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Environmental Fate and Potential Biological Effects of the Anti-Parasitic Chemo-therapeutant Emamectin Benzoate (active ingredient of SLICE®) in the Canadian Marine Environment

Michael G. Ikonomou

Fisheries and Oceans Canada
Institute of Ocean Sciences
9860 West Saanich Road
Sidney, BC V8L 4B2

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Canada

Foreword

This series documents the scientific basis for the evaluation of aquatic resources and ecosystems in Canada. As such, it addresses the issues of the day in the time frames required and the documents it contains are not intended as definitive statements on the subjects addressed but rather as progress reports on ongoing investigations.

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ABSTRACT

SLICE® is one of the chemotherapeutants used to control sea lice infestations at marine finfish aquaculture facilities in Canada. Concerns regarding the uptake and potential effects of the active ingredient of SLICE®, Emamectin Benzoate (EB), in non-target organisms have been raised by several stakeholder groups. EB was measured at parts per trillion (ppt) levels in water samples collected in the immediate vicinity of two salmon aquaculture sites in British Columbia following SLICE® treatment. EB was also detected at parts per billion (ppb) levels in sediment samples and in the muscle tissue of spot prawns collected within a 150 m radius of the net-pen. Sediment concentrations were related to the specific oceanographic conditions at each site. EB was found to be present in sediments up to 1.5 years following SLICE® treatment. Potential sub-lethal effects of EB on spot prawn were examined in a series of laboratory exposure experiments. Results suggest that short-term exposure to EB can impact this non-target crustacean.

1. BACKGROUND

SLICE® is the tradename of a therapeutant manufactured by Schering-Plough Animal Health Corporation which is marketed as an effective treatment against juvenile motile pre-adult and adult stages of sea lice species on farmed salmon. Sea lice are ectoparasitic copepods that attach to salmonids, and other marine species, and feed off the skin, mucous and blood of the fish, leaving open lesions that become susceptible to bacterial or viral infections and potentially leading to mortality of the host. Two species of louse are the target of this treatment; *Lepeophtheirus salmonis* (primary target) and *Caligus elongatus* (in BC the second species of interest is *C. cleminsi*). The active ingredient in the treatment is emamectin benzoate (EB), which is delivered to salmon through medicated feed. EB acts as a disruptor of neurotransmitter activity (chloride channel activator) in the target organism. Investigation into the potential deleterious effects of this insecticide on other non target marine crustaceans is warranted.

In Canada, SLICE® is one of the chemotherapeutants used to control sea lice infestations at marine finfish aquaculture operations. In British Columbia (BC), SLICE® is the only chemotherapeutant used for sea lice control. It is a pre-mix coating applied to fish food pellets and is administered as a drug under veterinary supervision. Since 1999, SLICE® has been prescribed by veterinarians in BC on a case by case basis through Heath Canada's Emergency Drug Release (EDR) program. In 2009, SLICE® received full approval from the Veterinary Drug Directorate of Health Canada for usage in Canada.

The recommended dose rate for SLICE® for sea lice control is 50 µg/kg of body weight per day for seven consecutive days (thus for each kilogram of fish treated, 350 µg of EB will be required over seven day treatment period). It can be used up to three times per year (maximum five treatments in two year grow out cycle). Based on these parameters the total EB used for a single treatment of a typical farm containing 280,000 fish, 2.50 kg each is approximately 250 g. This could translate to some 20 kg of annual use if the 80 farms currently active in BC (see below) were to treat once a year. Historical data indicate that the annual consumption of SLICE® on salmon farms in British Columbia has been reported by the Agricultural Branch of the BC Ministry of Agriculture and Lands (BCMAL) as 2.4 kg in 2000, 4.2 kg in 2001 and 8.9 kg in 2002. According to the BC Ministry of Environment, the total reported use of SLICE®™ in 2003 for BC was 4.95 kg. BCMAL noted that 7.35 kg of EB was prescribed in 2003 according to the data the Ministry collected from feed mills. These figures suggest that a substantial quantity of EB and other ingredients of SLICE®™ enter the ecosystem surrounding aquaculture operations in British Columbia and the waters off Canada's west coast.

Reported benefits of the use of SLICE® are the effective maintenance of a healthy culture population and reduced risk of aquaculture operations contributing to, and negatively affecting, wild populations of migratory salmon in the vicinity of farms. However, the use of SLICE®, and other chemotherapeutants, has also been identified as an environmental concern by local communities and stakeholders (e.g., First Nations, fishers) with respect to the potential effects on the surrounding benthic environment and adjacent valued ecosystem components (e.g., shellfish beds, prawn populations, salmon food chain).

Over the past decade the salmon aquaculture operations in BC have increased substantially. Between 1984 and 1989, farm sites in BC increased from 10 to 135 and at any given time, there are 80 active sites, stocking primarily Atlantic Salmon (*Salmo salar*)[1]. In 2009 in BC there were 18 companies operating some 131 sites covering some 4,575 hectares of total site area. The

[1] Province of British Columbia. *B.C. agriculture and seafood statistics publications*.

amount of SLICE® used by the industry over time is expected to increase as the number of sites used for salmon aquaculture has been increasing steadily.

The environmental behavior of EB in the ecosystem surrounding salmon aquaculture sites in BC is largely unexplored. Studies conducted elsewhere have shown that the insoluble nature of EB and its affinity for particulate organic matter causes most of this material to settle to the seafloor where it becomes a localized risk to the epifaunal and infaunal benthos. Preliminary data we have obtained from our recent studies conducted in BC show that:

1. EB is present in the water column during and following SLICE® treatment;
2. EB is present in the surface sediments during and following treatment up to 150 m from the farm site, and concentrations vary depending on site dynamics;
3. EB is detected in the surface sediments even one year post treatment;
4. EB profiles in sediment cores collected near impacted areas suggest that EB is persistent and there is no evidence of degradation;
5. EB is detected in the tissue of biota sampled near treated sites; and
6. in laboratory experiments, sub lethal effects were observed on spot prawns exposed to EB at environmentally relevant concentrations.

The purpose of this report is not to provide a comprehensive review of the environmental fate and effects of EB and it metabolites but to present the most recent data from our ongoing study on this subject, and to reflect on the six key factors identified in the previous paragraph. The physical and chemical properties and the registration, history of use and application regimes of this compound (in Canada and elsewhere) have been reported (Bright and Dionne 2005). Additionally, in the open literature, one can find several different methods for the detection and quantitative determination of EB and its desmethyl metabolite (AB) in environmental samples (Ikonomou and Surrdige 2013; see below). The structures of EB and AB are shown in Figure 1. In conjunction with this project we developed a highly sensitive analytical method based on liquid chromatography and electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) for the determination of EB and AB in sea water, marine sediments and tissue samples. The method developed was superior to all methods reported in the open literature in terms of sensitivity and versatility. Ikonomou and Surridge (2013) describes the method and compare its performance to existing methods. References dealing with the physical and chemical properties of EB, the methods developed and used for the determination of EB in environmental samples, and the toxicological studies associated with this pesticide are included in this article and in Veldhoen et al. (2012).

The release of toxic chemicals such as EB into water bodies and their impact on the marine environment is of concern to Fisheries and Oceans Canada (DFO). Understanding the pathways to which aquatic organisms are exposed to EB and other contaminants represents an important aspect of DFO research and provides evidence that may be considered by risk assessors and chemical regulators. Science knowledge is needed to support informed ecosystem-based environmental regulation and decision making of the aquaculture sector.

The results presented in this report are from an on-going study that started in 2009 and was conducted in collaboration with several partners including: Fisheries and Oceans Canada, the Pacific Salmon Forum, the BC Ministry of Environment, Environment Canada, the University of Victoria, the Pacific Prawn Fishermen's Association, Marine Harvest Canada, and Mainstream Canada. The study examines the following:

1. The fate and concentration of emamectin benzoate (EB) and its desmethyl metabolite in the surrounding environment of selected finfish farm sites following the application of SLICE®.

The two sites selected for the study are shown in Figure 2. Measurements of concentrations in localized sediments and water column have been carried out to provide an assessment of the potential effect on ecosystem functioning.

2. The uptake and potential toxicological impacts of EB and its desmethyl metabolite in the Pacific Spot Prawn, *Pandalus platyceros*, under both laboratory and field conditions. This dual approach will provide a standardized framework for toxicity assessment and evaluation of 'real world' environmental conditions against which the toxicity measures can be compared. The outcomes of the laboratory study provide a framework and the context in which field derived measurements can be assessed. The potential toxicological impacts are being assessed using novel techniques based on genomics.

The overall findings of these studies will provide the most detailed and comprehensive assessment to date of the potential effects of the application of SLICE® at marine cage finfish farm locations on the surrounding benthic environment and resident prawn populations. The measured environmental EB concentrations will be used to test, calibrate and implement the DEPOMOD model to predict the behavior of EB in relevant aquatic ecosystems. These findings will be useful in developing policy towards the regulation of SLICE® utilization.

2. ANALYSIS AND RESPONSE

2.1. TO WHAT EXTENT HAS EB BEEN DOCUMENTED IN THE ABIOTIC AQUATIC ENVIRONMENT IN CANADA?

2.1.1. Water

There are no data on EB concentrations or its metabolites in the water column during and/or following SLICE® treatments near marine finfish aquaculture operations in Canada. Some simulated calculations have been performed in the lower Bay of Fundy but those have looked at chemotherapeutants applied to the water column (azamethiphos, cypermethrin). Their findings, however, can not be extrapolated to EB, which is administered in the feed and has very different physicochemical properties in comparison to other pesticides and/or dye tracer used in these simulation studies.

As part of our study, unfiltered water samples were collected near the two salmon farms treated with SLICE®. These were collected from a vessel in trace clean 4-L amber jugs using a dip technique in which bottles were strapped to buckets on poles and submerged approximately 1-2 feet below the surface. Water sample collection was undertaken every three days during SLICE® treatment and then less frequently up to 4 months following treatment. Site A sample collection was directed along two transects extending east and west from the net-pens, at distances of 0, 30, 60, 100 and 150 metres (and at a Reference station) as confirmed with Global Positioning System (GPS) integrated marine software. Triplicate samples were collected at the Reference station first, then from lowest to highest expected concentration along each transect. At Site B water was only collected at 0 meters at the Southwest corner of the farm. All 4-L jugs were stored in sealed totes for transport. When received at the Institute of Ocean Sciences (IOS) they were stored at -20ºC until analysis.

