encoding for translation, transcription regulation, and apoptosis relative to control groups (Veldhoen et al. 2012). This chapter focuses on EMB dose responses in survival and molting of spot prawns using laboratory sediment exposures in addition to assessing the uptake of EMB in spot

prawn abdomen muscle tissue. Three exposure durations (ten, 30 and 45 days) were conducted using a range of sediment EMB concentrations (20 – 4000 $\mu\text{g kg}^{-1}$).

4.1 Methods and materials

4.1.1 Specimen collection

Spot prawns were collected with commercial traps from Patricia Bay in Saanich Inlet, Victoria BC, on January 12, 2011 for the ten-day exposures, April 7, 2011 for the 30-day sediment exposures, and October 24 and November 1, 2011 for the 45-day sediment exposure experiments. The collection site had no known history of EMB exposure. Traps were baited with commercial prawn bait and set for three hours before being hauled. Prawns were held on ship in aerated tanks for 15 minutes, transferred to a 500 L aerated tank on a truck and transported within one hour to the University of Victoria Outdoor Aquatics Facility. Prawns were held communally in a 1000 L tank with flow through seawater obtained from Cattle Point, BC, that was filtered, UV-treated and maintained at a temperature of 10.5 °C and 30 ppt salinity. Prawns were held in light-limited conditions, with shelter, at a loading density of one prawn per litre water and fed commercial feed pellets (4 mm, Skretting Vitalis) to satiation every other day.

4.1.2 Acute sediment toxicity tests

Premix 0.2% SLICE[®] was obtained from Intervet Schering-Plough Animal Health. Exposure tanks were prepared by mixing 2050 g of 2 – 20 μm silica sand with 450 mL of filtered seawater in 16 L polycarbonate tanks. Before each use, all tanks were washed with laboratory detergent, sodium hypochlorite and sodium thiosulphate, and then rinsed three times with test water. SLICE[®] premix was added to each tank and the sediment was homogenized with a stainless steel spoon for ten minutes. A 100 g sample of sediment was retained from each tank and frozen at -20°C until being shipped for chemical analysis to the Fisheries and Oceans Canada Institute of Ocean Sciences laboratory in Sidney, BC. Tanks were filled with 16 L of filtered seawater and placed via randomized block design in a water bath so an internal tank water temperature of 10 – 13 °C was maintained. Tanks were aerated with air stones to a dissolved oxygen concentration between 80 – 90% and the sediment was allowed to settle for 24 – 48 hours. For the 30

and 45-day exposures half the seawater was replaced weekly in each tank. Tanks were held in constant low light conditions for the duration of the experiment to simulate light levels at the water depth that prawns regularly inhabit. Prawns were observed daily for morbidity, mortality and molting. Upon death, or the conclusion of the experiment, prawns were measured, weighed, and sex and body condition was determined. Sex was determined by examining the appendix masculine and appendix interna on the second pleopod (modified swimming appendage on the abdomen). An assessment of body condition included examining prawns for broken or missing appendages, damage to the eyes, and the presence of any black spotting on the shell. Prawns that would be used for subsequent behavioural experiments to determine sublethal effects of EMB exposure were not assessed until the conclusion of all experiments to avoid handling stress.

4.1.2.1 Ten-day acute sediment toxicity test

The ten-day sediment exposure study was undertaken between January 20 and March 12, 2011. Each EMB test concentration of 20, 200, 2000, and 4000 $\mu\text{g kg}^{-1}$, and control was replicated 12 times (Table 12). Ten prawns, male or transitional stages that had an average carapace length of 33.77 mm (± 0.07 SE) and weight of 22.89 g (± 0.12 SE), were placed in each tank replicate at the start of the experiment. The average loading density of each tank was 14.4 g prawn L^{-1} seawater. Prawns were not fed for the duration of the ten-day experiment.

Table 12. Experimental conditions in each acute toxicity test.

Exposure duration	Exposure concentration ($\mu\text{g kg}^{-1}$)	No. replicates	No. prawns/tank	Avg. prawn weight \pm SE	Avg. prawn length \pm SE	No. feeding intervals
10 days	0, 20, 200, 2000, 4000	12	10	22.89 (0.12)	33.77 (0.07)	None
30 days	0, 20, 200, 1000, 2000, 4000	6	4	17.47 (0.24)	30.69 (0.14)	Pellet and squid every 7 days
45 days	0, 20, 40, 200, 500, 1000, 2000	6	4	23.06 (0.30)	32.50 (0.16)	Squid every 5 days

4.1.2.2 Thirty-day acute sediment toxicity test

The 30-day sediment exposure study was undertaken between April 20 and May 23, 2011. Each EMB test concentration, 20, 200, 1000, 2000, and 4000 $\mu\text{g kg}^{-1}$ and control was replicated six times. Tanks were split into four equal sections using vexar so prawns would be physically separated. Four prawns were haphazardly selected for each tank and were either male or transitional stages and had an average carapace length of 30.69 mm (± 0.14 SE) and weight of 17.47 g (± 0.24 SE). Prawns were fed every seven days (four times throughout the experiment) with a squid and commercial feed pellet diet. Uneaten food was removed from the tanks after three days. A Molt Impact Index (MII) (Table 13), adapted from Waddy et al. (2010), was used to compare the molting success of spot prawns exposed to the different EMB concentrations.

Table 13: Molt Impact Index (MII) for quantifying the effect of emamectin benzoate on molt success in spot prawns.

MII	Description
1	Molted successfully and survived until end of study period
2	Molted successfully and died between two to six days after ecdysis
3	Molted successfully and died within 24 hours of ecdysis
4	Died during ecdysis, unsuccessful molt

4.1.2.3 Forty-five day acute sediment toxicity test

The 45-day sediment exposure study was undertaken at UVic Outdoor Aquatics Facility between November 7 and December 22, 2011 to determine the uptake of EMB in tissue over this time period. Due to high mortality in test prawns preceding the experiment and in all EMB exposures after the experiment commenced, mortality and molting could not be assessed. This same mortality response was observed in 2009 when prawns were obtained from Saanich Inlet from October to November indicating this may be a sensitive period during the lifecycle and survival after capture is reduced. This has not been previously documented in the literature.

To assess uptake of EMB in prawn tissue each of the six EMB test concentrations: 20, 40, 200, 500, 1000, and 2000 $\mu\text{g kg}^{-1}$ were replicated six times. Similarly to the 30-day tests, four prawns were placed in each tank, individually separated with vexar.

Prawns selected were either male or transitional stages and had an average carapace length of 32.50 mm (\pm 0.16 SE) and weight of 23.06 g (\pm 0.30 SE). Prawns were fed every five days with a squid diet. At the conclusion of the experiment spot prawns were immediately frozen to -20°C until chemical analysis at the Fisheries and Oceans Canada Institute of Ocean Sciences lab in Sidney, BC.

4.1.3 Chemical sample analysis

Sample clean up procedures and detection of EMB residues in spot prawn abdomen tissue and laboratory prepared sediments was conducted according to the methodology of Ikonomou and Surridge (2011). Final sample extracts were analyzed by high performance liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS). Instrumentation used included a Dionex P680 (Dionex, Sunnyvale, CA) HPLC system and Sciex API 5000 triple quadrupole mass spectrometer using multiple reaction monitoring (MRM) mode acquiring positive ions. HPLC parameters are in Table 14 and MRM mass spectrometer parameters in Table 15. One analysis method modification is the TurboIonSpray® optimized conditions used were: ionspray voltage: +5500V, curtain gas 20 au (arbitrary units), nebulizer gas flow 30 au, turbo-ion gas flow 20 au, collision gas 4 au, and turbo-ion gas temperature 500°C .

To provide an additional level of detection confirmation a second MRM transition was observed and chromatographic retention times were compared to authentic standards for further identification. Acquisition, quantitation, and data processing of all MRM signals were achieved using Analyst software (1.4.0). A nine-point calibration curve was generated for each analyte and used for quantitation. Instrument linearity was demonstrated (r^2 were >0.99) over a concentration range of 0.020 - 25 ng/mL. Results were acceptable if percent recovery of performance standards were within 40 to 110 % of the expected value. Method quality assurance and control included two duplicates, one matrix spike, one lab blank, and bracketing verification standards in each sample batch of 20 samples. The level of detection (LOD) and level of quantification (LOQ) of EMB in sediments is respectively 0.053 and 0.177 $\mu\text{g kg}^{-1}$ and for the desmethyl metabolite, LOD and LOQ in sediment are 0.080 and 0.266 $\mu\text{g kg}^{-1}$. In prawn tissue samples the EMB

LOD and LOQ are 0.041 and 0.090 $\mu\text{g kg}^{-1}$, and for the desmethyl metabolite are 0.058 and 0.142 $\mu\text{g kg}^{-1}$.

Table 14: High performance liquid chromatography (HPLC) parameters

HPLC Column:	Reverse phase C ₁₈ column (Xtera C ₁₈ -MS, 4.6 x 30 mm, 5 μ particle size, Waters) and guard column (Opti-Guard® C ₁₈ , 1mm, chromatographic specialties)
Mobile phase:	A: methanol B: 10 mM ammonium acetate in HPLC water
Gradient Program:	Time 0-2: 70% A, 30% B Time 2-2.1: 90% A, 10% B Time 11.8-11.9: 70% A, 30% B
Column Temp:	35°C \pm 0.1°C
Flow Rate:	0.3 mL / minute
Injection Volume:	10 μ L
Run Time:	15 minutes

Table 15: MRM mass spectrometer parameters

Analyte	Precursor ion (m/z) Qualifying ion (q)	Precursor Ion Identity	Precursor - product transition (m/z)	Retention Time (min)	Collision energy (V)	Declustering potential (V)
EMB	886.4	[M+H] ⁺	886.4→158.2	5.8	48	50
	886.4(q)	[M+H] ⁺	886.4→126.4			
Desmethyl metabolite	872.6	[M+H] ⁺	872.6→144.6	5.7	35	50
	872.6 (q)	[M+H] ⁺	872.6→112.4			
Terbumeton	226.5	[M+H] ⁺	226.5→170.0	3.2	25	20

4.1.4 Statistical analysis

All analyses were completed using statistical computing software *R* (R Development Core Team 2011). Differences were considered to be significant if $\alpha < 0.05$. Model selection was undertaken using Akaike information criterion (AIC) and a likelihood ratio test to determine the best-fit models.

For the ten-day sediment toxicity test a generalized mixed effects model (GLMM) fit by maximum likelihood was used to compare the relationship between EMB exposure treatment and within tank molting frequency, on the response of prawn mortality. Prawn individuals in the same tank could physically and chemically interact with one another so

could not be considered as independent; therefore the data reflect mortality per tank. A random effect for repetition was included in the model to account for the possible variance resulting in the temporal range due to staggered start dates of each rep. To test whether EMB exposure affected molting rates in prawns a GLMM was fit to compare the relationship of EMB exposure treatment on molting frequency within the tanks.

For the 30-day sediment toxicity tests the LC_{50} was determined using the MASS library in R (MASS package, Venables and Ripley 2002). Two Cox proportional hazards (PH) models were fit to the data to determine the effect of EMB exposure on days until death of prawns and days until molting. These data were right-censored as not all prawns died or molted before the predetermined end date of the study period. For both models a cluster function was used to specify non-independent observations, i.e. prawns exposed in the same tank. The number of prawns that molted at the end of 30 days in each EMB exposure group was compared with a poisson distributed GLMM with repetition included as the random effect to account for the possible variance resulting from the spatial randomized block design and staggered rep start dates. A generalized linear model (GLM) with quasipoisson distribution correcting for over dispersion was fit to compare molting success, the molt impact index (Table 13), between the different exposure groups.

To compare the uptake of EMB in prawn tissue following a 45-day exposure period at different EMB concentrations, a linear mixed effects model fit by maximum likelihood was used. The concentration of EMB in tissue was log transformed to achieve normality and equal variance assumptions. A random effect of tank was used to account for non-independent prawns exposed in the same tank. Only prawns that survived past 40 days were included in the analysis.