A distinct spatial distribution of EB concentrations was observed in the water samples with EB decreasing with increasing distance from the net-pen; see data presented in Figure 3. EB concentrations on the West and East side of the site were very similar, which is expected based on the water flow patterns of this site. EB concentrations in the water column were highest during SLICE® treatment and remained high several days post treatment; see data presented in Figure 4. The data shown are for samples collected in the West transect, but the samples

collected in the East transect had similar profiles. Trace amounts of EB were detected in the water samples up to about a month post treatment, at which point no measurable concentrations were observed as we reached the method's limit of detection (LOD) of 7 pg/L (defined as the concentration corresponding to the average response plus 3 standard deviations for a blank sample, based upon a nine-point weighted calibration curve; see Ikonomou and Surrdige 2013). As shown in Figure 4, the highest EB concentrations measured in the water samples were at the parts per trillion (ppt) or ng/L level, around 500 pg/L. As expected, the highest EB concentrations were detected in close proximity to the net-pen, i.e., within a 50 m radius, and dropped substantially at greater distances; see Figure 4. In conjunction with EB, the desmethyl metabolite (AB) was measured in all samples examined. However, all AB concentrations were below the method LOD; see Figure 3.

It is clear from the data presented in this section that ppt concentrations of EB are present in the water column during SLICE® treatment and several days thereafter. The concentrations are highest within a 50 metre radius of the farm, then drop off substantially with distance. No measurable concentrations were found one month post treatment.

2.1.2. Sediment

Prior to our study there were no reliable data from Canada demonstrating the environmental fate and transport of EB in the marine environment. Findings from studies conducted in Europe indicate that, in the period during and after treatment with SLICE®, EB is released into the marine environment (either associated with excess feed not consumed by treated fish, or in the fecal material excreted by the fish) and, due to the insoluble nature of the compound and its affinity for particulate organic matter, settles on the seafloor beneath and around the treated farm site.

Some 10 years ago Parker and Mallory (2003) collected sediments in the vicinity of a salmon farm in the Bay of Fundy, New Brunswick in 2002. However, they were not able to measure EB concentrations in the sample collected as the analytical method used had a high detection limit of 0.4 µg/g (i.e., parts per million). Data for EB in sediment are also available from other studies conducted in east coast of Canada but those are not very informative as the concentrations reported are close to the method detection limit and, in almost all cases, the analytical methods used are not reliable.

Sediment samples from our study were analysed for EB and AB residues using ultra-trace analytical methodology based on liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS) instrumentation. The limits of quantitation (LOQ) achievable using this methodology (defined as the concentration corresponding to the average response plus 10 standard deviations for a blank sample; see Ikonomou and Surridge 2013) are in the sub parts-per-billion (<ppb) range. As with the water samples, sediment samples were collected from a vessel using a winch operated Van Veen sediment grab sampler. Collection occurred along two transects extending east and west from the net-pens, at distances of 0, 30, 60, 100 and 150 metres (and at a Reference station) as confirmed with GPS integrated marine software. From each benthic grab, replicate samples were prepared by removing the top 1 cm of sediment and storing in 100-ml trace clean amber jars. Samples were collected at the Reference station first, then from lowest to highest expected concentration along each transect. At Site B, sediment samples were collected using a hand operated 10lb Van Veen sampler at 0, 100 and 300 metres southwest from the net-pens. All sediment samples were transported in coolers and frozen at -20ºC until analysis.

The EB concentrations measured in surface sediment samples collected from the Reference site and Farm Site A are presented in Figure 5. The EB levels measured at the Reference site

were close to the limit of quantitation and were consistently low throughout the entire sampling period. The EB concentrations were the highest underneath the net-pen, i.e., W0 and E0, with the highest concentration reached at 35 ppb (or ng/g) some three weeks after SLICE® treatment commenced.

Detectable EB concentrations were measured on January 15, a few days after treatment commenced, with the highest level measured at E0 at 5 ppb. The sediment EB levels gradually increased from the commencement of treatment, reaching the highest concentrations some three to four weeks after SLICE® treatment and then gradually decreasing over time. It is evident from the data that the same (bell-shaped) EB concentration profile is observed throughout the sampling period as a function of distance from the net-pen. The highest concentrations are measured in the immediate vicinity of the net-pen (0 to 60 metres in both directions) and the levels measured at 100 and 150 metres from the net-pen are very low, approaching LOQ levels. The EB concentrations were measurable four months after treatment and at distances of 150 metres from the treated farm. The spatial profiles observed suggest that EB could be present at distances greater than 150 metres but at lower concentrations (ppt or less). These findings suggest that EB emerging from the salmon farms after SLICE® treatment is primarily sequestered in the sediments in close proximity to the fish farm, i.e., within a 60 to 100 metre radius of the farm site. The EB profiles observed in the sediments of Site A reflect closely the solids accumulation in the vicinity of that Site, as predicted by the DEPOMOD model; see results presented in Figure 7.

It is important to note that four months after SLICE® treatment there were detectable amounts of EB in the vicinity of the farm site. This is a unique finding as it does not parallel results obtained from similar studies conducted in Europe and elsewhere. Although it is likely that new “EB free” material (excrement, unconsumed fish feed, etc.) would have been deposited in the locations sampled after SLICE® treatment was terminated, the EB concentrations measured were unexpectedly high. One possible reason for this could be the sediment sampling technique employed. As noted previously, samples from each benthic grab were prepared by removing the top 1cm of sediment and storing in 100 ml trace clean amber jars. It is possible that within that 1cm of sediment sample collected, both “old” (EB contaminated sediment) and “new” (EB free sediment) material was collected. We are in the process of using mathematical model calculations and data from sediment core samples collected at that site to better understand the origin of EB concentrations detected in surface sediments four months after SLICE® treatment was terminated at this particular farm site.

Depending on the oceanographic conditions at a specific farm site the EB concentrations measured in the corresponding sediments could be very different. EB concentrations measured in the sediments collected along the east and west transects of Site A were substantially higher than those measured at a different location in the Broughton Archipelago; see data presented in Figure 6. Both sites (A and B, see Figure 2) were treated with similar amounts of SLICE®, yet the concentrations measured in the corresponding sediments before, during and after SLICE® treatment were very different. At the high flush site (Site B) the EB concentrations measured even underneath the net-pen (at 0 m) were very low and close to the method detection limits. This was the case during all time intervals sampled at this site. The data suggest that in highly flushed sites, like Site B, the EB containing particles are “washed” away by the currents and minimal quantities of EB are found in the sediments underneath the net-pens, whereas the opposite appears to be true for poorly flushed sites such as Site A.

2.2. HOW MUCH EB ENDS-UP IN THE SEDIMENTS FOLLOWING A FULL CYCLE OF SLICE® TREATMENT AT A SPECIFIC LOCATION?

A typical SLICE® prescription for sea lice control on a specific biomass of farmed Atlantic Salmon in British Columbia is as follows:

- Farm inventory: 280,000 Atlantic Salmon
- Average Weight: 2.50 kg
- Biomass: 702,800 kg
- Dosage: 50 ug EB per kg fish biomass per day for 7 days
- Total EB used: 124.9 kg of 0.2% SLICE® premix (~250 g of EB)

If we assume that most of the EB is distributed in the top 1 cm of sediment and within a radius of 100 m from the net-pen one can calculate the total volume of sediment contaminated with EB. Knowing that the top 1 cm of sediment has an average of 50% humidity the sediment volume is then converted into dry weight of sediment. From the sediment EB concentrations obtained from this study we can assume an average EB concentration of 7 ppb (ng/g) within the 100 m radius. Roughly, this translates to a total budget of 2.8 g of EB in the top 1 cm of sediment. This calculation was based on the data from a poorly flushed site (i.e. Site A). Based on the information available from this study, it seems that up to 1% of the EB used in a single SLICE® treatment at a typical farm site will end up in the sediments in the immediate vicinity (within 100 m radius) of the farm site.

2.3. TO WHAT EXTENT HAS EB BEEN FOUND IN AQUATIC BIOTA IN CANADA?

The uptake and potential health effects of EB (the active component of SLICE®) and its metabolite AB in non-target organisms, benthic species in particular, has been of concern to several stakeholder groups. Such concerns generally stem from reports indicating that, in the period during and after treatment with SLICE®, EB is released into the marine environment (either associated with excess feed not consumed by treated fish or in the fecal material excreted by the fish) and, given the insoluble nature of the compound and its affinity for particulate organic matter, settles on the seafloor beneath and around the treated farm site. Since the target organism (sea lice) is of the same sub-phyla as other commercially valuable species, and the specific mode of action of the chemical involves interaction with a phyla-common nerve transmission process, there is potential for the effect of a SLICE® treatment to extend beyond the farm site. Previous studies on this issue (e.g., Scottish Environmental Protection Agency Annual Monitoring) have found detectable concentrations of EB (and other chemotherapeutants) in sediments sampled near salmon aquaculture sites, and EB has been found in crustaceans immediately following treatment and in scavenging organisms several months after SLICE® treatment; however, the potential uptake and effect on resident benthic organisms is unclear).

In this study we examined the uptake and potential toxicological impacts of EB and its desmethyl metabolite by the Pacific Spot Prawn (*Pandalus platyceros*), under both laboratory and field conditions. Spot Prawns were selected for this study because they are a marine invertebrate species and are of significant commercial importance in western Canada.

The laboratory exposure experiments were conducted in conditions replicating the natural environment in the vicinity of salmon farms following a SLICE® treatment. The aim was for these experiments to provide the data necessary to assess potential biological effects of EB on prawns in the field, thus formulating the basis of a field study and risk assessment. Details on

the structure and implementation of the laboratory study are provided in Appendix A. In the laboratory study we comprehensively examined the uptake and biological effects of EB and its desmethyl metabolite (AB) by the Pacific Spot Prawn. Clean marine sediments were spiked with EB at a range of concentrations (ppb to low ppm range) and prawns were exposed over a wide range of time periods (up to 8 days) and concentrations. Representative water, sediment and prawn samples were collected at the beginning, and at predetermined time intervals throughout the experiment. These were analyzed for both EB and AB to assess the dissolved concentration and thus the bio-availability of EB. EB is known to be particle bound but depending on the environmental conditions some fraction may be dissolved in the water column. Following the exposures the prawns were examined for potential toxicological impacts using novel techniques based on genomics. Organisms were examined for mRNA and analyzed for selected gene transcripts to determine if molting, reproduction, stress and other sub-lethal effects indicators were altered in the presence of EB. The findings of the genomics work were summarized in a manuscript that has been published in the open literature (Veldhoen et al. 2012).

To determine the EB tissue load in exposed animals, spot prawns exposed to increasing nominal concentrations of EB were examined for the presence of the insecticide and its metabolite in muscle tissue following 8 days of treatment. Even at these relatively short exposure times significant levels (up to 100 ppb) of EB were measured in the tissue of the exposed animals. Multiple individual prawn samples were analyzed for each of the exposures, and the mean values and standard deviation data obtained from the measurements are presented in Figure 8. For all the laboratory experiments, sediment and overlying water samples were collected and analyzed for each of the tanks used in the experiments. In all cases measurable amounts of EB were observed in the water column. As an example, an 8 day exposure to 100 ppb sediment-associated EB results in approximately 0.5 ppb EB in the overlying sea water and 0.45±0.15 ppb (µg/kg dry weight) EB in spot prawn muscle tissue. The aim of these measurements was to establish an overall understanding on the partitioning of EB (sediments and water) during the exposure experiments and to postulate potential uptake pathways of EB by the spot prawn. The EB metabolite AB was also measured in all these samples and its relevance to measured EB concentrations is discussed later in this report.

In the field-based study we examined the uptake and biological effects of EB and its metabolites by local prawn populations in the vicinity of two salmon farms following the application of SLICE®. Both “free-range” and contained prawns were examined.

2.3.1. Wild prawn samples

Prawns were collected using standard commercial methods summarized by deploying traps along the ocean floor baited with salmon diet pellets for short periods of time (3 hr to 48 hr). Sampling locations at the farm site were determined precisely with GPS integrated marine navigation software. Using a commercial prawning vessel, traps were deployed/recovered from the bottom using a rope and winch system. With a target of 6 prawns per site, live captured prawns were quickly sampled for genomics in the following manner: the first abdominal segment was removed and a small dorsal section of tissue (1 cm x 5 mm) was excised using a scalpel. Excised samples were immediately stored in RNA-Later solution and kept on ice for 24 hours prior to freezing. The remaining prawn tissue was stored in Ziploc bags and frozen for EB analysis. Site A prawn collection occurred at specified distances (0, 100 and 300 metres) along a westerly transect from the farm in order to parallel the east-west current directions at this site. Control prawns were collected at a site distant from the farm.

2.3.2. Contained prawn samples

In addition to the timed collection of wild prawns, a containment trap holding live prawns was deployed immediately at the pen perimeter (0 m). Standard prawn traps (2' x 2' x 8") were designed as containment traps by sealing the entry ports to prevent entry and escape of prawns. Containment at 0 metres would allow us to examine prawns exposed to an area of high deposition proximal to the farm and would provide control over the movement of the prawns (since the wild prawns are free to move in and out of the deposition areas). Additionally, it has been observed by commercial prawners that prawn concentrations decrease near salmon farm sites during SLICE® treatment, so containment traps would ensure the collection of samples. Approximately 100 prawns were collected at the control site and deployed in a containment trap at the farm, on the ocean floor. A containment trap with approximately 100 prawns was also deployed at the control site and would represent the control prawns for both wild and contained samples. Prawns were sampled from each of the containment traps at the same time intervals and in the same manner that wild prawns were sampled. Some of the containment trap sampling was burdened with unforeseen problems at both Site A and B and we did not manage to obtain all samples planned.

Both "free-range" and contained prawns were collected from the field study at both of the sites (A and B) that were treated with SLICE®, and from reference/control sites. These were collected at different time intervals and at times reflecting pre-, during and post-SLICE® treatment. The aim of this sampling strategy was to collect water, sediment and prawn samples at predetermined locations and at the same time. A total of several dozen wild prawn muscle tissue samples were analyzed and the mean EB concentrations obtained for each of the locations and/or sampling times are presented in Figures 9 and 10. EB concentrations were measured in all samples examined but were generally in the low ppb range. EB levels measured in the prawns from the control sites were in the sub-ppb range and close to the method limit of detection (LOD). It is important to note that the EB concentrations measured in the wild prawns were substantially lower in comparison to those examined from the laboratory exposure studies; see data presented in Figure 8.

As with the sediment samples, the EB concentrations in the prawns collected at the low flush Site A (see data in Figure 9) were higher in comparison to those collected at the high flush Site B (see data in Figure 10). EB concentrations above the LOD were measured in some of the samples associated with the control site associated with Site B. After evaluation of the sediment and biota data collected from that site and taking into consideration the water flow dynamics of that general area it seems that this location, although some 500 m away from Site B, could be impacted by the "organics plume" originating from the net-pen located at Site B.

As with the lab study, a large number of representative tissue samples were examined for mRNA and analyzed for the selected gene transcripts to determine if molting, reproduction, stress and other sub-lethal effects indicators were altered in the presence of EB. Unfortunately, due the wide range of variables associated with the field study and some of the difficulties experienced in obtaining all the desired samples from the field, the genomics data are inconclusive. Our aim was to repeat the field study during the 2010/2011 SLICE® treatments by the farm operators but we were not able to achieve that goal. In order to add value to the genomics data already obtained, and to get a better perspective on the potential biological impacts of SLICE® on spot prawns, we will attempt to repeat the field study.

2.4. IS EB TOXIC TO AQUATIC BIOTA?

The data presented in the previous section clearly show that EB is bio-available and bio-accumulates in the muscle tissues of spot prawns collected in the vicinity of salmon farms

treated with SLICE®. This is the first finding of its kind in Canada and forms the foundation for a more substantive evaluation for fully assessing the bio-accumulation, bio-magnification, route of uptake, and potential biological effects of EB and its metabolites in spot prawn, a commercially valuable species of the west coast of Canada.

Data obtained from the laboratory exposure experiments showed that significant levels of EB were detected in the tail muscle tissue of all exposed animals. Animals selected for the experiment did not have eggs and were of similar weight. Significant mortality was observed within 8 days of EB treatment at concentrations between 0.1 and 0.8 mg/kg and there was no effect of EB on molting. Several novel prawn cDNA sequences were isolated from the tail muscle by directed cloning and subtractive hybridization of control versus EB exposed tissues. Quantitative PCR analyses revealed significant alterations in the levels of mRNAs encoding the 60S ribosomal protein L22, phosphoenolpyruvate carboxykinase, spliceosome RNA helicase WM6-like, a small heat shock protein, and the intracellular signal mediator histidine triad nucleotide binding protein 1. Differential expression of these mRNA transcripts suggests that protein synthesis, gluconeogenesis, RNA localization and splicing, transcription regulation, apoptosis, and stress pathways may have been impacted. The mRNA encoding the molting enzyme, ß-N-acetylglucosaminidase, was not affected by EB treatment. However, the expression of this transcript was extremely variable, making it unsuitable for effects assessment. The results suggest that short-term exposure to EB can impact this non-target crustacean. Full details on our study examining the biological effects of EB on spot prawn as assessed by genomics based approaches are discussed in Veldhoen et al. (2012).

In another laboratory exposure study we examined the acute toxicity of EB and AB on a non-target organism, the amphipod crustacean *Eohaustorius estuaries*. These findings have been described in a recent publication by Kuo et al. (2010). The 10-d LC50 values for EB and AB (i.e., the concentrations required to kill 50% of test animals within 10 days) were 0.185 and 0.019 mg/kg wet weight sediment (0.146 and 0.015 mg/kg dry wt), respectively, for *E. estuaries* while no obvious decay patterns were observed for either EB or AB during the 10-day period.

Toxicological studies examining the impact of EB on species other than the ones described above have been conducted by researchers from other parts of the world where SLICE® is used in finfish aquaculture. However, these may not be of direct relevance to resident species found in the coastal areas of Canada, and are not discussed in this report. Additionally, there are no reports in the peer-review literature where one can find reliable data on the environmental concentrations of EB (in water, tissue or biota) and toxicological data directly related to chemical measurements. Although the use of SLICE® has been identified as an environmental concern by local communities and stakeholders with respect to the potential effects on the surrounding benthic environment and adjacent valued ecosystem components (e.g., shellfish beds, prawn populations, salmon food chain), to date very little reliable data have been generated to address such concerns.

2.5. HOW STABLE AND/OR PERSISTENT IS EB IN THE ENVIRONMENT?

Previous studies on the bioaccumulation potential of EB in Bluegill Sunfish (Chukwudebe et al. 1996) reported AB as a major metabolite detected following a 28-day exposure study. In addition, a separate study (Kim-Kang et al. 2004) using Atlantic Salmon reported 0-17% conversion of labeled [3H]-EB to AB while monitoring over a 90-day period following the final dose. These independent studies both indicate AB as the major degradation product of EB. Therefore, environmental assessment of AB in addition to EB is essential when measuring environmental concentrations. EB is largely excreted un-metabolized in the feces within one day after treatment; however, tissue levels decrease slowly, which is believed to result in the prolonged effectiveness of EB against sea lice.

One of the goals of our study was to measure, in conjunction with EB, the metabolite AB in all samples collected. The principle behind this idea was to assess potential degradation of EB to its principal metabolite in the three environmental compartments examined (water, sediment and biota) as a function of time. As mentioned above, no measurable concentrations of AB was found in any of the water samples examined; however, measurable concentrations of AB were observed in tissue and sediment samples. When plotting EB versus AB concentrations using all the field sediment and tissue samples collected, significant correlations were obtained in both instances with R^2 values of 0.521 and 0.465, respectively. To evaluate potential conversion of EB into its metabolite AB over time we plotted the AB:EB concentration ratio as a function of time for both the sediment and the tissue samples (Figures 11-14). The sediment samples show an increase in AB concentrations relative to EB as a function of time; see data presented in Figure 11. The same trend is observed in the prawn tissue samples, specifically those collected in the containment traps underneath the net-pen of Site A; see data presented in Figure 12. The prawns from the containment traps had similar AB concentrations to the ones collected within 10 m of the net-pen at site A, yet the AB:EB ratio of the latter samples did not increase as sharply over time as for the contained prawns; see data presented in Figure 13. The AB concentrations in the prawns collected at the Reference site were some 10 times lower in comparison to those collected at Site A. For those prawns the AB:EB ratio did not change over time; see data presented in Figure 14. The highest AB concentration increase over time was observed in the prawns contained underneath the net-pen of Site A; see Figure 12. Based on the AB concentrations measured in the corresponding sediments it is clear that these animals were exposed to ppb levels of AB as well. Judging from the AB:EB profiles observed in Figures 11 and 12, and the remaining prawn tissue AB profiles, the data suggest that spot prawns do not seem to convert a significant amount of the parent compound EB into its metabolite AB over a 4 month exposure period.