4.2 Results

4.2.1 Sediment sample chemical analysis

The difference in the levels of emamectin benzoate measured in sediments for the target doses between 20 to 4000 $\mu\text{g kg}^{-1}$ ranged from 0.4% to 29.0% (71 – 103% of target) and the average coefficient of variation was 26% (Table 16). Laboratory percent recoveries of EMB were between 70 and 90% and thus it was not necessary to correct the data

according to recovery levels. EMB levels were detected in some of the control sediments as a result of contamination from previous trials. Contamination levels were low (range between 0.1 and 3.4 $\mu\text{g kg}^{-1}$) in 78% of the exposure tanks and this treatment will still be considered the control that other treatments will be compared to.

Table 16: Target and actual emamectin benzoate (EMB) and desmethyl metabolite concentrations in sediment exposures in acute toxicity tests

Target [EMB]	Actual [EMB] $\mu\text{g kg}^{-1}$ wet weight (\pm SE)	Actual [metabolite] $\mu\text{g kg}^{-1}$ wet weight (\pm SE)	No. Samples Analyzed	Percent of target (coefficient of variation)
0	0.44 (\pm 1.9)	< LOD	18	-
20	17.7 (\pm 3.1)	< LOD	7	88.6 (46.7)
40	40.2 (\pm 1.9)	< LOD	5	100.4 (10.5)
200	205.2 (\pm 36.7)	< LOD	6	102.6 (43.8)
500	472.8 (\pm 28.5)	< LOD	5	94.6 (13.5)
1000	808.3 (\pm 85.9)	1.2 (\pm 0.7)	5	80.8 (23.7)
2000	1419.0 (\pm 114.0)	2.1 (\pm 1.3)	5	71.0 (18.0)
4000	3329.7 (\pm 367.4)	8.7 (\pm 2.3)	5	83.2 (24.7)

4.2.2 Ten-day acute sediment toxicity test

Mortality of spot prawns increased as molting frequency increased (Figure 15; GLMM, Likelihood Ratio Test, $\chi^2 = 54.24$, $df = 1$, $p < 0.001$) and there was no effect of EMB treatment exposure (Likelihood Ratio Test, $\chi^2 = 3.57$, $df = 4$, $p = 0.467$). Regardless of treatment, 64% of prawns that died during the ten-day experiment had recently molted. There was no difference in the number of prawns that molted in each EMB exposure group (Figure 15; GLMM; Likelihood Ratio Test, $\chi^2 = 6.10$, $df = 4$, $p = 0.192$).

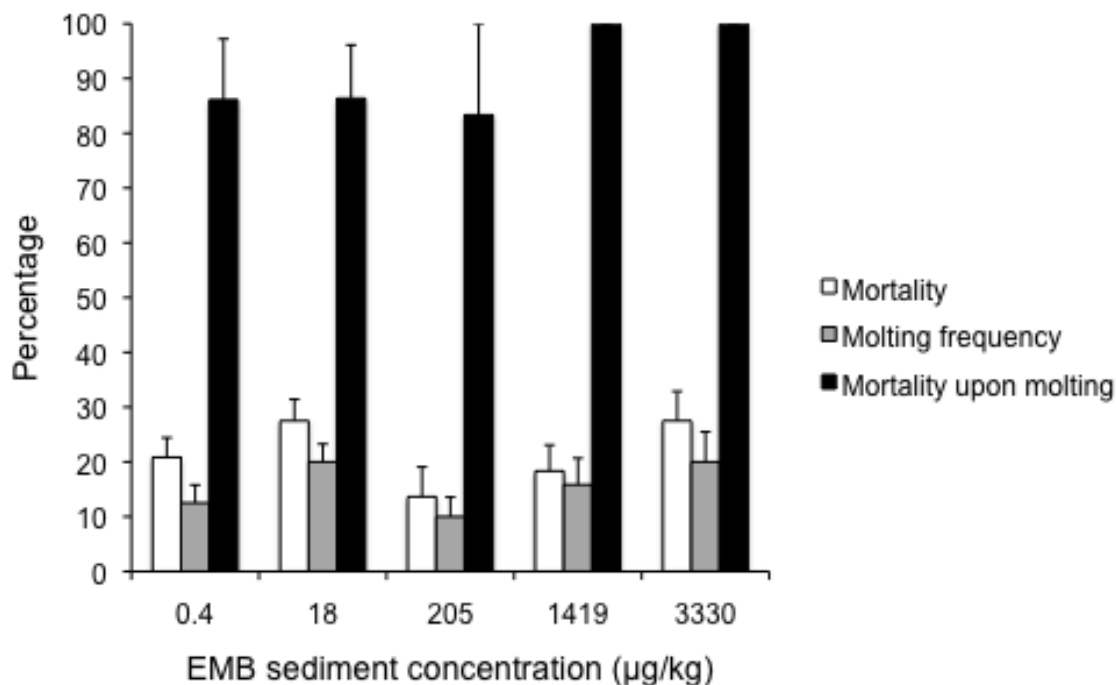


Figure 15: Mortality, molting frequency and mortality upon molting percentage of spot prawns in each emamectin benzoate (EMB) exposure group over a ten-day sediment exposure. Error bars are standard error. Mortality of spot prawns increased as molting frequency increased and there was no effect of EMB treatment exposure on molting frequency or mortality.

4.2.3 Thirty-day acute sediment toxicity test

The 30-day LC_{50} was estimated to be $735 \mu\text{g kg}^{-1}$ (± 1.7 SE) of EMB. Mortality occurred in all exposure groups. In estimating time to death there was no difference in the mortality response of prawns between the 0.4 to $205 \mu\text{g kg}^{-1}$ exposure groups or between the 808 to $3330 \mu\text{g kg}^{-1}$ exposure groups so they were pooled. Prawns in the higher exposure group ($808 - 3330 \mu\text{g kg}^{-1}$) had 6.97 times the daily hazard of death compared to lower exposures (Figure 16; Cox PH model, $z = 4.64$, $p < 0.001$). Prawns that molted during the exposure period increased the daily hazard of death by a factor of 3.36 times (Figure 16; Cox PH model, $z = 4.90$, $p < 0.001$). The Molt Impact Index was significantly higher for prawns exposed to EMB concentrations of $808 \mu\text{g kg}^{-1}$ and greater compared to lower doses groups (Table 17; GLM Deviance = 13.368, $df = 1$, $p < 0.001$), however,

there was no significant difference in the Molt Impact Index between the three high exposure concentrations ($p > 0.05$). There was no difference in the number of prawns that molted in each EMB exposure group (GLMM; Likelihood Ratio Test, $\chi^2 = 8.9649$, $df = 5$, $p = 0.111$), or the mean time to molt (Cox PH model; Likelihood Ratio Test, $\chi^2 = 6.032$, $df = 5$, $p = 0.303$), however, the risk of molting declined in larger prawns (Cox PH model, $z = -3.75$, $p < 0.001$). Prawns in high EMB concentrations groups were observed not to feed on either feed pellets or squid during the exposure period, however this was not quantified.

Table 17: Number of spot prawns exposed to different EMB treatments in the sediment over a 30-day period that molted, died and the average time until these events occurred. *Significantly different than 0.4 $\mu\text{g kg}^{-1}$ group ($p < 0.001$; Cox proportional hazards model).

EMB Treatment ($\mu\text{g kg}^{-1}$)	n	Molt	Mortality	Mean time (days \pm SE)		Mean Impact on Molt Success (\pm SE)
				Molt	Mortality	
0.4	24	10 (42%)	4 (17%)	17 \pm 3	20 \pm 5	1.3 \pm 0.3
18	24	7 (29%)	3 (13%)	16 \pm 3	15 \pm 3	1.3 \pm 0.3
205	24	6 (25%)	3 (13%)	10 \pm 4	19 \pm 1	1.8 \pm 0.5
808	24	12 (50%)	13 (54%)*	13 \pm 2	14 \pm 1	2.5 \pm 0.3*
1419	24	10 (42%)	14 (58%)*	17 \pm 3	17 \pm 2	3.1 \pm 0.2*
3330	24	9 (38%)	19 (79%)*	17 \pm 3	18 \pm 2	3.2 \pm 0.2*

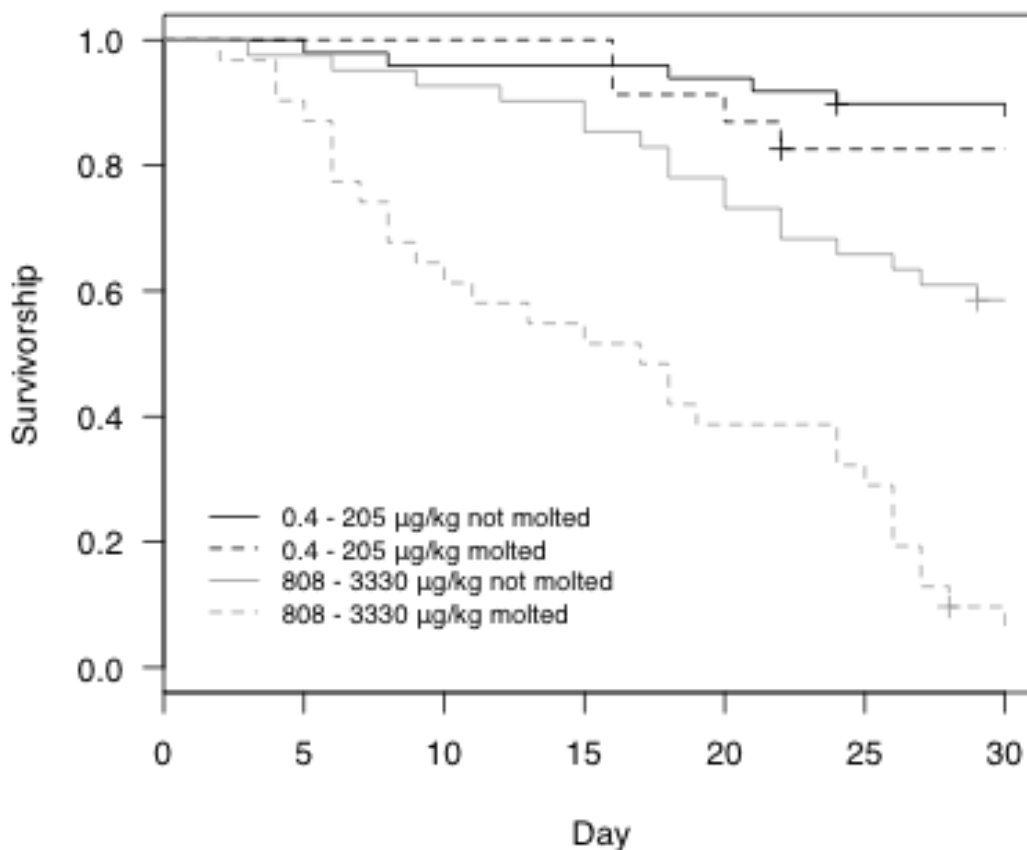


Figure 16: Survivorship of spot prawns exposed to EMB in sediment over a 30-day period. Prawns in the higher exposure group (808 – 3330 µg kg⁻¹) had decreased survivorship compared to lower exposures (0.4 to 205 µg kg⁻¹) over the 30-day period. Prawns that molted during the exposure period increased the daily hazard of death.

4.2.4 45-day acute sediment toxicity test

The uptake of EMB in prawn tissue increased with increasing EMB sediment exposure concentration (Figure 17; Linear mixed effects model, Likelihood Ratio Test $\chi^2 = 36.812$, $df = 5$, $p < 0.001$). There was no difference between the tissue concentration of prawns exposed to 18 or 40 µg kg⁻¹ ($p = 0.194$), and no difference between prawns exposed to 808 or 1419 µg kg⁻¹ ($p = 0.606$). Desmethyl metabolite residues were found in the abdomen tissue of all prawns exposed to 472 µg kg⁻¹ EMB and greater and only detected in several prawns exposed to 40 and 205 µg kg⁻¹.