The data collected from this study, the sediment data in particular, suggest that: a) EB is persistent in the marine environment; and b) no substantial amounts of EB are converted into the AB metabolite under the conditions of this study. Data presented in Figures 5 and 6 clearly show that EB is present in the sediments in the vicinity of the farm sites treated with SLICE® some 4 months post treatment. The concentrations decreased over time post treatment but these data do not show if the decrease observed was due to EB degradation into some metabolite or due to dilution, i.e., new organic material deposit on the top of the organic matter containing EB. The sampling techniques we used for the sediment collection of this study do not allow for such a differentiation to be made.

Very close to the time when we collected the last samples from Site A (i.e., five months post SLICE® treatment) the salmon from this site were harvested and the net-pens were not restocked. Approximately one year following the harvesting of the fish of Site A we revisited this location and collected sediment samples at exactly the same locations as in the previous sampling campaign. At the same time we also collected sediment samples at Site B and from a number of other sites in the general area where salmon aquaculture is prominent. The sites samples and the corresponding EB concentrations measured are presented in Figure 16. There were measurable EB concentrations in the sediments of Site A 1.5 year post SLICE® treatment. The concentrations measured in the latest sampling campaign had decreased in comparison to what measured the previous year but EB was still present in the sediments. EB was detected in the sediments of all the other sites sampled none of which an active site with salmon in the net-pens. Furthermore the AB:EB ratios measured in these samples (data not shown) were no different than the ratios presented in Figure 11. These data clearly show that EB is persistent in the sediments for long periods of time and it does not get metabolized to AB. It should be noted here that there are no data in the open literature which show the environmental fate of EB in

marine sediments. Data of this kind are essential as they formulate the basis for risk assessment evaluations.

2.6. ARE THERE RESEARCH NEEDS THAT WOULD HELP INFORM MANAGERS ABOUT EB FATE AND EFFECTS IN THE CANADIAN AQUATIC ENVIRONMENT?

- Research on the toxicological effects of EB on spot prawns, including apparent mortality at lower EB concentrations and lack of a dose-response relationship (which may reflect other causes of mortality such as trauma or cannibalism). This would include a repeat of the field and exposure studies conducted previously, with the aim of filling data gaps from the first study and making full use of new and existing genomics data, which may help to understand the mechanisms(s) behind (sub-)lethal effects, as well as chronic exposure and full life cycle exposure studies.
- Research on mechanisms of EB uptake in spot prawns.
- Research on health endpoints of concern to population health, including neurological development and behavior, immune function and resistance to disease, reproductive health, and growth, reproduction and survival of the pacific spot prawn.
- Research on the comparative vulnerability of species other than the spot prawn to EB, and the influence of sex, age and life history parameters.
- Research on the spatial distribution and budgets of EB and AB in areas where salmon aquaculture has taken place.
- Research on EB toxicity in the context of multiple stressors in the environment, including the presence of other contaminants, changes in food web structure, and changes in climate.
- Research on the degradation and metabolic pathways of EB in the marine environment, including the identification of any metabolites other than AB.
- Research on the persistence and stability of EB in the environment to establish residence time of EB and its degradation and metabolite products in aquatic food webs in impacted areas.
- Research on the spatial transport of EB away from finfish farms and into areas of critical habitat or spawning habitat for aquatic biota.
- Develop models to predict the EB distribution in the aquatic environment following full cycle SLICE® treatment at fin fish aquaculture sites.
- Use the data generated from the toxicological studies to calculate bioaccumulation and biomagnifications factors and develop sediment guidelines for EB and its metabolites and degradation products.

3. CONCLUSIONS

The goal of this project was to generate a component of the science knowledge relating to the environmental behavior of EB. Such knowledge is required to support informed ecosystem-based environmental regulation and decision making of the aquaculture sector at both the federal and provincial levels. The main conclusions that can be drawn from this study are as follows:

- Following SLICE® treatment at the two study sites, EB concentrations in surface sediment were found to range from 0.12 ppb (the limit of quantification, or LOQ, of the analytical

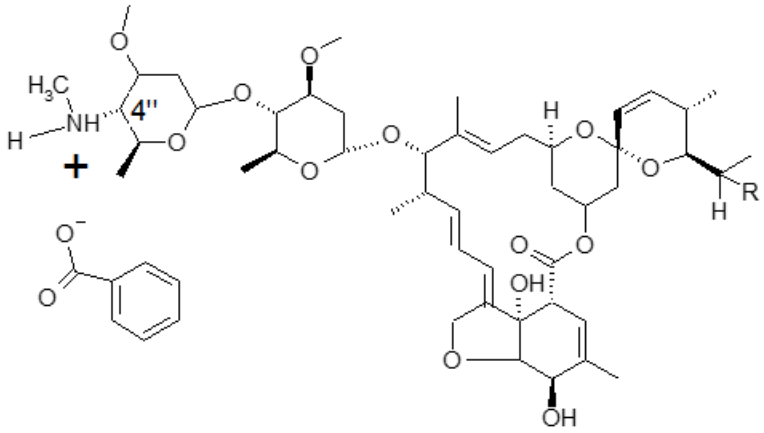
method) to 35 ppb within a radius of 150 m from the farm site. The EB sediment concentrations were substantially lower at Site B than at Site A, which may be attributed to different hydrodynamic and/or biophysical conditions at the two sites.

- EB was found in surface sediments where SLICE® has been used. EB residues on the order of 3 ppb remain in the sediment around study Site A for an extended period (>1.5 years). Site B and four neighboring sites were also sampled at the same time with levels of EB measured between 0.12 ppb (method LOQ) and 6.5 ppb. It is recommended that future work links the history of SLICE® usage with sediment profiles at these and other salmon farm sites in BC.
- Conversion of EB to its main conversion product AB was observed in sediment collected at Site A. The concentration of AB was less than 30% of the EB concentration measured in the same sample over a 115 day period following SLICE® treatment. This ratio did not change in samples collected up to 1.5 years later at Site A, or at the other sites examined.
- EB was detected in sub-surface water samples at levels between 0.006 ppt (method LOQ) and 0.635 ppt at both study sites during SLICE® treatment. EB appeared to dissipate quickly over time and was not detected in sub-surface water 4 to 5 weeks after treatment. AB was not detected in water samples.
- EB was measured at between 0.09 ppb (method LOQ) and 3.1 ppb in the muscle tissue of spot prawns collected in the vicinity of salmon farms treated with SLICE® over a period of 100 days post-treatment. AB was also detected, at approximately 30% of the EB concentration.
- The laboratory-based examination of spot prawn using static aquarium conditions indicates that short-term (8 day) exposure to sediment containing >100 ppb EB can alter the expression of specific genes (mRNA abundance patterns) in muscle tissue. It is not possible to draw direct linkages between these studies and field measurements of EB in water, sediment or biota. Additionally, research involving standard toxicological measurements (LOAEC, NOAEC, LC50), different (e.g., pre-molt) life-cycle stages, and environmentally relevant EB concentrations is needed to assess the impact of EB on prawns and other susceptible organisms, fisheries and ecosystems.
- Gene expression analysis was performed on wild and contained prawns collected near salmon farm sites. However, there was not enough information to interpret the results of these analyses. It is recommended that additional gene expression work be undertaken in the future.
- The available sampling techniques were adequate for an initial study to assess the fate of EB. Recommendations for future studies include greater use of the particle tracking model DEPOMOD and sampling strategies that provide additional information (including EB levels in suspended particles and bottom waters) for determining the environmental concentrations and fate of EB.
- Liquid chromatography-tandem mass spectrometry provides the sensitivity, specificity and accuracy required to measure EB, AB and other chemicals of concern in environmental and biological samples. This technology is available to DFO researchers through the Laboratory of Expertise in Aquatic Chemical Analysis (LEACA) at the Institute of Ocean Sciences in Sidney, B.C.

The results of the study show that (i) EB can remain and so potentially build up in benthic sediments close to salmon farms, depending on the frequency and extent of SLICE® usage and local site conditions; and that (ii) EB is bioavailable and can be measured in the muscle tissues

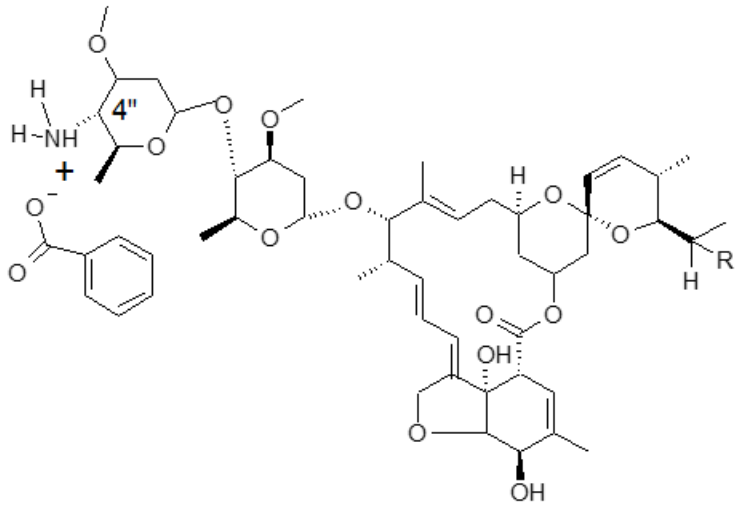
of spot prawns near salmon farms treated with SLICE®. EB released into the environment following the application of SLICE® dissipates quickly, and most of the EB reaching benthic sediments remains localized within a short distance (150 m) of the farm site. However, it was not possible to extrapolate measurements made at the two study sites to other aquaculture sites in BC, as there are insufficient data to determine a relationship between site conditions and the environmental fate of EB or its potential impact on fisheries and ecosystems. Further research is needed to assess the persistence of EB in aquatic ecosystems, as well as the bioaccumulation and potential biological effects of EB and its metabolites in spot prawns and other non-target organisms.

4. FIGURES



Emamectin Benzoate, 4''-Deoxy-4''-(methylamino)avermectin B1 Benzoate salt

EB1a, R=C_2H_5, major > 90% EB1b, R- CH_3, minor < 10%



Desmethyl Metabolite, 4''-deoxy-4''-epi-aminoavermectin B1 Benzoate salt

AB1a R=C_2H_5, major >90% AB1b R=CH_3

Figure 1. Structures of emamectin benzoate (EB) and its desmethyl metabolite (AB).