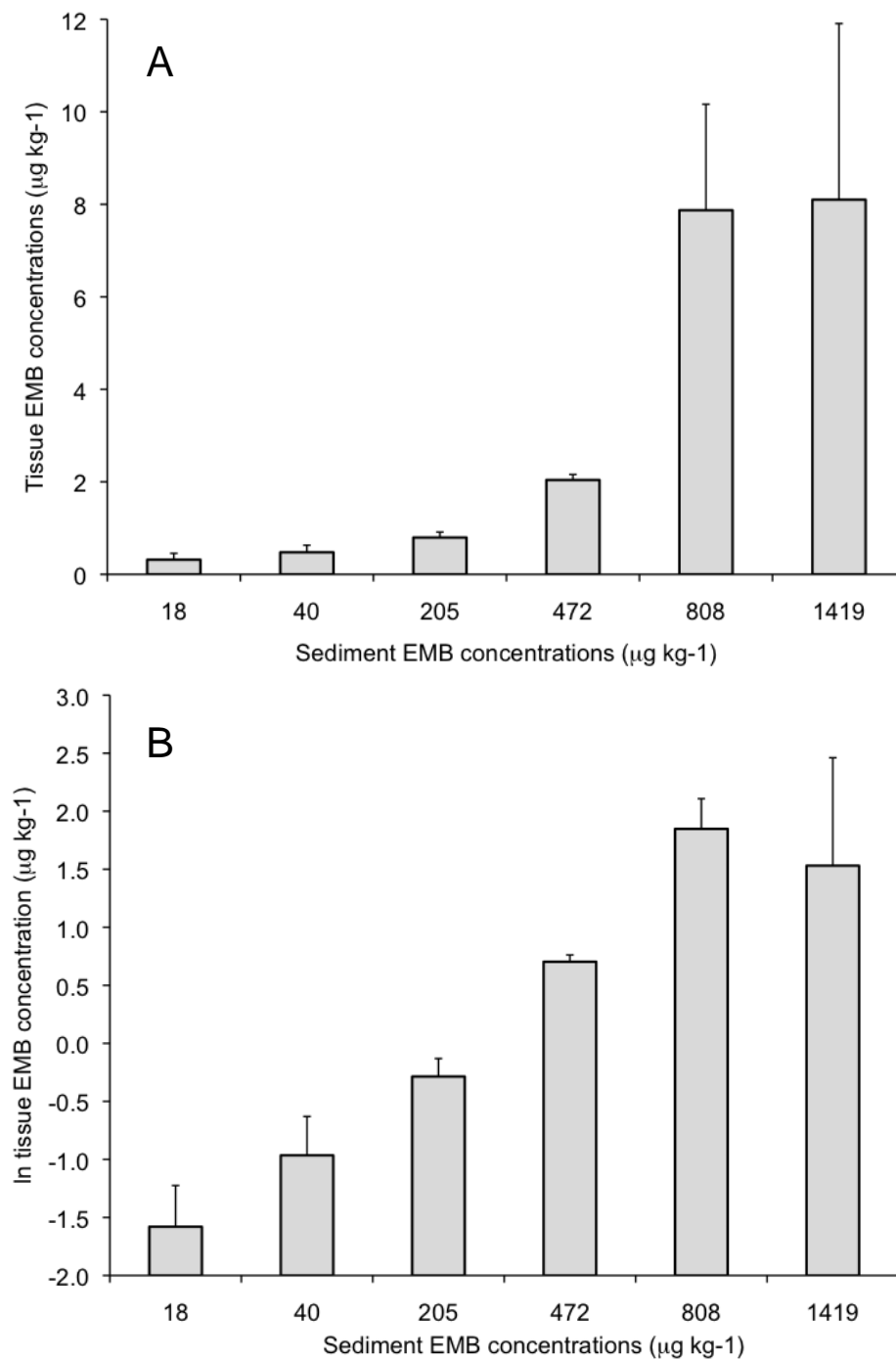


Figure 17: Average EMB tissue concentrations (A) and the natural log of EMB tissue concentrations (B) of prawns that survived past 30 days at EMB sediment exposures of 18 (n=7), 40 (n=5), 205 (n=6), 472 (n=7), 808 (n=7), and 1419 $\mu\text{g kg}^{-1}$ (n=3). Error bars are standard error. The uptake of EMB in prawn tissue increased with increasing EMB sediment exposure concentration

4.3 Discussion

Over the ten-day EMB exposure period within tank molting frequency was the best predictor of prawn mortality. The effects of molting can be largely attributed to cannibalism as recently molted individuals were left vulnerable to predation by tank mates and dead individuals displayed significant signs of scavenging. Cannibalism to some degree in spot prawn is not an uncommon observation, especially of susceptible recently molted individuals (King 1997). Since no dose response of EMB exposure on molting rates was observed, and the effects of molting so strongly predicted prawn mortality, the mortality effects associated with EMB exposure were undetected. High molting rates of spot prawns in captivity were also observed by Stoner (2012) who had 41% of prawns molt within 30 days of live capture.

EMB dose was an important predictor of prawn mortality over the 30-day exposure period when prawns could not physically interact with one another. Prawns exposed to concentrations of EMB equal to and greater than $808 \mu\text{g kg}^{-1}$ exhibited depressed survivorship compared to lower dose groups, especially if molting occurred during the exposure period. The majority of prawns exposed to EMB doses $205 \mu\text{g kg}^{-1}$ and less molted successfully and survived until the end of the experiment, whereas prawns exposed to higher EMB concentrations died within 24 to 144 hours of ecdysis. While no significant differences were observed between the Molt Impact Index of the three highest EMB treatments, 40% of the prawns exposed to $808 \mu\text{g kg}^{-1}$ that died following ecdysis lived several days after molting, while only 10% of prawns in the $1419 \mu\text{g kg}^{-1}$ treatment survived to the following day, and none survived more than 24 hours in the $3330 \mu\text{g kg}^{-1}$ group. Reduced molting success resulting from avermectin exposure has been reported for copepods (Willis et al. 2003), insects (Strong and Brown 1987) and American lobsters *Homarus americanus* (Waddy et al. 2010). Ovigerous lobsters displayed decreased molt success when orally delivered four to eight successive low EMB doses every two weeks compared to lobsters delivered one or two higher doses, resulting in the same cumulative exposure. These results from lobsters may indicate that the effects from EMB accumulate over repeated exposures. Several explanations why high doses of EMB may prohibit successful molting in spot prawns include reduced energy reserves, nerve impulse disruption, and the loss of a protective barrier.

Physiologically, molting is a very energy intensive process, requiring the formation and mineralization of a new exoskeleton, muscle regeneration, and forgoing sustenance in the time leading up to and following ecdysis (Drach 1939). Undergoing the molting process while compensating for toxicity effects from EMB exposure may undermine an individual's energy reserves, resulting in death during or following ecdysis. On a cellular level avermectin compounds increase the nerve cell membrane permeability in crustaceans allowing an influx of chloride ions, resulting in the loss of cell function, nerve impulse disruption, and hyperpolarization of nerve and muscle tissue (McKellar and Benchaous 1996; Roy et al. 2000; Wolstenholme and Rogers 2005; Mellin et al. 1983; SEPA 1999). The molting process requires the proper functioning of the androgenic glands and neurosecretory cells to release a synchrony of hormones and thus a successful molt may be inhibited for prawns exposed to high levels of EMB due to nerve impulse disruption. A final explanation for unsuccessful ecdysis in prawns exposed to high EMB doses could be the loss of the hard exoskeleton. During molting it takes several days for the new exoskeleton to harden and often during this time crustaceans, such as crabs, will burrow in sand to avoid predation. Burrowing in sand containing EMB with a vulnerable exoskeleton could increase the potential for EMB uptake, though there is no evidence in the literature. EMB tissue concentrations of spot prawns in the 30-day exposure were not assessed, however future research should compare EMB tissue loads of exposed prawns that had recently molted to prawns that have not undergone ecdysis.

In both the ten-day and 30-day exposures no difference in molting rates of prawns in different EMB treatments groups was observed. Veldhoen et al. (2012) also found EMB treatment did not affect molting frequency in spot prawns exposed to EMB through sediment over eight days. In addition no EMB-related changes in the mRNA expression of the enzyme β -N-acetyl-glucosaminidase (NAG) that aids in the molting process were observed. Increasing EMB dose exposure, delivered orally, has been found to increase premature molting in overigerous lobsters observed for over a year (Waddy et al. 2002; Waddy 2003; Waddy, Merritt, et al. 2007b), however, the exact mechanism remains unknown. Waddy et al. (2002; 2010) have hypothesized that EMB affects molt timing and process by hindering the synthesis or release of the molt-inhibiting neuropeptide (MIH), consequently allowing the molting glands to release ecdysteroid molting

hormones, causing premolt. The extended observation time in the lobster studies or the method of EMB delivery may explain why similar results were not observed in spot prawn.

Survival of spot prawns captured from Saanich Inlet during September to November in both 2010 and 2011 for the laboratory experiments was compromised. Prawns held in the holding tanks were observed to be lethargic and would not feed, unlike prawns collected in the winter and spring. While these results were not quantified it does indicate a need to determine the most vulnerable time of year for spot prawn survival and suggest management to avoid SLICE[®] treatments during this time period. Currently the majority of salmon farms treat with SLICE[®] in the fall season in order to ensure decreased loads of sea lice on farms during the spring juvenile salmon out migration period.

Concentrations of EMB residues observed in prawn abdomen tissue increased with EMB concentration exposure. Likely factors that affect EMB accumulation in tissue are the route and duration of exposure. Potential pathways of how EMB accumulates in abdomen muscle tissue in laboratory exposures includes: adsorption through the gastrointestinal tract membrane from the consumption of EMB bound to sediment particulates during feeding, adsorption through the exoskeleton, and uptake through water by adsorption across the gill membrane during respiration. Due to the variables tested the most likely uptake route can not be postulated, though Veldhoen et al. (2012) observed a significant amount of aqueous EMB to partition from the exposure sediment into the seawater, lending support to its importance as a potential pathway. In this experiment silica sand was used as the exposure medium, which would have low binding capacity with EMB due to the absence of organic material compared to the high organic matter that is present in marine sediments. This may have lead to elevated EMB levels in the seawater of the exposure tanks. However, the tissue adsorption of xenobiotics by fish gills is much more restrictive than through the gastrointestinal tract (Wood and Part 1997; Trischitta et al. 1999; de Wolf et al. 2007). While the adsorption capacity of different tissues in spot prawns is unknown, if similar to fish there would be limited potential for EMB uptake through the gills. Furthermore, due to its physical properties (large

molecular weight and size, and the high number of hydrogen bond acceptor) EMB is not likely to be a molecule to passively diffuse across biological membranes.

Prawns exposed for 30 to 45 days to EMB levels of $18 \mu\text{g kg}^{-1}$, which are comparable to *in situ* sediment concentrations in the depositional area near salmon farms, had average tissue loads of $0.317 \mu\text{g kg}^{-1}$ wet weight (± 0.138 SE) or $1.337 \mu\text{g kg}^{-1}$ dry weight (± 0.619 SE). Studies examining tissue EMB residues in species in the vicinity of salmon farms have found 0.09 to $3.1 \mu\text{g kg}^{-1}$ dry weight in spot prawns caught near BC salmon farms over 100 days post treatment (DFO 2012b) and 2 to $5 \mu\text{g kg}^{-1}$ wet tissue weight in crustacean species near Scottish farms one week after SLICE[®] application (Telfer et al. 2006). Crustaceans in the vicinity of treating salmon farms seem to accumulate higher EMB tissue levels than spot prawn in these laboratory sediment exposures. Treatment groups exposed to EMB levels in the laboratory between 472 and $808 \mu\text{g kg}^{-1}$ over 30 to 45 days attained EMB tissue levels similar to those observed in the field studies. In addition to sediment and water exposure, crustaceans near salmon farms are also exposed to salmon feed and production fish feces containing much higher concentrations of EMB which they may uptake through ingestion. The higher accumulation of EMB in wild spot prawns *in situ* could also be due to longer EMB exposure durations if prawns are remaining near treating farms over several years as EMB can persist in the environment for over 1.5 years (DFO 2012b) and treatments can occur on farms as frequent as two times a year to every other year. Spot prawns have limited migration ranges (Bower, Meyer, and Boutillier 1996; Boutillier and Bond 2000; Bower and Boutillier 1990) so there is the potential that individuals near salmon farms may experience long-term chronic exposures over their entire lifecycle.