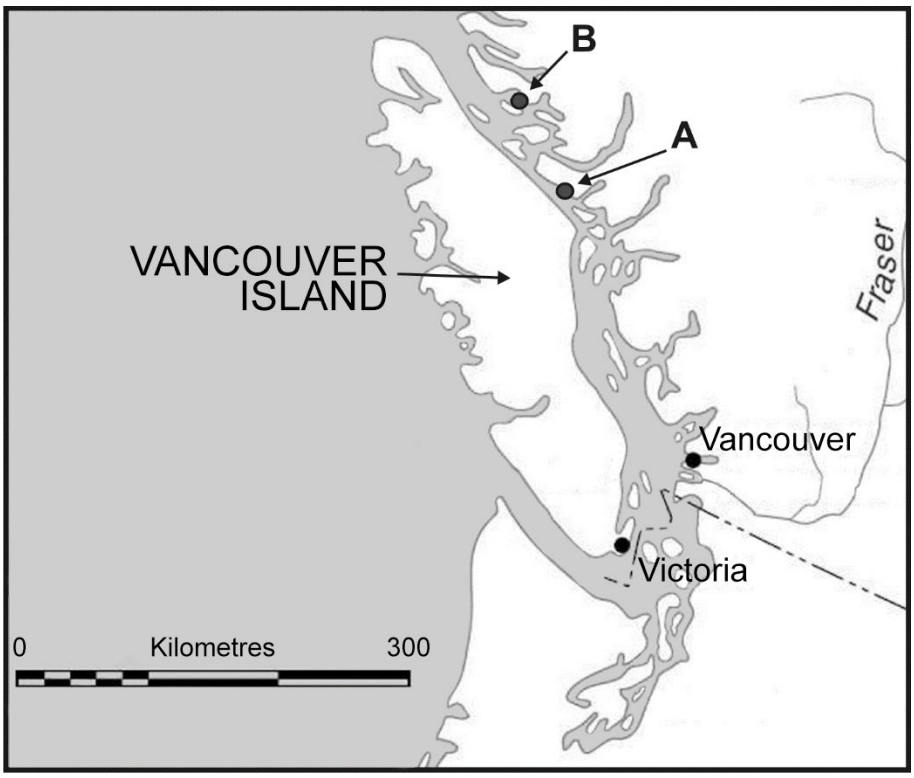


Figure 2. Location of Salmon farms (A and B) that were treated with SLICE® and where water, sediment and tissue samples were collected for this study.

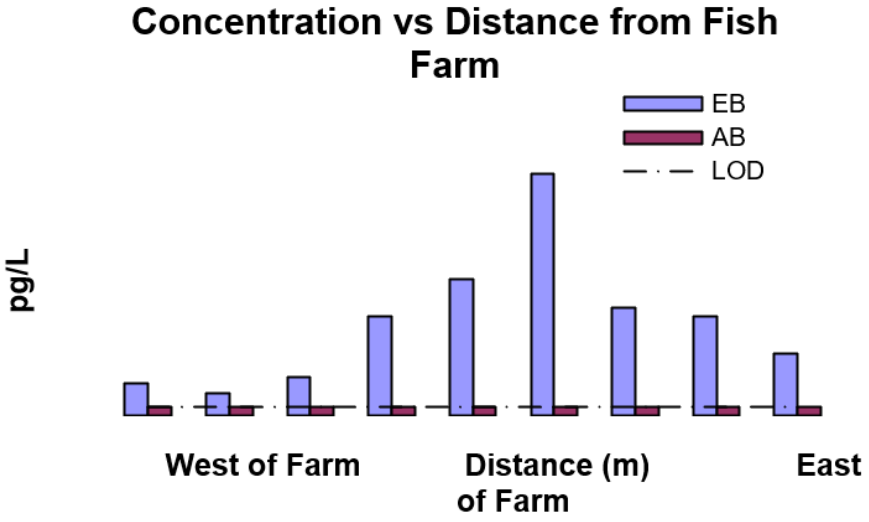


Figure 3. Concentrations of EB and AB in water samples collected at site A during day 3 of the 7 day SLICE® treatment.

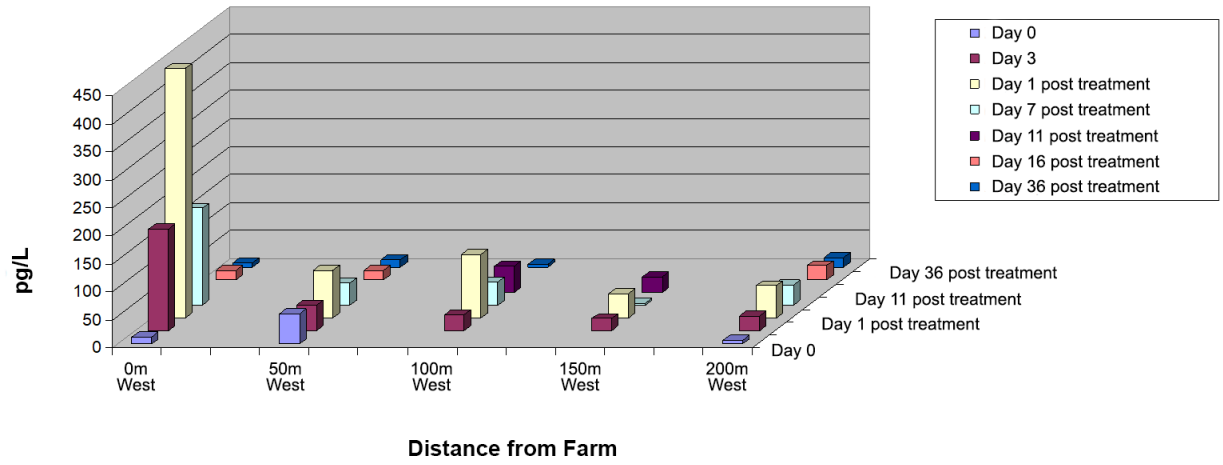


Figure 4. Concentrations of EB measured in the water samples collected in the west transect at different distances from the net-pen of site A during and post SLICE® treatment. Values presented here are mean concentrations obtained from the analysis of two to three samples collected in each of the locations.

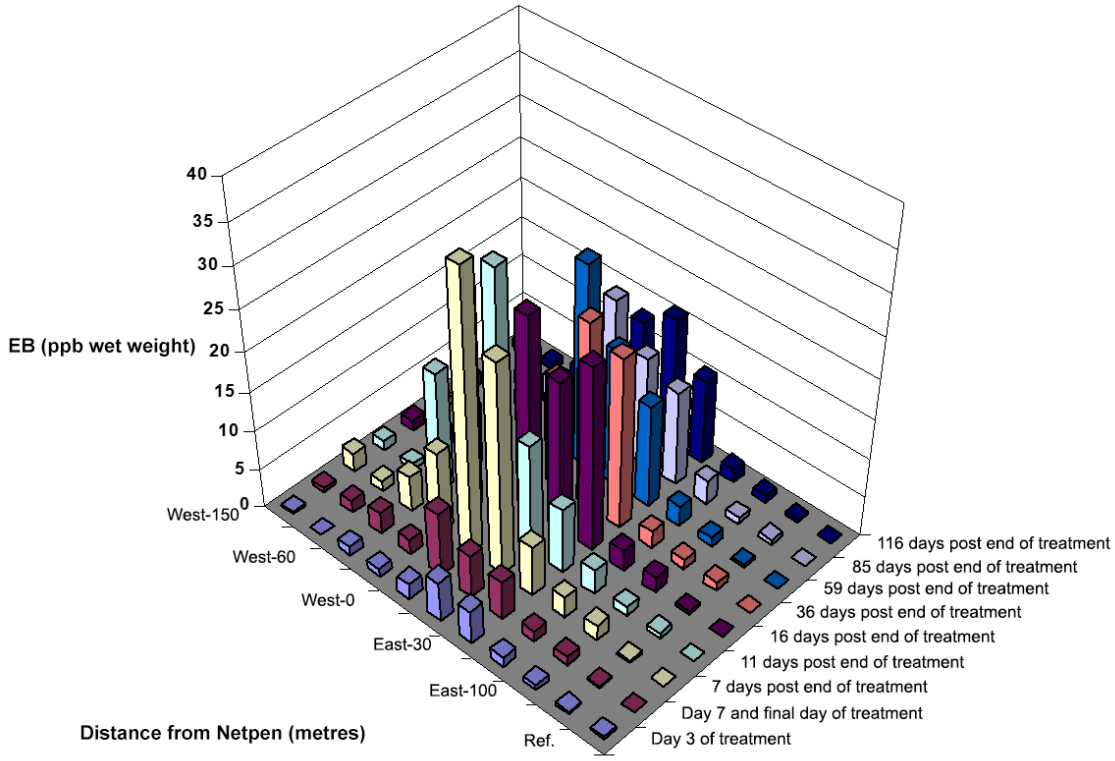


Figure 5. Emamectin Benzoate concentrations (ppb wet weight) as measured in surface sediment samples collected at farm Site A along east and west transects over four months of sampling. Samples were collected at specific distances east and west from the farm at 0 m, 30 m, 60 m, 100 m and 150 m as well a reference (Ref) site. Average moisture level for all samples was determined to be 69.7% ± 11.4%. Treatment commenced on 12 January 2009.

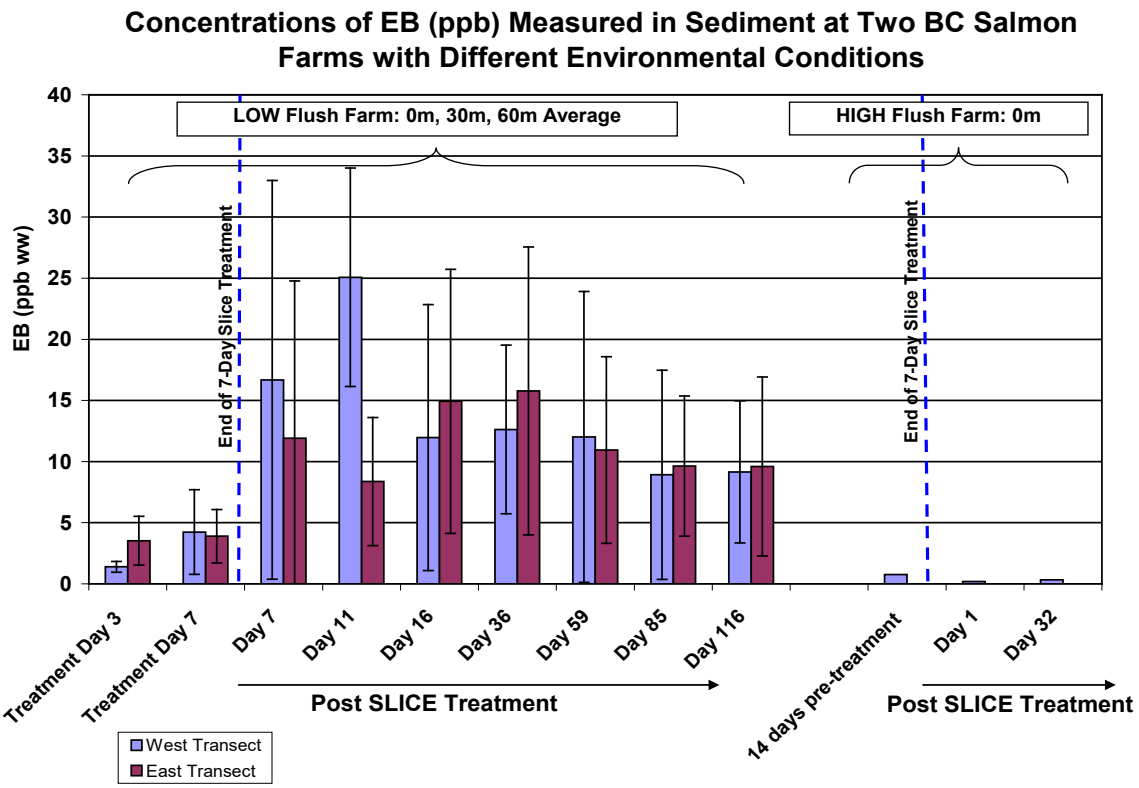


Figure 6. Emamectin Benzoate concentrations (ppb wet weight) as measured in surface sediment samples collected at farm Sites A (low flash farm) and B (high flush farm) before, during and post SLICE® treatment. For Site A the same data presented in Figure 5 are included here for comparison purposes. To better visualize the EB concentration differences between the two sites, the EB concentrations measured at 0, 30 and 60 m at each of the sampling times of Site A have been averaged and mean concentrations as well as the standard deviation resulted are shown in the Figure. The same treatment was applied to the data from both the East and West transects of Site A.

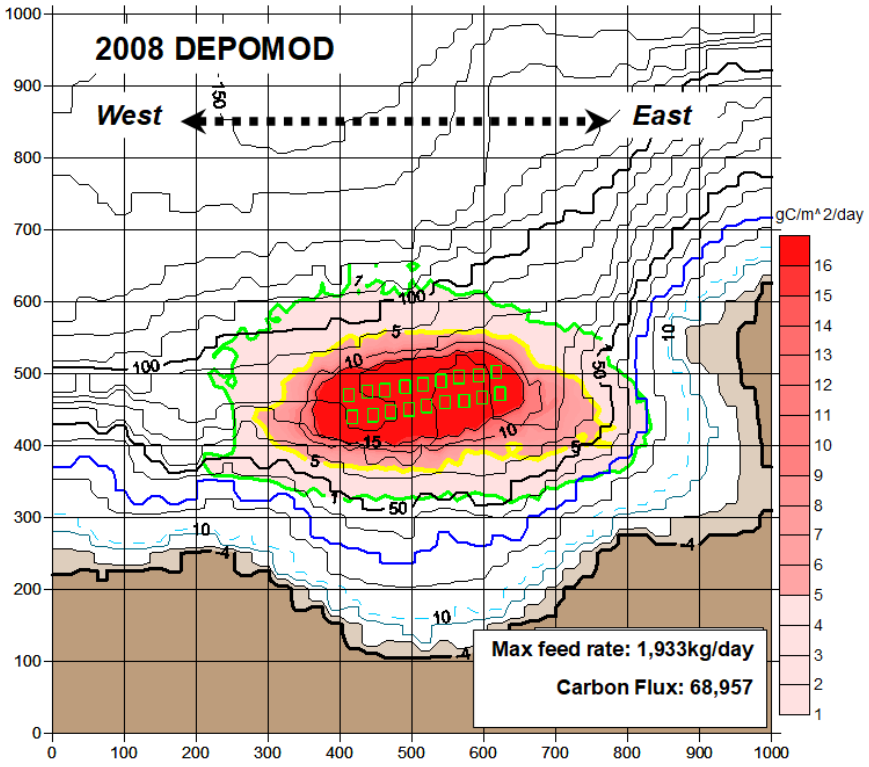


Figure 7. DEPOMOD modeling for solids accumulation at Site A salmon farm in 2008. The 2008 salmon pen orientation remained the same in 2009 when this study was conducted.

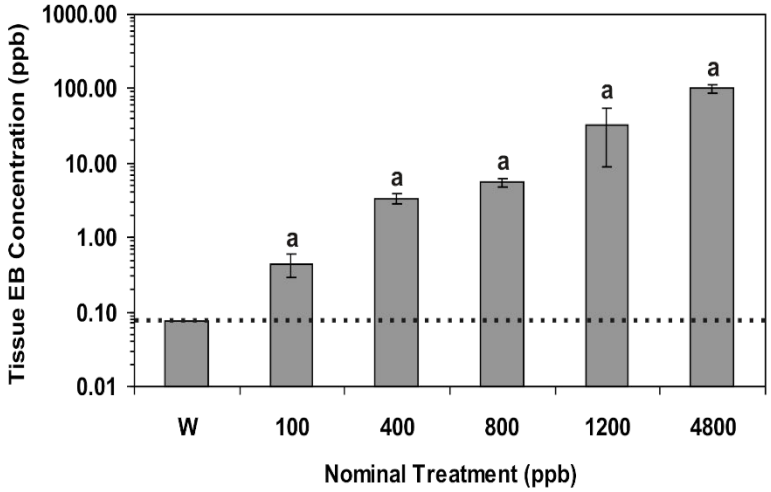


Figure 8. Spot prawn muscle tissue EB concentrations relative to the nominal application concentration at 8 days of exposure. Limit of detection = 0.075 ppb dry weight, is indicated by the dotted line. Significant differences in EB tissue concentration compared to the sea water control (W) were determined by the Mann-Whitney U test where (a) $p<0.05$.

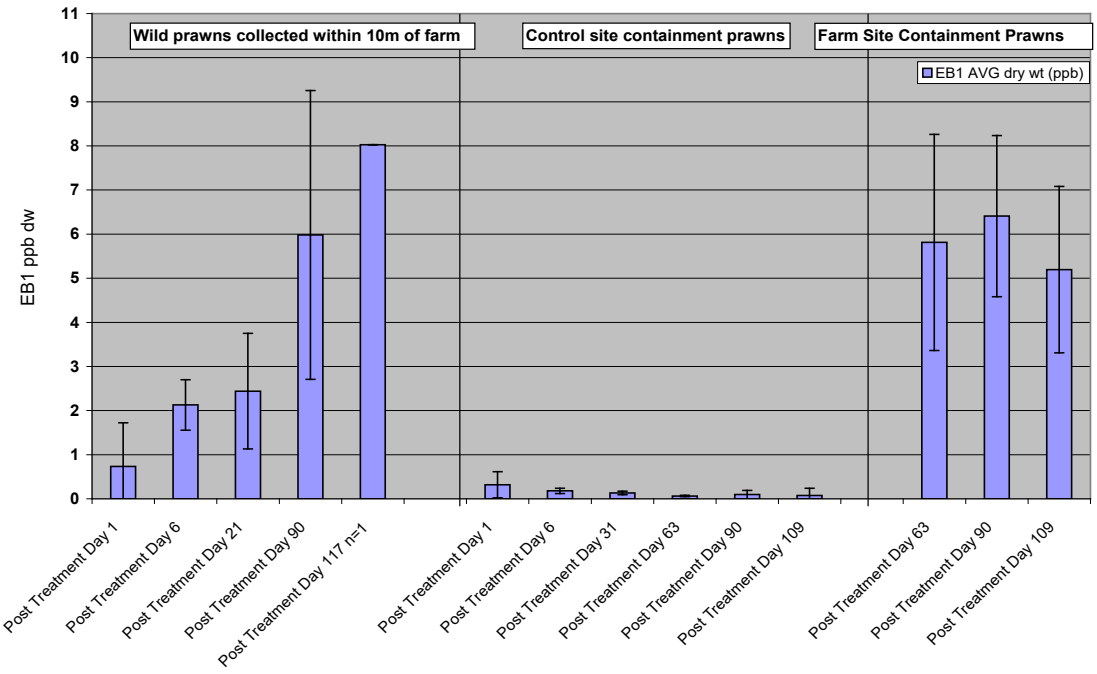


Figure 9. Mean EB concentrations measured in wild spot prawn muscle tissue (ppb, dry weight) collected in the vicinity of Site A, pre-, during and post-SLICE® treatment.

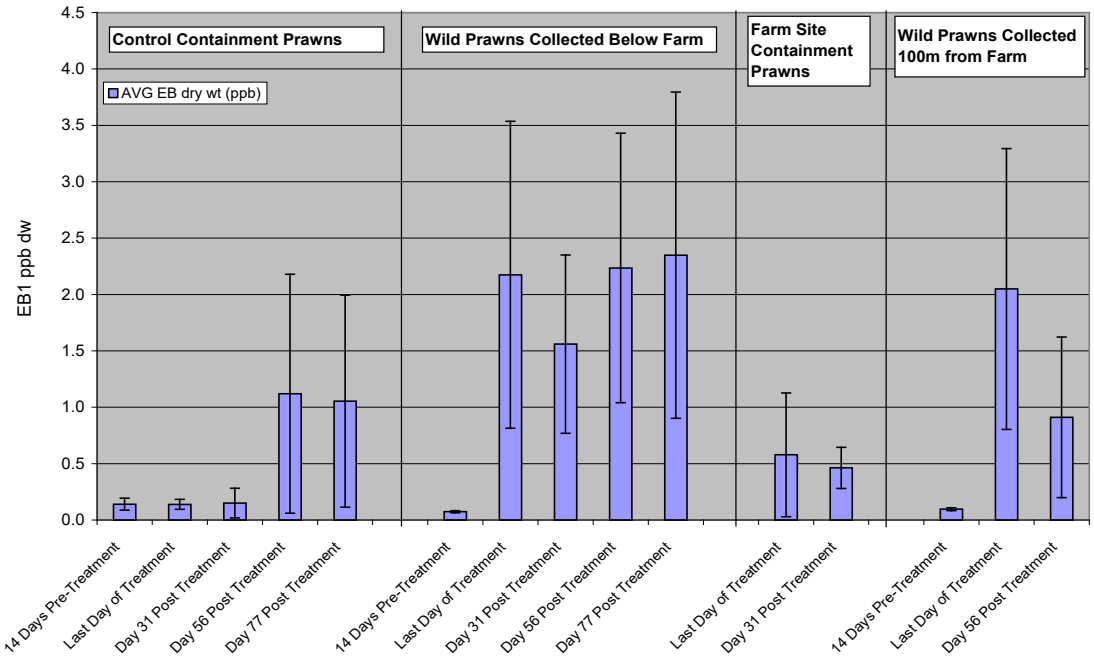


Figure 10. Mean EB concentrations measured in wild spot prawn muscle tissue (ppb, dry weight) collected in the vicinity of Site B, pre-, during and post-SLICE® treatment.