The effects of long-term EMB exposure on the individual scale and on the population scale remain unknown. Waddy et al. (2010) have demonstrated that the effects of long-term chronic low EMB doses on ovigerous lobsters in a laboratory setting can be more detrimental than the effects from short-term high doses. The 30-day EMB sediment exposure LC_{50} for spot prawns was estimated to be $735 \mu\text{g kg}^{-1}$ (± 1.7 SE). EMB residues in settled particulate material collected in sediment traps deployed near treating salmon farms in Scotland observed concentrations of 75 to $366 \mu\text{g kg}^{-1}$ wet sediment weight (Telfer et al. 2006), however, hydrodynamic conditions, bathymetry and increased

nutrient loads in the depositional footprint near salmon farms will affect the dispersal and dilution of EMB, as well as its bioavailability. The maximum concentration that has been observed in sediment underlying salmon farms in BC is $35 \mu\text{g kg}^{-1}$ wet sediment weight. Reported EMB sediment exposure LC_{50} values for other crustaceans are limited to two species of amphipods, *Corophium volutator* and *Eohaustorius estuarius* (McHenery 1999; Mayor et al. 2008). These exposures were over a ten-day period and the LC_{50} values ranged between 153 and $193 \mu\text{g kg}^{-1}$. If this experiment with spot prawn had been limited to ten days the resulting LC_{50} would have been concentrations greater than $3330 \mu\text{g kg}^{-1}$. With an additional 20 days of EMB exposure the LC_{50} value receded to $735 \mu\text{g kg}^{-1}$ indicating that the duration of exposure does seem to be an important factor affecting the toxicity of EMB in sediment to spot prawns. Long-term chronic effects on the population level *in situ* may be occurring at concentrations much lower than LC_{50} values observed in laboratory exposures.

This research presents results of the biological impacts of EMB sediment exposure to spot prawn. Exposure durations over ten days such as these present novel information that is important in understanding the effects of longer term exposure, as these are the conditions that are present in the vicinity of treating salmon farms. Important conclusions that can be made from this research include that high exposure doses of EMB can cause increased mortality and decreased molting success in spot prawns. EMB can accumulate in the tissues of spot prawns, however, the possibility of depuration from tissue remains unknown. Initial data suggests that exposure duration is also an important factor in EMB toxicity. These results lend support to the development of further research on the impacts of low EMB dose long-term chronic exposures on marine species that would aid in identifying whether the long-term prophylactic use of SLICE[®] to treat sea lice outbreaks is a sustainable solution for the salmon farming industry and for the BC coastal environment.

Chapter 5. Biological effects of emamectin benzoate acute sediment exposures on spot prawn food detection and orientation capability

5.0 Introduction

The detection of chemical cues by crustaceans is important for food recognition and tracking (Hazlett 1968; Carr 1978; Keller, Powell, and Weissburg 2003; Horner, Weissburg, and Derby 2004; Daniel, Fox, and Mehta 2008), avoiding predators (Berger and Butler 2001; Mackie and Grant 1974), courtship behaviour and reproduction (Dunham 1978; Hardege and Terschak 2011; Gleeson 1982; Raethke, MacDiarmid, and Montgomery 2004), larval release (Forward, Rittschof, and Vries 1987; Tankersley et al. 2002), and planktonic settlement (Clare and Matsumura 2000; Forward et al. 2003). The primary chemoreceptors in the crustacean olfactory system are setae called aesthetascs located on the first antennae (antennules), that are innervated with sensory neurons (Laverack 1964; Cate and Derby 2001; Sandeman and Sandeman 2003). The nervous system is crucial to the transmission of water-borne cues sensed by the aesthetascs to the olfactory lobe.

In decapod crustaceans the presence of a chemical stimuli elicits a hierarchical sequence of feeding behaviour responses: (1) detection of chemical stimuli by the chemoreceptors on the antennules, mouthparts, and pereopods* (Figure 18), (2) orientation or change of position towards the chemical stimuli, (3) locomotion towards or displacement from the chemical stimuli, (4) initiation of feeding, and (5) continuation or termination of feeding (Dethier et al. 1960; Lindstedt 1971; Lee & Meyers 1996a; 1996b). Each of these response phases have specific associated behaviours. During the detection phase typical behavioural responses include the antennule flick and wipe, maxilliped beat, dactyl wave and wipe, and head bob (Lee and Meyers 1996a). Antennular flicking and the wipe grooming behaviour allow crustaceans to access odorants and then remove them from sensory receptors. Downward flicking of the

* Modified walking appendage on the cephalothorax

flagella on the antennules assists in odour detection by reducing the boundary layer surrounding the aesthetascs, thus removing stagnant seawater and allowing fresh water to exchange (Snow 1973; Gleeson, Carr, and Trapido-Rosenthal 1993). Antennular grooming behaviour is conducted by spontaneous repetitive wiping of the antennae through the setae on the third maxillipeds, which serves to reduce accumulating debris on and between the antennular aesthetascs and nonaesthetasc chemosensilla (Barbato and Daniel 1997; Daniel, Fox, and Mehta 2008). During the orientation phase crustaceans will display behaviours including a dactyl rake, probe or dig with the pereopods, and a turn. These pereopod probing movements assist in searching for prey in the immediate vicinity (Lee and Meyers 1996b). During the locomotion phase crustaceans will walk, run, search, or search frantically towards a chemical stimuli. Finally during the feeding initiation phase crustaceans will either grab, lunge, pounce, hold or taste the source, and during the continuation of feeding phase crustaceans will either ingest or reject the source (Lee and Meyers 1996b).

The ability for crustaceans to detect odorants can be affected by environmental conditions, including pollutants (Blinova and Cherkashin 2012). Since EMB affects nerve impulse transmission the ability for exposed spot prawns to accurately process and respond to food odorants may be compromised. To date no published research has quantified the sublethal effects of EMB sediment exposure on food detection, orientation, or locomotion of exposed individuals. Through two laboratory experiments the ability of spot prawns to detect, orientate, and move towards a food stimulus will be assessed after ten and 30-day EMB sediment exposures.

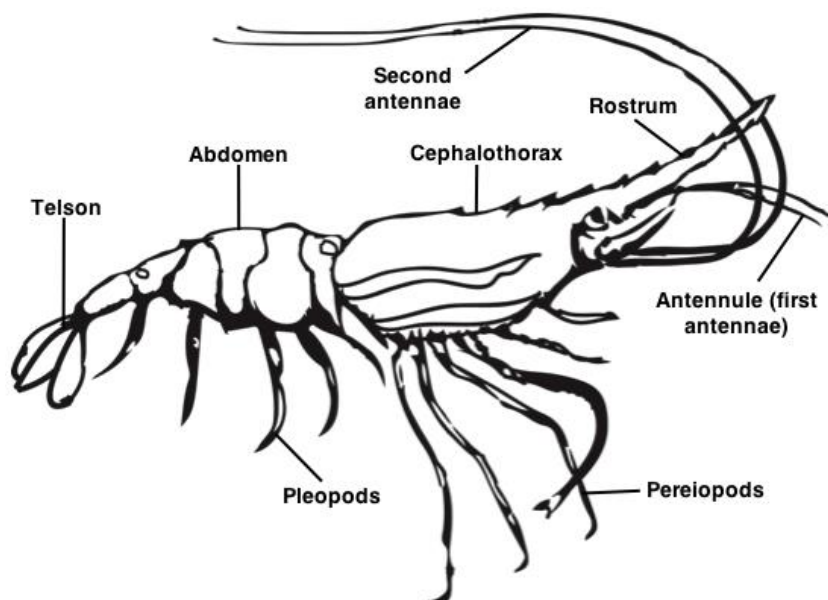


Figure 18: Spot prawn body diagram

5.1 Methods and Materials

5.1.1 EMB exposure

Premix 0.2% SLICE[®] was obtained from Intervet Schering-Plough Animal Health. To prepare the 16 L polycarbonate exposure tanks 2050 g of 2 – 20 µm silica sand was mixed with 450 mL of filtered seawater. Before each use all tanks were washed with laboratory detergent, sodium hypochlorite and sodium thiosulphate, and then rinsed three times with test water. The sediment was homogenized with a stainless steel spoon for ten minutes upon the addition of measured amounts of SLICE[®] premix. From each tank a 100 g sample of sediment was retained and frozen at -20°C until shipped for chemical analysis to the Fisheries and Oceans Canada Institute of Ocean Sciences laboratory in Sidney, BC. Tanks were filled with 16 L of filtered seawater and placed via randomized block design in a water bath for 24 – 48 hours to allow the sediment to settle. Tank seawater was maintained at 10 – 13 °C and 80 – 90% dissolved oxygen concentration with air stones. For the 30-day exposures half the seawater was replaced weekly in each tank. For the exposure durations prawns were in constant low light conditions and observed daily for morbidity, mortality and molting.

For the ten-day EMB exposures, twelve tanks were prepared for each of the five test exposure treatment concentrations, 0, 20, 200, 2000, and 4000 $\mu\text{g kg}^{-1}$ that each contained ten prawns (average carapace length 33.77 mm (± 0.07 SE) and weight 22.89 g (± 0.12 SE)) held communally. Prawns were not fed for the duration of the exposure, so were starved for ten days prior to the behaviour experiments. Within six hours of the conclusion of the ten-day exposure, 30 prawns from each of the five exposure treatment concentrations (five from each tank rep; only 25 prawns available exposed to 205 $\mu\text{g kg}^{-1}$ concentrations), were randomly selected and tested to determine the dose response of EMB on food stimuli detection and orientation. Thirty different prawns from each of the five treatments levels of ten-day sediment exposures were subjected to Y-tube olfactometer trials to test the effects of EMB exposure on the ability of prawns to orientate and navigate towards a food stimulus.

Six tanks of each EMB concentration of 0, 20, 200, 1000, 2000, and 4000 $\mu\text{g kg}^{-1}$ were prepared for the 30-day exposures. Four prawns (average carapace length 30.69 mm (± 0.14 SE) and weight 17.47 g (± 0.24 SE)) were held in each tank, physically separated in their own vexar compartment and fed every seven days (four times throughout the experiment) with a squid and commercial feed pellet diet. All surviving prawns from the 30-day sediment exposure experiment were subjected to the food detection and orientation experiment. Due to greater mortality in high EMB dose 30-day exposure groups the sample size for each treatment was uneven.

5.1.2 Food stimulus preparations

A food stimulus solution was prepared by mixing 588 g (± 9.08 SE) chopped Pacific sardines (*Sardinops sagax*), in four litres of filtered seawater. The solution was allowed to sit for 24 hours at 10.5°C, and then filtered (5 micron mesh size) to remove all solids and frozen until use.

5.1.3 Detection and orientation behavioural response

Experiments were conducted in a dark room with a single red bulb (60W, 130 V, 0.46 Amps) and a video camera to record behaviour so as to eliminate observer effects on prawn behaviour. In a randomized block design prawns from each exposure group were

individually acclimatized in a glass tank (30 x 20 x 18 cm) for five minutes. For minutes six and seven the number of antennular flicks and the number of times the first and second antennae were wiped through the third maxilliped were recorded. At minute eight 3 mL of sardine solution was added to the water surface in the centre of the tank using an eye dropper. At minute nine the number of antennular flicks and antennae wipes were recorded again for two minutes. The period of time after the introduction of the food stimulus (minute eight) to exhibiting dactyl probing of the periopods was recorded. At the end of minute ten prawns were removed, measured, weighed, and assessed for condition which included examining for broken or missing appendages, damage to the eyes, and the presence of any black spotting on the shell. Between each trial the tank was rinsed with sodium hypochlorite, sodium thiosulphate, and then clean seawater three times.