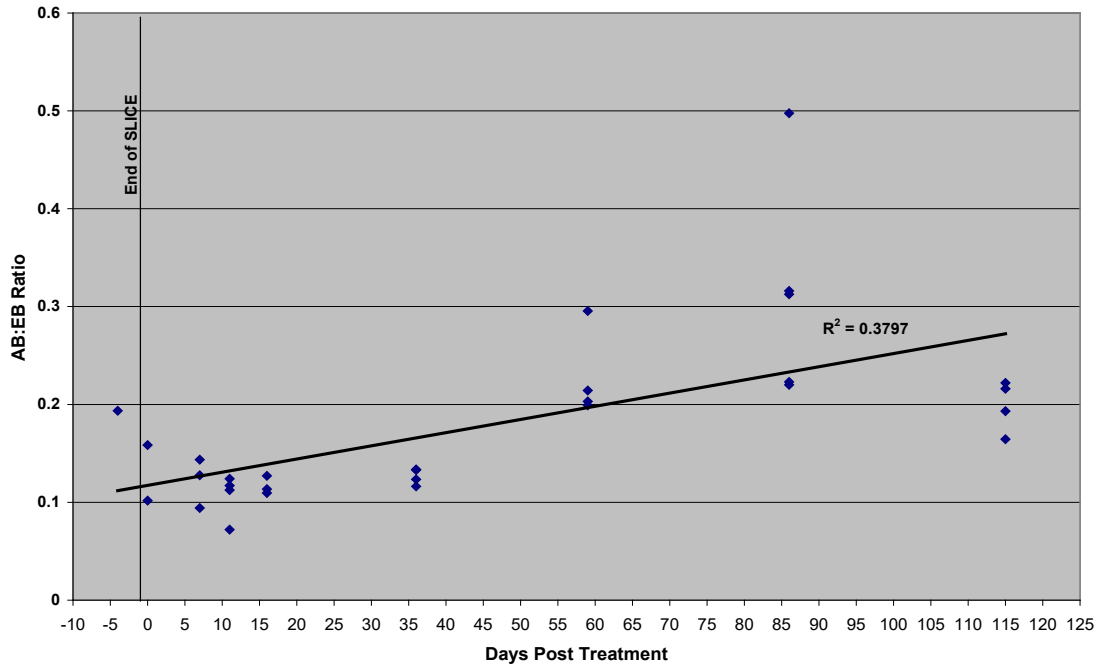


Figure 11. AB:EB ratio measured in Site-A sediment samples collected within 60 m of farm during and post SLICE® treatment. AB concentrations ranged between 3 and 25 ppb dry weight.

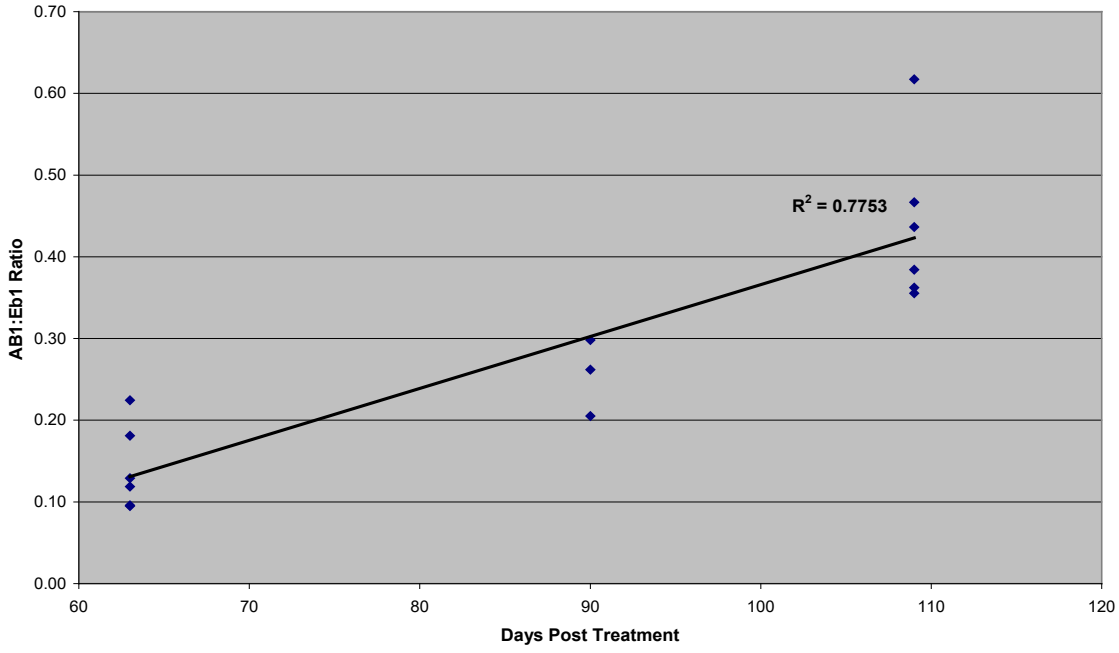


Figure 12. AB:EB ratio measured in wild prawns from control containment traps (ppb dry weight) - deployed in the close vicinity of Site A. Samples collected up to 110 days following SLICE® treatment. AB concentrations ranged between 0.3 and 3.3 ppb dry weight.

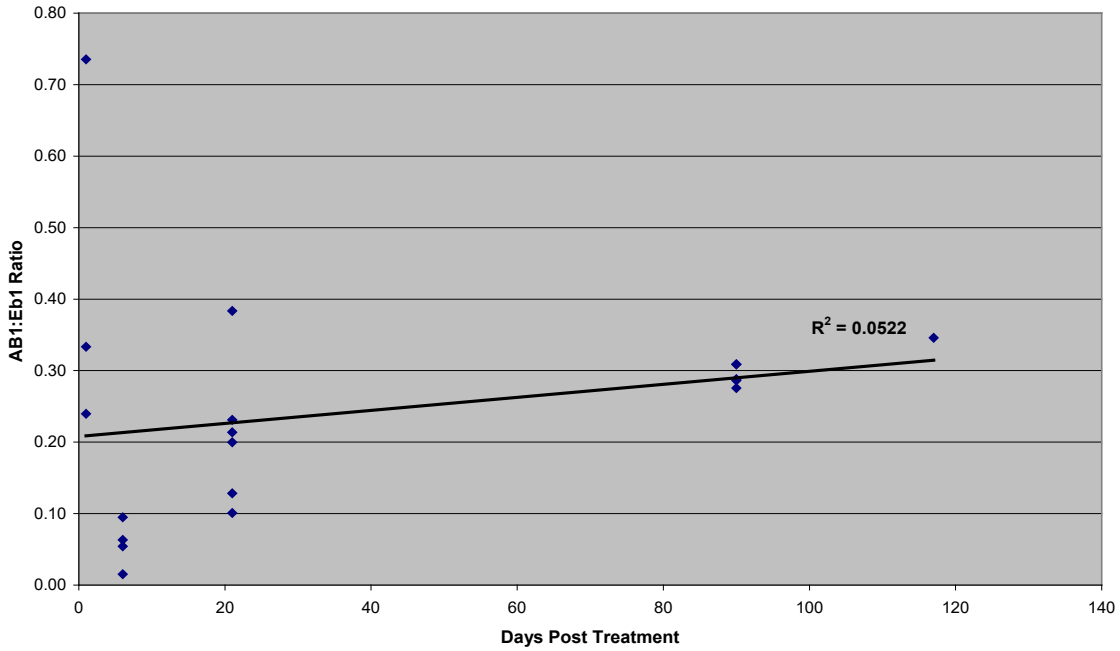


Figure 13. AB:EB ratio measured in wild prawns ("free-range") collected within 10 m of the net-pen at Site A. Samples collected up to 120 days following SLICE® treatment. AB concentrations ranged between 0.1 and 3.4 ppb dry weight.

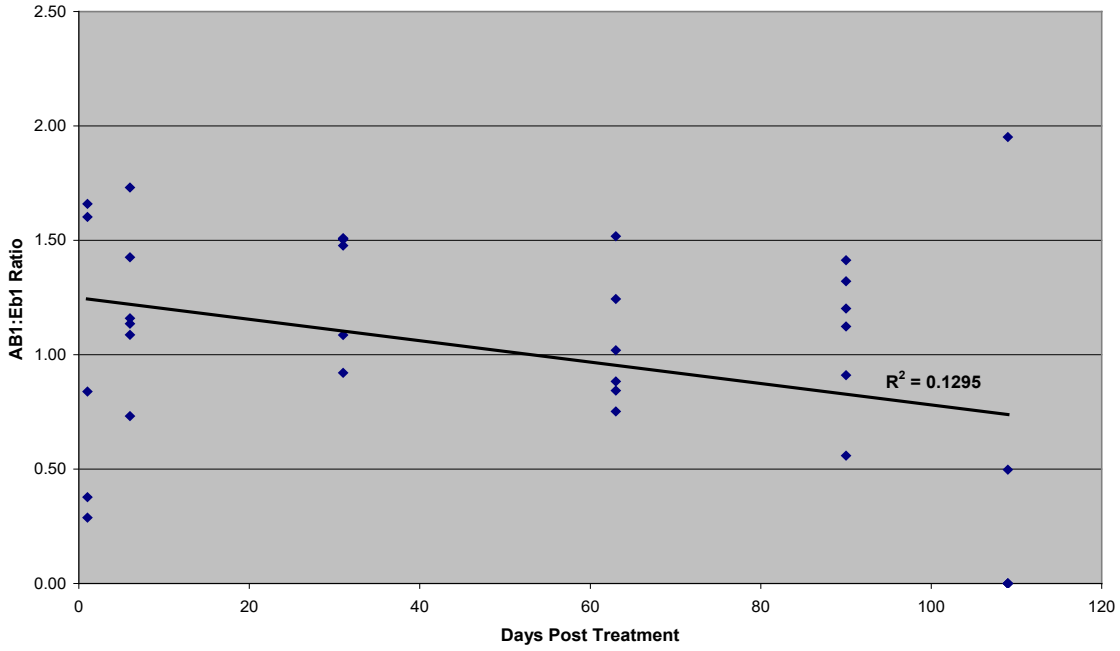


Figure 14. AB:EB ratio measured in wild prawns from control containment traps (ppb dry weight) - deployed at a reference/control location. Samples collected at the same time intervals as those collected at Site A. AB concentrations ranged between 0.1 and 0.3 ppb dry weight.

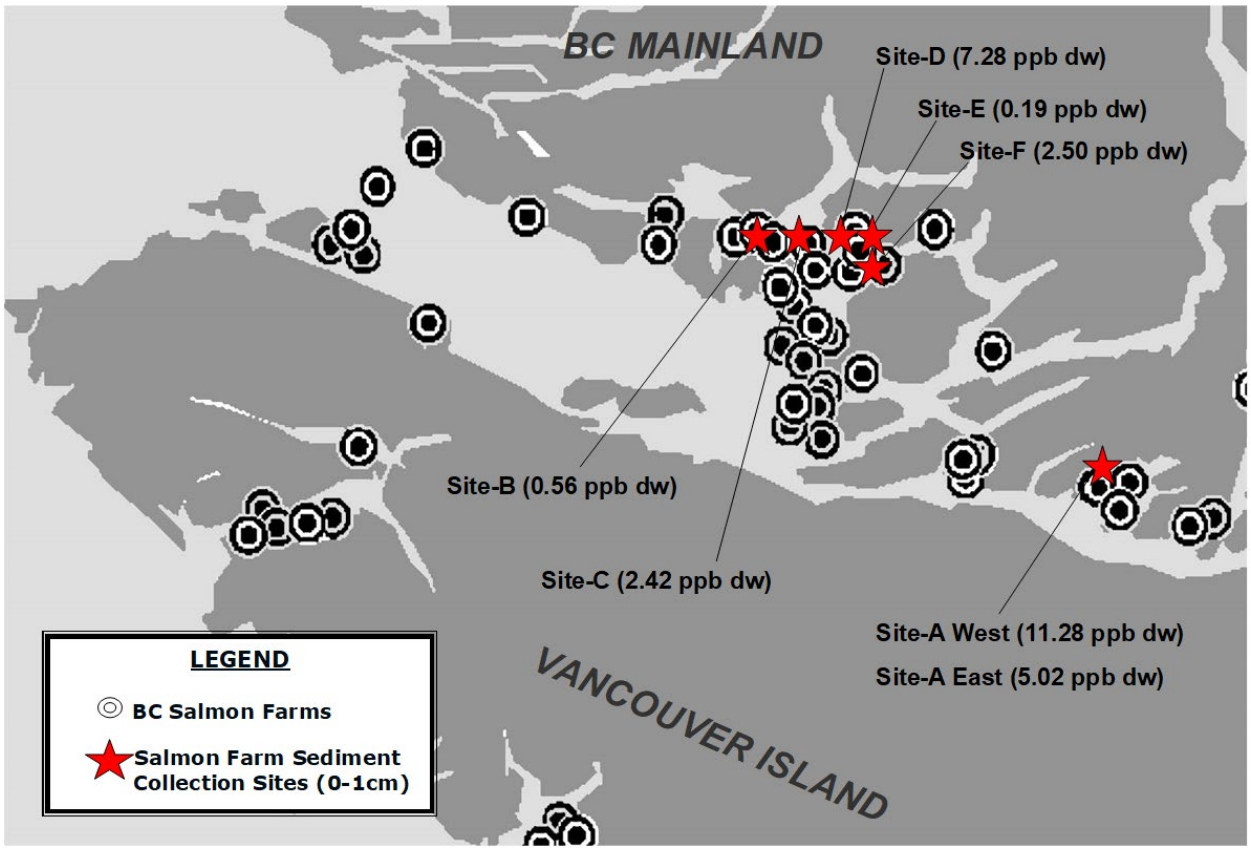


Figure 15. EB concentrations (ppb dry weight) measured in the surface sediments of Sites A and B some 1.5 years post-SLICE® treatment. Also shown here are EB concentrations collected in surface sediments at other locations in the Broughton Archipelago where salmon aquaculture is prominent.

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APPENDIX A. DETAILS OF THE LABORATORY EXPOSURE STUDY

In this component, via a set of controlled laboratory experiments, prawns were exposed to EB. Marine sediment samples were spiked with EB at concentrations (ppb range) that are expected to be found in the natural environment in the vicinity of finfish farm sites following the application of SLICE®. Sediments were placed in specially designed aquaria and 30 litres of filtered sea water were added at a temperature of 6-8ºC. EB present in the sediments is expected to reach equilibrium with the water column in a very short period of time. This is a static testing system and does not require replacement of fluids. The number of prawns added to each aquarium was designed to maintain a loading density of 0.5 g/L. There were a minimum of 5 replicates per concentration and the minimum exposure time was 96 hours. Experiments with longer exposure times were conducted as well.

The laboratory part of the study involved:

- Exposure experiments conducted in controlled laboratory conditions;
- Exposure of prawns to different concentrations of EB in aquaria specially designed to replicate conditions in the natural environment;
- Performing a time series experiment where prawns were exposed to known concentrations of EB at different time intervals;
- Quantifying the concentration of EB in prawns from all exposure experiments. In parallel, quantifying the concentration of EB in water and sediment samples collected in pre-determined time intervals and representative of all exposure experiments;
- Frequent collection of representative prawn samples from each aquarium for genomic work. Biological effects relating to stress, molting and detoxification capacity were assessed using genomic probes that were developed via this project; and
- Examination and modeling of the potential uptake of EB by the test organisms based on the experimental conditions used.

The total numbers of samples generated from this part of the study are summarized below. Also below are given details on how the lab study was conducted.

Table 1. Sample collection summary for SLICE® laboratory exposure study at the Pacific Environmental Science Centre (PESC).

DESCRIPTION	# SAMPLES
TOTAL WATER SAMPLES	210
TOTAL SEDIMENT SAMPLES	140
TOTAL TANK RINSE (MeOH) SAMPLES	24
TOTAL # of PRAWNS	321

A.1. EB EXPOSURE EXPERIMENTS

This work was performed in the laboratory of Dr. Graham van Aggelen at the Pacific Environmental Science Centre (PESC) of Environment Canada in North Vancouver, BC. The aquaria used for this study are available at PESC and are used routinely for a wide range of toxicology studies where organisms are exposed to contaminants at different experimental conditions.

Test Animals

The test species were the spot prawn, *Pandalus platyceros*.

Justification

P. platyceros were selected for this study because they are a marine invertebrate species and are of significant commercial importance in western Canada.

Description

Approximately 500 prawns were collected in the ocean waters near the Institute of Ocean Sciences (IOS) and transferred to PESC. There they were held in a 500L tank with a fresh supply of sea water and acclimated for 10 days prior to testing. Approximately 20% prawn mortality was observed during the initial stages of acclimation, dropping to 5% during the later stages. During the holding period the prawns were fed thin cut squid pieces and food pellets. Prawns were of mixed sex and collected from an area where they had not been exposed to EB or any other product with a similar mechanism of action. The animals were examined by a qualified taxonomist to confirm the identity of the test species.

Health Status

Clinically healthy prawn were acclimated to the test facility for a period of 10 days prior to the start of the trial. Individuals that appeared to be diseased or of questionable health status were not used in the exposure experiments.

Holding Conditions

Glass tanks were for the exposure experiments. EB was spiked into "clean marine" sediment using existing sediment and spiking protocols and apparatus at PESC. EB sediment concentrations were 100, 400, 800, 1200 and 4800 ppb. Sediments were placed onto grids positioned at the bottom of 40L aquariums to a depth of 2-3 cm. Thirty liters of filtered sea water at a temperature of 6-8ºC were added to each aquarium. The testing temperature was maintained at 6-8ºC for the length of the experiment. This is a static testing system and does not require replacement of fluids. The initial weights of each prawn were recorded and 10 prawns were introduced to each replicate concentration. There were a minimum of 5 replicates per concentration and the minimum exposure time is going to be 96 hours. Control groups were exposed to clean sediment only and underwent the same handling. This was done for both the concentration range experiments and the time series experiments.

Dissolved Oxygen Concentration

The dissolved oxygen concentration in the water at test initiation for both the range-finding and definitive studies was at 90 percent of saturation. The flow was set to deliver a minimum of 1.5 mL/minute to each tank. The water within each test chamber was gently aerated. The dissolved oxygen concentrations in the control(s) and all treatment chambers remained above 60 percent of saturation throughout the exposure period.

Room and Tank Identification

The room containing the experimental tanks was labeled with the study number. Tanks within the room were labeled with the tank number, replicate number, and treatment code.

Tank Management

Main system water supplies and experimental temperature were monitored by a 24-hour alarm system. Staff were on-call at all times in case of problems. Management and environmental conditions were identical for each tank.

Husbandry Conditions

All tanks were cleaned, disinfected and rinsed before stocking with prawn. Daily husbandry was in accordance with the relevant Standard Operating Procedure unless otherwise indicated.

Prawn were treated in individual exposure tanks.

Records of daily ratio, prawn behaviour and mortality were maintained.

Prawns were observed daily during the course of the study. Any deviations from normal behavior and appearance were recorded.

No medication was administered.

Any dead or moribund prawns were removed from the tanks, weighed and subjected to gross examination. Numbers of dead or moribund prawn observed each day in each tank were recorded on standard data collection forms. Histological samples were collected from dead or moribund prawn.

Sampling for Chemical Analyses and Genomic Measurements

Representative samples of spiked sediment collected to verify concentration and homogeneity of mixture were collected at set time intervals and were analyzed for EB concentrations.

Overlying water was collected at set time intervals and was analyzed for EB concentrations.

Organisms were collected from each of the tanks and were prepared for genomic and chemical analyses. The Genomics work was performed at the University of Victoria and chemical analysis was performed at the Institute of Ocean Sciences.

Prawns that died during the course of the study were frozen at -20ºC. After completion of the corresponding experiment, an equal number of surviving prawns from each treatment group were frozen. Edible tissues from prawns that died during the study and those that survived were analyzed for EB residues.