5.1.4 Locomotion behavioural response

5.1.4.1 Y-tube olfactometer construction

A Plexiglas Y-tube olfactometer 15 cm tall and 15 cm wide with an outflow arm 76 cm long and inflow arms 86 cm long (Figure 19) was used to observe the locomotion and navigational response of EMB exposed and non-exposed spot prawns to a food stimulus. A line was drawn on each of the arms at 45 cm distance from the arm junction. Seawater flowed through the Y-tube at a rate of 800 mL min⁻¹ and food stimulus was introduced to one arm via an IV tube at a rate of 5 mL min⁻¹ while plain seawater in an IV was released at the same rate into the other arm. Plastic straws 15 cm long were stacked into the end of each arm to create laminar flow. An insertion chamber with a gate allowed water to run through the chamber to the back of the Y-tube and out. To prevent vertical stratification of the food stimulus in the Y-tube there were two water outtakes, one near the floor of the Y-tube and one near the water surface. By adding food dye to the inflow arms it was determined that water flow was laminar with little horizontal mixing of the water downstream from the bifurcation point to the end of the outflow. A mixture of sand and marine enamel paint was applied on the bottom of the Y-tube to increase traction for the prawns. Experiments were conducted in light-limited conditions with three red lights (60W, 130 V, 0.46 Amps) 60 cm overhead positioned at the top, bottom and middle of

the Y-tube. A video camera recorded prawn activity to remove the potential for the effect of observer presence on behaviour.

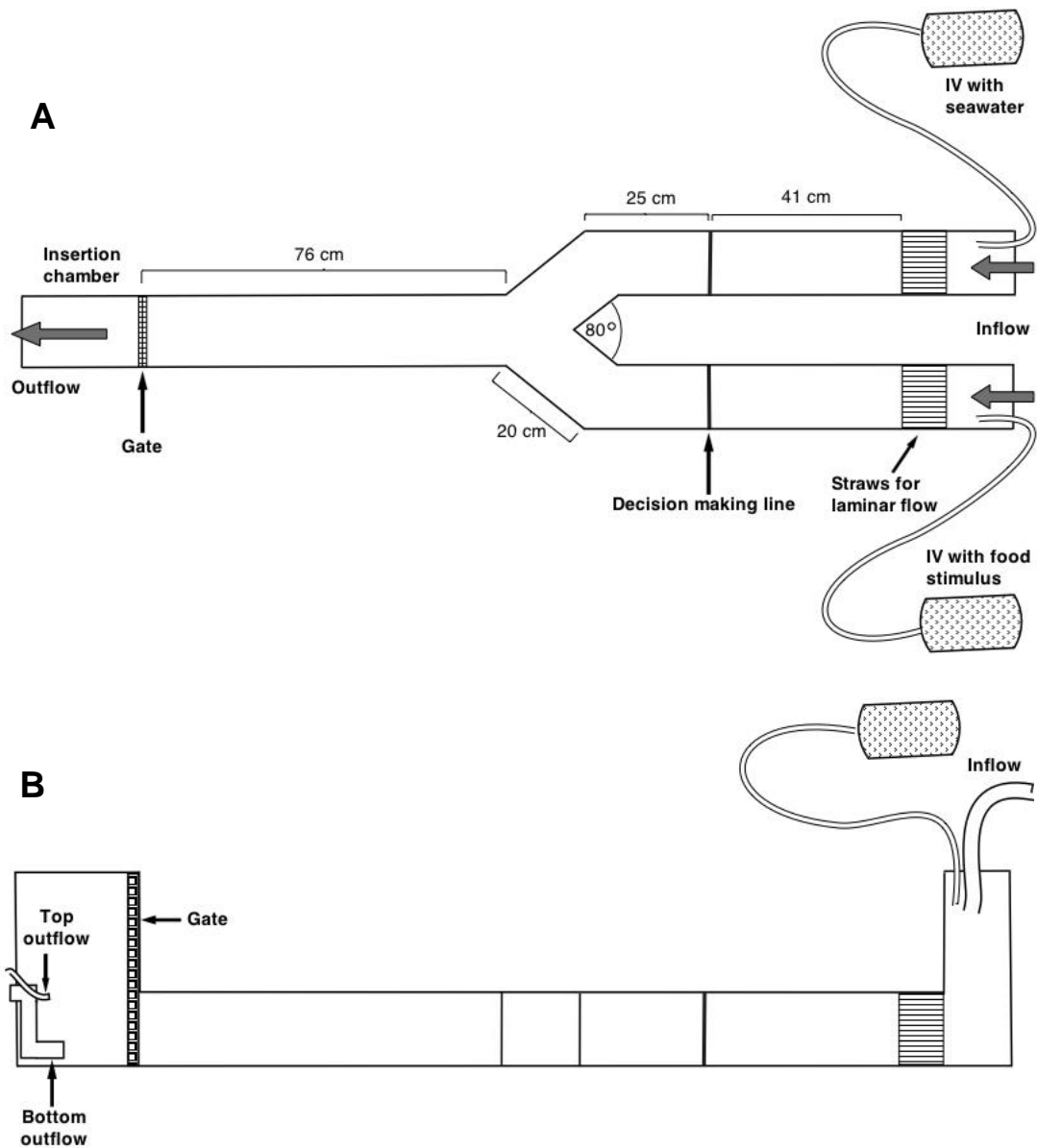


Figure 19. A top down (a) and side view (b) of Y-tube experimental set up. All dimensions are to scale.

5.1.4.2 Y-tube experiment

The prepared sardine food stimulus solution was added for 20 minutes before a prawn was introduced to the insertion chamber to allow the solution to run the full length of the apparatus (as established with previous tests with food dye). A prawn was then

introduced to the insertion chamber and acclimatized for ten minutes. The gate was raised, allowing the prawn access to the rest of the Y-tube for 15 minutes. If the prawn left the insertion chamber this was considered to be an active response. If a prawn crossed the decision line on one of the arms they were marked to have made a choice. Other observations included the time upon leaving the insertion chamber, the time to make a choice, and the distance and route travelled. After the 15 minute trial the prawn was removed, measured, weighed, and sex and condition was determined. Five minutes after the previous trial, a new trial would start. Three trials would occur before the Y-tube was drained and cleaned with sodium hypochlorite and then rinsed with sodium thiosulphate, freshwater three times, and then seawater. The Y-tube would then be filled with seawater and the food stimulus would be randomly switched between the arms to remove possible positional effects.

5.1.5 Statistical analysis

All analyses were completed using the statistical computing software *R* (R Development Core Team 2011). Differences were considered to be significant if $\alpha < 0.05$. Model selection was undertaken using Akaike information criterion (AIC) and a likelihood ratio test to determine the best-fit models.

5.1.5.1 Detection and orientation behavioural response

Antennular flicking of prawns exposed to different EMB concentrations were compared before and after the addition of a food stimulus using a negative binomial distribution generalized linear mixed effects model (GLMM) with a random effect to account for correlated observations on the same individual twice (ADMBglmm package; Fournier et al. 2012). A GLMM was also applied to model antennular grooming data. To account for the overdispersion and a large number of zeros in the response variable, the number of antennular grooms in two minutes, a hurdle model (ADMBglmm package) was used to model separately the count component with a negative binomial distribution using all the non-zero values, and the zero-inflated component using a binomial distribution comparing whether a prawn did not display antennular grooming during the two minutes (zero values) or if they did (all positive values).

GLMM with poisson distribution fit by maximum likelihood (lme4 package, Bates et al. 2011) was used to compare the number of prawns in each exposure group that displayed dactyl probing within the observed three minute time period following the introduction of the food stimulus. A random effect of tank was included in the model to account for prawns that were not independent of one another, (i.e. exposed in the same tank). A parametric survival model with exponential distribution was used to compare the mean time to dactyl probing after the introduction of food stimulus of prawns exposed at the different EMB concentrations.

5.1.5.2 Locomotion behavioural response

GLMM fit by maximum likelihood were used to compare the number of prawns in each exposure group that had no activity versus were active (left the insertion chamber), made no choice versus made a choice (passed the Y junction), and choose the control seawater branch versus the branch with the food stimulus. A parametric survival model with exponential distribution was used to compare the mean time to a prawn becoming active. A parametric survival model with a weibull distribution (hazard decreases with time) was used to compare the mean time to a prawn making a choice. Survival models were chose based on AIC comparisons. A non-parametric Kruskal-Wallis with multiple comparisons was used to compare the distance that active prawns moved throughout the Y-tube during the 15 minutes.

5.2 Results

5.2.1 Sediment sample chemical analysis

The difference in the levels of emamectin benzoate measured in sediments for the target doses between 20 to 4000 $\mu\text{g kg}^{-1}$ ranged from 0.4% to 29.0% (71 – 103% of target) and the average coefficient of variation was 26% (Table 18). EMB levels were detected in some of the control sediments as a result of contamination from previous trials. Contamination occurred in 78% of control exposure tanks and levels were low (range between 0.1 and 3.4 $\mu\text{g kg}^{-1}$), therefore treatment will still be considered the control that other treatments will be compared to.

Table 18: Target and actual emamectin benzoate (EMB) and desmethyl metabolite concentrations in sediment exposures in acute toxicity tests

Target [EMB]	Actual [EMB] $\mu\text{g kg}^{-1}$ wet weight (\pm SE)	Actual [metabolite] $\mu\text{g kg}^{-1}$ wet weight (\pm SE)	# Samples Analyzed	Percent of target (coefficient of variation)
0	0.44 (\pm 1.9)	< LOD	18	-
20	17.7 (\pm 3.1)	< LOD	7	88.6 (46.7)
200	205.2 (\pm 36.7)	< LOD	6	102.6 (43.8)
1000	808.3 (\pm 85.9)	1.2 (\pm 0.7)	5	80.8 (23.7)
2000	1419.0 (\pm 114.0)	2.1 (\pm 1.3)	5	71.0 (18.0)
4000	3329.7 (\pm 367.4)	8.7 (\pm 2.3)	5	83.2 (24.7)

5.2.2 Detection and orientation behavioural response

For prawns exposed to EMB for a ten-day period, the introduction of a food stimulus significantly increased the rate of antennular flicking (Figure 20; GLMM, $df = 1$, deviance = 8.34, $p = 0.004$) while it did not in spot prawns in the 30-day trials ($p > 0.05$). Spot prawns in the 3330 $\mu\text{g kg}^{-1}$ exposure treatment had significantly depressed antennular flicking compared all other treatment groups in both the ten-day ($df = 1$, deviance = 14.44 $p < 0.001$) and 30-day trials (GLMM, $df = 1$, deviance = 10.402, $p = 0.001$). In the 10-day exposure the rate of antennular flicking increased in larger prawns (GLMM, $df = 1$, deviance = 9.54, $p = 0.002$), a trend not observed in the 30-day data.

The number of spot prawns that displayed antennular grooming behaviour was greater after the addition of the food stimulus in both the ten-day (Figure 20; GLMM, $df = 1$, deviance = 26.18, $p < 0.001$) and 30-day trials (GLMM, $df = 1$, deviance = 25.61, $p < 0.001$). In the 30-day trials the number of times antennae were groomed in prawns that were actively displaying the behaviour was significantly higher after the addition of a stimulus compared to before (GLMM, $df = 1$, deviance = 34.17, $p < 0.001$) a trend not observed in the ten-day data (GLMM, $df = 4$, deviance = 4.58, $p = 0.3334$). Exposure to higher EMB concentrations significantly reduced the number of prawns that displayed antennular grooming behaviour in the 1419 and 3330 $\mu\text{g kg}^{-1}$ treatments in the ten-day trial ($df = 4$, deviance = 39.68, $p < 0.001$) and the 808 – 3330 $\mu\text{g kg}^{-1}$ treatments in the 30-day trial ($df = 1$, deviance = 12.93, $p < 0.001$) compared to all lower exposure groups.

For prawns actively displaying grooming behaviour the number of times the antennae were cleaned in the two minute observation period was not different between EMB treatments in either the 10-day ($df = 4$, deviance = 4.58, $p = 0.3334$) or 30-day experiments ($df = 5$, deviance = 7.96, $p = 0.158$).

Spot prawns exposed to 0.4 to 1419 $\mu\text{g kg}^{-1}$ sediment over the ten-day period were not significantly different in the proportion (80 – 90%) that displayed dactyl probing after the addition of a food stimulus or in the time until dactyl probing display ($p > 0.05$) with a predicted response time of 1.27 minutes (Figure 21). Significantly less (50%) spot prawns in the 3330 $\mu\text{g kg}^{-1}$ treatment displayed dactyl probing within the observation period (GLMM, Likelihood ratio test, $\chi^2=8.731$, $df = 1$, $p = 0.003$) with a longer predicted response time of 3.93 minutes (Parametric survival model, $z = 4.07$, $p < 0.001$). Spot prawns in the 0.4 to 205 $\mu\text{g kg}^{-1}$ 30-day exposure groups were not significantly different in the proportion (80%) that displayed dactyl probing after the addition of a food stimulus (GLMM, Likelihood ratio test, $\chi^2 = 1.6139$, $df = 2$, $p = 0.446$) or in the time till display, 1.24 minutes (Figure 21; Parametric survival model, $z = 2.44$, $p = 0.295$). Fifty-five percent of prawns in the 808 $\mu\text{g kg}^{-1}$ exposure group displayed dactyl probing within a predicted 3.04 minutes, while all of prawns in the 1419 to 3330 $\mu\text{g kg}^{-1}$ treatment exhibited dactyl probing in 0.42 minutes on average.

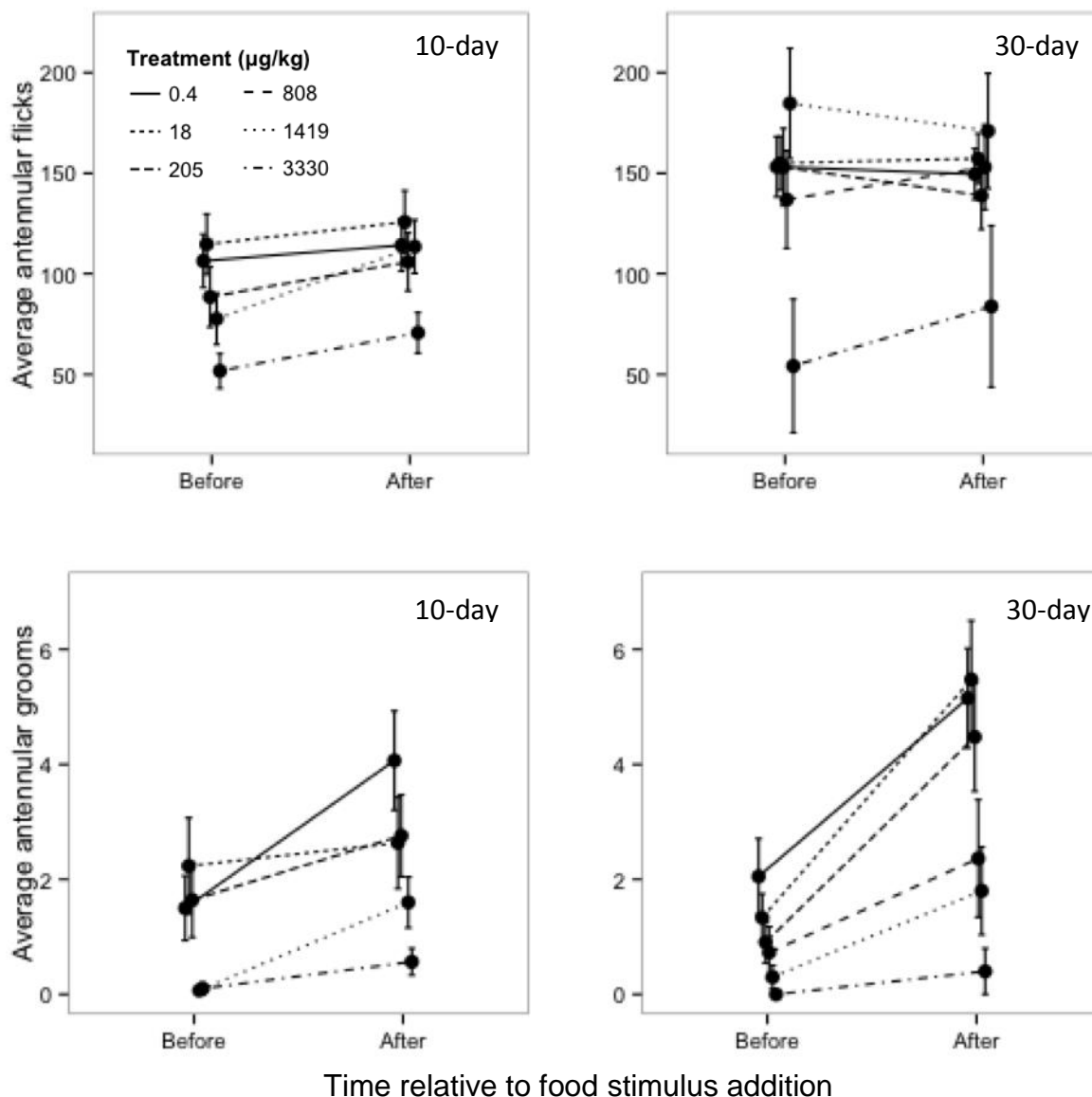


Figure 20: Average antennular flicking and grooming by spot prawn, previously exposed to sediment with concentrations of EMB ranging from 0.4 to 3300 $\mu\text{g kg}^{-1}$ for ten and 30 days, before and after the introduction of a food stimulus. Error bars are standard error. Ten-day: food stimulus significantly increased the rate of antennular flicking and grooming, spot prawns in the 3330 $\mu\text{g kg}^{-1}$ treatment had significantly depressed antennular flicking, significantly less spot prawns in the 1419 and 3330 $\mu\text{g kg}^{-1}$ treatments displayed antennular grooming behaviour; 30-day: food stimulus significantly increased the rate of antennular grooming, prawns in the 3330 $\mu\text{g kg}^{-1}$ treatment had significantly depressed antennular flicking, significantly less prawns in the 808 – 3330 $\mu\text{g kg}^{-1}$ treatments displayed antennular grooming.

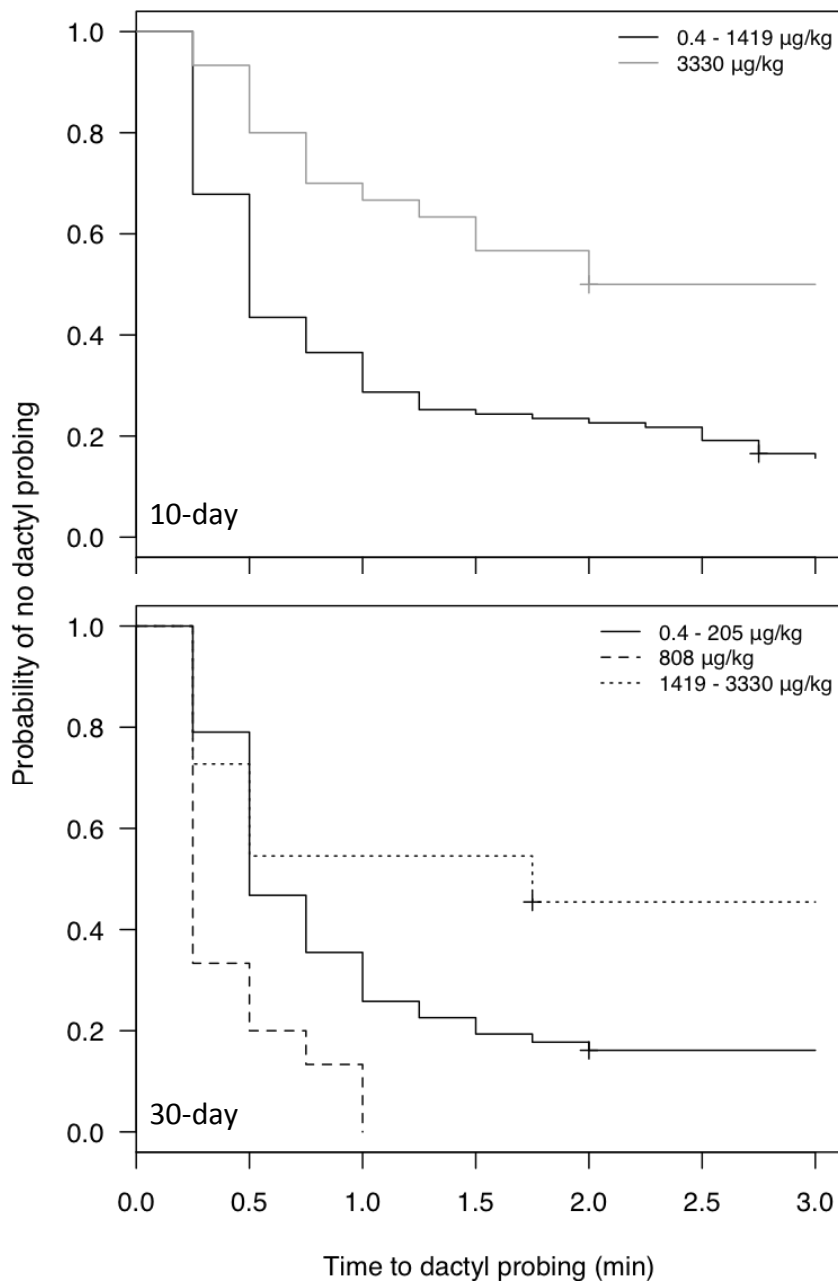


Figure 21: The time for spot prawns exposed to different concentrations of EMB for ten and 30 days to respond to a food stimulus by probing the sediment with the dactyl of the periopods (walking legs). 10-day: Significantly less spot prawns in the 3330 $\mu\text{g kg}^{-1}$ treatment displayed dactyl probing compared to lower exposures and had a longer predicted response time; 30-day: Significantly less spot prawns in the 808 $\mu\text{g kg}^{-1}$ treatment displayed dactyl probing compared to lower exposures and had a longer predicted response time, while all prawns in the 1419 to 3330 $\mu\text{g kg}^{-1}$ treatment exhibited dactyl probing in the shortest response time.

5.2.4 Locomotion behavioural response

There was no difference in the level of activity of prawns exposed to different EMB concentrations (Figure 22; GLMM, Likelihood ratio test, $\chi = 6.796$, $df = 4$, $p = 0.147$). The length of time until activity in the $3330 \mu\text{g kg}^{-1}$ exposure group, predicted to be 30.67 minutes, was significantly longer than the 14.75 minutes predicted for all lower dose exposure groups (Figure 23; Parametric survival model, $z = 2.43$, $p = 0.008$).

None of the $3330 \mu\text{g kg}^{-1}$ exposure prawns passed the Y-junction making a choice, while a significantly greater proportion of prawns in the other exposure groups made a choice, though were not different from one another (GLMM, Likelihood ratio test, $\chi = 24.414$, $df = 1$, $p < 0.001$). For the $0.4 - 1419 \mu\text{g kg}^{-1}$ exposure groups there was no difference in the predicted length of time, 26.14 minutes, for prawns to make a choice between the food stimulus or control seawater arm (Parametric survival model, $p = 0.550$). There was also no difference in the proportion of prawns from each exposure treatment that chose the food stimulus arm over the control seawater arm (GLMM, Likelihood ratio test, $\chi^2 = 3.8149$, $df = 3$, $p = 0.282$).

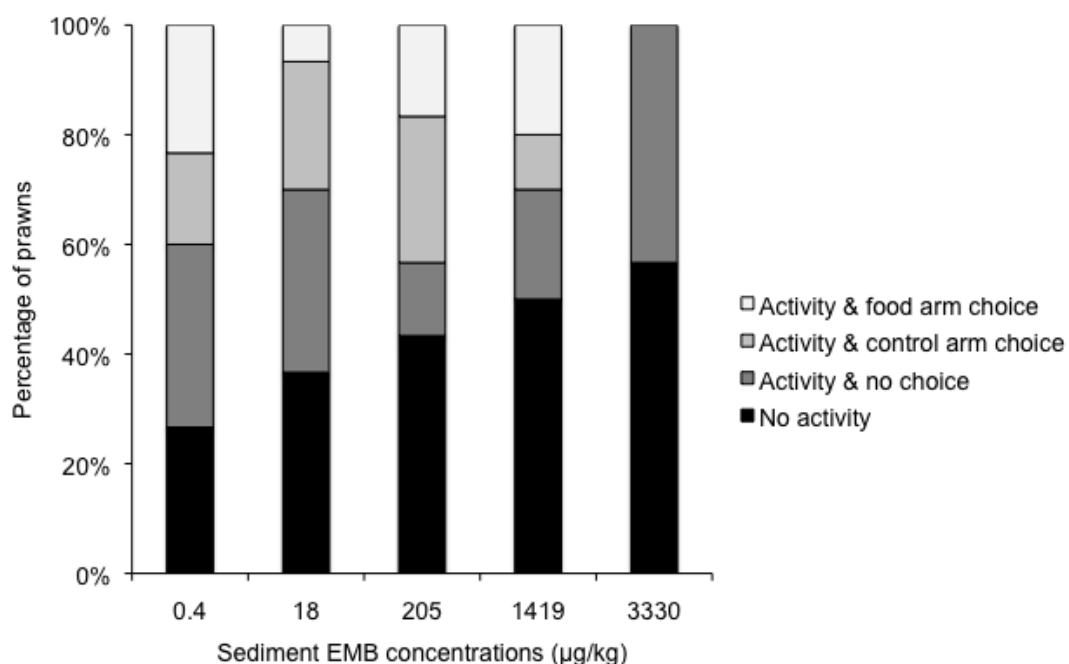


Figure 22: Percentage of prawns in each EMB treatment that were active and made a choice during the locomotion behavioural response experiment. There was no difference in the proportion of prawns that were active, made a choice, or what arm they chose in the different EMB concentrations, except none of the $3330 \mu\text{g kg}^{-1}$ treatment made a choice.

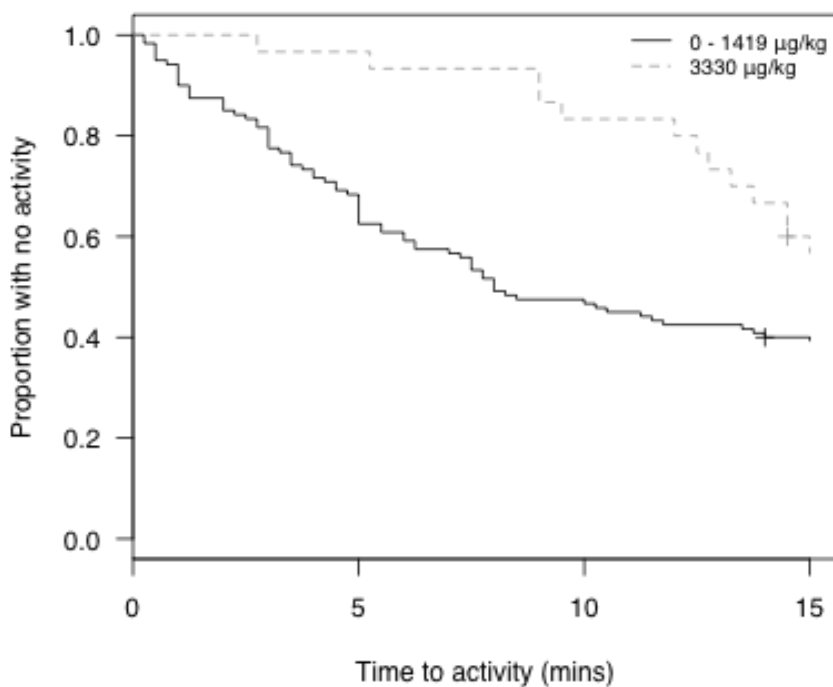


Figure 23: Length of time for spot prawns, exposed to different EMB treatments over a ten-day period, to become active (leave the insertion chamber in the Y-tube) in the presence of a food stimulus. The predicted length of time until activity in the 3330 µg kg⁻¹ exposure group, was significantly longer than for all lower dose exposure groups

For active prawns that were exposed to doses 1419 µg kg⁻¹ and less, there was no difference in the total distance travelled in the Y-tube, however, prawns exposed to 3330 µg kg⁻¹ travelled significantly less than other treatments (Figure 24; Kruskal-Wallis multiple comparisons, $\chi^2 = 23.952$, $df = 4$, p-value < 0.001).

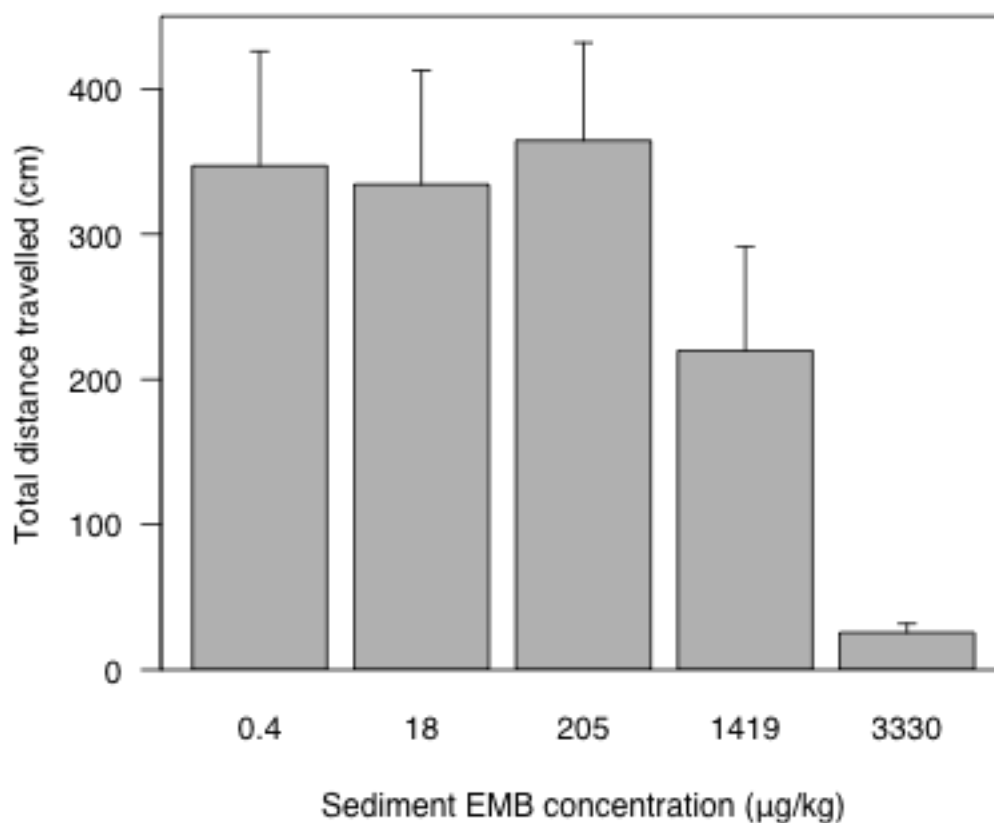


Figure 24: Total distance travelled in the Y-tube by spot prawns exposed to different EMB concentrations over a ten-day period. Error bars are standard error. Spot prawns exposed to 3330 µg kg⁻¹ treatment travelled significantly less than other treatments.

5.3 Discussion

EMB toxicity effects on the olfactory response of spot prawns to chemical stimuli was only observed in the highest EMB exposure groups tested. In the detection and orientation behavioural experiments, while no difference was detected in antennular flicking rates in exposure groups 0.4 to 1419 µg kg⁻¹ in both the ten and 30-day exposure trials, a significantly depressed flicking rate was observed in prawns in the 3330 µg kg⁻¹ group. In the ten-day trials spot prawns increased the rate of antennular flicking after the addition of the food stimulus, however, because no control tests were run adding plain

seawater as a substitute for the seawater-sardine mixture it is not possible to differentiate whether prawns were responding to the food stimulus or to the disturbance of the water.

Antennular grooming behaviour is not exhibited as frequently by spot prawns as flicking behaviour, and often during a two minute observation period no grooming behaviours would be documented. Spot prawns were more likely to exhibit grooming after the addition of a food stimulus, while flicking occurred more continuously regardless of the stimulus. These observed trends likely occurred because antennular flicking is utilized to sample and detect odourants from the water column (Snow 1973; Gleeson, Carr, and Trapido-Rosenthal 1993) so would be required both before and after the introduction of a stimulus, while grooming of the antennae removes odourants from antennae receptors to make them accessible to new stimuli (Barbato and Daniel 1997; Daniel, Fox, and Mehta 2008), so this behaviour would be employed after the addition of a food stimulus when odourants are present. Exposure to high doses of EMB ($> 808 \mu\text{g kg}^{-1}$) significantly reduced antennular grooming behaviour in spot prawns in both ten and 30-day trials indicating that these prawns may have been compromised in their olfaction detection ability. If antennae are not regularly groomed microbes and detritus can accumulate and damage the aesthetasc receptors, potentially impairing olfactory functionality (Bauer 1977).

In the ten-day trials the proportion of spot prawns exposed to $3330 \mu\text{g kg}^{-1}$ that displayed dactyl probing after the addition of a food stimulus was significantly reduced compared to all other treatment groups and there was a threefold increase in the predicted response time. Interestingly a different trend was observed in the 30-day trials with spot prawns in the two highest dose groups, 1419 and $3330 \mu\text{g kg}^{-1}$, having the shortest predicted response time and the highest proportion of spot prawns respond, followed by the three lowest dose groups 0.4 , 18 and $205 \mu\text{g kg}^{-1}$, with the $808 \mu\text{g kg}^{-1}$ treatment group having the longest response time and the least number of prawns respond. This irregular dose response by spot prawns to EMB has also been observed by Veldhoen et al. (2012) where lower prawn mortality was observed in higher EMB dose groups compared to prawns exposed to middle doses. Veldhoen et al. (2012) hypothesize that high concentrations of EMB may induce early compensatory mechanisms that does not occur in prawns exposed to lower doses. It is also important to stipulate that the number

of surviving spot prawns in the 808, 1419 and 3330 $\mu\text{g kg}^{-1}$ exposure groups for the 30-day trials was low and thus the sample size of these groups ($n = 11, 10$ and 6 respectively) for the detection and orientation behavioural experiments were significantly lower than the lower exposure groups ($n = 20 - 21$). Experimentation with larger sample sizes is required to corroborate these observed trends.

In the locomotion behavioural experiment using the Y-tube olfactometer it was determined that spot prawns in the 3330 $\mu\text{g kg}^{-1}$ exposure group took twice as long to become active compared to exposure groups 0.4 to 1419 $\mu\text{g kg}^{-1}$ (though there was no significant difference in the proportion that were active between all treatments). The average total distance travelled by 3330 $\mu\text{g kg}^{-1}$ individuals was significantly lower with no prawns able to travel past the decision line to make a choice. The Y-tube was not successful in deciphering whether spot prawns were able to detect chemical stimuli and successfully locate the source as there was no difference in the proportion of prawns from each exposure treatment that chose the food stimulus arm over the control seawater arm. Most Y-tube olfactory experiments have been conducted on small insects and mites and thus this type of design may not be appropriate for larger marine invertebrates. Prawns are very mobile and it was observed that if prawns did not choose the correct arm initially they would continue to search for the location of the food source by travelling to the opposite arm. Greater success may have been achieved also if a L-sodium glutamate solution would have been used rather than a sardine-seawater mixture for the chemical stimulus as this would have resulted in a more consistent, evenly mixed solution that specifically contains only the compound that crustaceans respond to in chemical food cues (Barbato and Daniel 1997).

In this experiment it was not possible to distinguish if spot prawns in the high EMB exposure groups were unable to perceive food chemicals due to reduced behavioural olfactory responses (antennae flicking and grooming, and dactyl probing) or if they had the same detection capabilities as other treatment groups but had restricted movement to respond because of impacts on the nervous system or other toxicity effects. Increases in antennae flicking were observed in 3330 $\mu\text{g kg}^{-1}$ group after the addition of a food stimulus, albeit reduced compared to other groups, so some olfactory response is possible. Prawns in the 3330 $\mu\text{g kg}^{-1}$ exposure group in all experiments displayed

orientation problems that were not quantified, which included the inability to hold the abdomen and carapace off the bottom and travelling in circles. Orientation problems after avermectin exposure has been observed in both crustaceans (Burrige et al. 2004) and insects (Agee 1985).

The most relevant finding of this research was that at high exposure doses of EMB spot prawns displayed decreased activity of olfactory behaviours both before and after the introduction of chemical stimuli. While concentrations of this magnitude are not expected in the wild, spot prawns exposure *in situ* may be for much longer durations than those tested in the laboratory. Given the importance of the detection of chemical cues by crustaceans for food recognition, predator avoidance and reproduction, the impacts of chemicals that may disrupt these processes should be further investigated before widespread and long-term use. While the literature on acute toxicity of avermectins in regards to LC_{50} is extensive, the sublethal effects of long-term EMB exposure on marine crustacean species remain unknown.

Chapter 6. General Discussion

6.1 Overview of Results

Sea lice outbreaks on salmon farms remain a constant predicament in the British Columbia salmon aquaculture industry that can both lower product value and have wide-ranging environmental implications. The BC industry depends on the regular application of the chemotherapeutant SLICE[®], active ingredient emamectin benzoate (EMB), to control sea lice outbreaks on farms. EMB is a crustacean neurotoxin that is applied in feed to production fish over a seven-day period. Consequently, this type of in-feed treatment can result in EMB deposition in the benthic environment that has raised concerns regarding the impact on non-target species in the vicinity.

There has been significant research into the acute toxicity of many different organisms to various chemotherapeutants and pesticides used in the salmon aquaculture industry. These types of bioassays have been important in the development of risk prediction models, representative test organisms, and allowable residue limits in the marine environment. Pertinent research on lethal and sub-lethal effects of EMB exposure on Pacific Northwest species is extremely limited and toxicity benchmarks have still not been established for many species and their different life stages. Spot prawns were the Pacific Northwest species of interest in this study due to their ecological and commercial importance, delectability, as well as the interest and concern of commercial fishermen that have contributed to the formation and process of this research. The research presented here attempts to address current gaps important to regulatory criteria regarding chemical use in the BC aquaculture industry.

The primary objective of this research was to determine if EMB exposure had a measurable biological effect (lethal, sub-lethal and behavioural) on spot prawns in the laboratory and in the field. This thesis included four main components: a field survey conducted in the Broughton Archipelago, BC, to determine if EMB residues could be detected near actively treating salmon farms and whether farm proximity affected spot prawn size distribution (Chapter 2), and three laboratory experiments assessing effects of EMB pellet exposure on spot prawn mortality, molting and consumption rates (Chapter

3), mortality and molting of spot prawns exposed to EMB in sediment (Chapter 4), and effects of EMB sediment exposure on spot prawn food detection, orientation and locomotion (Chapter 5).

EMB residues were detected in marine sediment proximate to farm sites in the Broughton Archipelago before, during, and after SLICE[®] treatment. These results indicate that EMB is persisting between treatment applications, and in one farm was detected three years following the last treatment. Male and transitional stage spot prawns captured near farm sites attained a greater size and had increased body condition compared to reference sites. However, at two farm sites in Knight Inlet the size distribution and catch abundance of prawns changed over the sampling period, a trend not observed at reference sites, demonstrating that farm activity may alter prawn population dynamics. An important caveat though is that these observations were from a small sample size collected over one season with a broad survey focus making it difficult to interpret size and catch trends in relation to SLICE[®] treatment or any specific farm activity.

Laboratory results demonstrated that prawns will consume SLICE[®] pellets but only following a period of starvation. After the initial exposure to SLICE[®] pellets, feeding rates declined with subsequent exposures. The depressed consumption rates were unlikely a residual effect of EMB toxicity, but rather an aversion to the SLICE[®] pellets as prawns resumed feeding when offered a preferred diet. No effect of SLICE[®] pellet exposure on prawn mortality was observed. Sediment EMB exposure data demonstrate that high doses, above those observed in the depositional area near farms, cause the highest mortality. Similar results have been established with many other different species, though a novel finding of this study is that mortality was largely due to the inability of molting individuals to successfully complete ecdysis. Prawns are very vulnerable during period of molting, as exhibited by cannibalism of molting individuals in group exposures. Prawns exposed to high dose EMB concentrations displayed a significant reduction in olfactory behaviours to food stimuli. During all of these experiments EMB did not affected the rate of molting in prawns, as has been observed in American lobsters (Waddy et al. 2002). Since some control sediment exposures were unknowingly contaminated with trace EMB it was not possible to compare medicated versus non-

medicated spot prawns. However, because survival and successful molting ability was quite high in all low exposure groups and the greatest response was observed in extremely high exposure groups, it is still possible to draw relevant conclusions from this research.

6.2 Challenges

This research presented many challenges throughout the process, some seemingly insurmountable. One of the largest limitations throughout all aspects of this research was the lack of biological information on spot prawns. Though a difficult species to study due to its' inhabited depth and nocturnal behaviour, there remain many unanswered basic biology questions including migration ranges and main diet. While these limitations sometimes made it difficult to design appropriate methods relevant to the study animal, observations both in the lab and in the field often brought on new and interesting discoveries. It was discovered that holding prawns in captivity over long periods proved to be difficult due to disease outbreak, synchronous mass molting, and cannibalism. For further research on long-term EMB exposure on prawns it will be important to develop methodology to prevent the problems that were encountered during this research. One regret is that it was not possible to process the spot prawn tissue samples collected near farm sites for EMB residues due to equipment failure and analysis costs, as this would have added valuable information on EMB bioavailability and uptake. The final challenge to this research has been the heated politics revolving around sea lice, salmon farms, impacts on wild juvenile salmon, and effective management strategies. Cooperation between industry, government, academics, and the public to develop sound studies to assess the in-field risk of EMB to the BC marine environment is fraught with concerns over proprietary information and politics. Navigating this environment was challenging, albeit interesting, and while this research has produced important results there are many more critical questions that would require transparency between involved groups. Unfortunately the likely cause of why so few studies are being conducted in Pacific Canada on SLICE® impacts is because of these challenges.

6.3 Study implications and future directions

Health Canada's Pest Management Regulatory Agency (PMRA) was established in 1995 to provide regulation regarding the safe access and use of pest management tools that minimize hazards to human health and the environment. Specific toxicity endpoints — mortality, reproduction, and growth — are used by PMRA to determine the risk of a pesticide to the environment, and its regulation and application restrictions. In the laboratory toxicity tests, elevated levels of EMB sediment exposure were observed to affect prawn mortality, molting success, olfactory behaviours, and locomotion. This research has directly assessed the effects of acute EMB exposure on prawn mortality and indirectly the effects on reproduction and growth by measuring effects on molting and olfactory behaviours. Molting is a critical process for reproduction in some crustaceans (i.e. soft shelled mating in Dungeness crabs, protandric hermaphroditism in spot prawns) as well as growth and development. Olfactory detection and response behaviours are which are important for mate location, courtship behaviour, and larval release during reproduction and food recognition and tracking which ultimately will affect growth. Since it is known that after the application of SLICE[®] the environmental fate of EMB includes its' persistence for several years in marine sediment and the uptake by spot prawn inhabiting areas near salmon farms (DFO 2012b), these initial observations on the effects of EMB on spot prawn mortality, molting, and olfaction provide justification into further assessment on the effects of EMB (toxicity endpoints) on spot prawn.

Overall, EMB sediment concentrations that had a measurable impact on mortality, molting success, and behaviour in prawns, under the parameters tested, was 40 to 200 magnitude greater than levels present in the marine environment. Prawns exposed to EMB levels in the lab similar to those found in marine sediment near farms were not visibly affected by exposure. While exposure concentration is often the focus of acute toxicity studies, exposure duration in this case seemed to be an important factor in assessing EMB toxicity. Results from Waddy et al. (2010) indicate that small chronic EMB doses is more detrimental to lobsters than larger doses over one or two administrations. Though the EMB exposure tests in this research are some of the longest test durations in the literature, they still only provide data on relatively short-term EMB acute toxicity effects compared to multi-year chronic exposure that may occur in the

marine environment. The sub-lethal effects from chronic EMB exposure may be occurring in marine populations at orders of magnitude below concentrations causing mortality in laboratory based experiments.

In situ conditions are also very different from laboratory experiments as there may be compounding effects in addition to EMB toxicity. This could include sediment chemistry and other heavy metals and chemicals present in the depositional area near farms, resource limitation, inter- and intra-species competition, and predation. How spot prawns respond to SLICE[®] feed pellets may also be very different *in situ* compared to the laboratory. Spot prawns captured at farm sites during the field survey were of a larger size and in better condition than reference sites, indicating that prawns may be benefiting from fish pellet subsidies directly or indirectly. Food subsidies from farms may attract and keep spot prawns in the farm footprint, leaving them vulnerable during SLICE[®] treatment periods. In the laboratory spot prawns would consume SLICE[®] medicated pellets initially but quickly began to reject them on subsequent exposures. It is difficult to postulate if a similar response may be observed in prawns located near farms, especially if they are habituated to consuming unmedicated feed pellets year round.

Predictions of the effects of EMB chronic exposure on non-target species remain uncertain. If the sustained low-level EMB concentrations that spot prawns encounter near farms is detrimental to their ability to molt successfully or respond to chemical cues in their environment this would have wide ranging consequences for food detection, reproduction and predator avoidance. Alternatively it is possible that with the increased use of SLICE[®] over the past decade in BC spot prawns have adapted and populations are more resilient to repetitive EMB treatments, perhaps overall benefiting from farm nutrient subsidies.

To better understand chronic long-term EMB exposure, studies need to be conducted that better represent conditions in the marine environment that assess both sub-lethal and lethal effects. Long-term field surveys and fisheries models need to be developed that assess the effect of SLICE[®] treatments on non-target organisms on individual and population scales. Further to this it is important to establish the most sensitive life stages and time periods of ecologically and commercially important species

to avoid EMB treatment during these periods. Crustaceans can be particularly vulnerable during ecdysis and reproduction periods.

6.4 Conclusions

In Canada, SLICE[®] application in the aquaculture industry began in 1999. For ten years SLICE[®] was used regularly on an emergency basis, though it was not an approved chemotheraputant until 2009. Currently, SLICE[®] is the only approved chemical treatment for sea lice in BC. SLICE[®] can be used several times a year on farms and persists in marine sediment between treatments. SLICE[®] still seems to be effective in reducing sea lice outbreaks on farms in BC, though resistance of sea lice is being observed in Atlantic Canada. Deltramethrin (Alphamax[®]) is likely the next candidate for sea lice treatment.

Many different products have been developed to treat sea lice outbreaks on salmon farms and include organophosphates, chitin synthesis inhibitors, pyrethroids, hydrogen peroxide, and avermectin compounds. Historically, initial recurrent use of the same chemical successfully controlled outbreaks but eventually would lose its effectiveness as sea lice develop resistance, or it might be realized that the chemical is toxic to production fish (Horsberg and Hoey 1989; Jones, Sommerville, and Wootten 1992; Tully and McFadden 2000; Haya et al. 2005). Once chemicals are no longer effective the industry will need to switch to the application of a new product, depending on jurisdictional approval. The scientific process is often not able to keep pace with this continuous rotation, making it difficult to develop long-term field studies.

The precautionary principle guides Canada's environmental policy, including the Canadian Environmental Protection Act (Environment Canada 2010; 2012), and as defined by the United Nations (1992) states that:

“Where there are threats of serious or irreversible damage, lack of full scientific certainty shall not be used as a reason for postponing cost-effective measures to prevent environmental degradation.”

The first peer-reviewed study regarding the toxicity effects of EMB on a species located in BC was published in 2010, 11 years after SLICE[®] use in Canada began. Since then only a few studies have followed. Further research on EMB toxicity has been established in European countries. Results overall indicate that over short-term exposures mortality

of sensitive species at concentrations representative of *in situ* conditions is negligible; however, the lethal, sub-lethal and behavioural effects of long-term exposure in marine species remain inconclusive and largely unknown. This research attempts to provide baseline data on the biological effects of EMB exposure on spot prawn both in the field and the laboratory, and while a small contribution, will hopefully provide a springboard for future research. Many profitable fisheries and ecologically important areas, such as Rockfish Conservation Areas, overlap with salmon aquaculture operations in British Columbia and so further research in understanding the toxicity of SLICE[®] application to Pacific Northwest non-target species is critical in maintaining a sustainable aquaculture industry, sustainable fisheries, and healthy coastal marine ecosystems.

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