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Feeding response in marine copepods as a measure of acute toxicity of four anti-sea lice pesticides

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ABSTRACT

Anti-sea lice pesticides used in salmon aquaculture are released directly into the environment where non-target organisms, including zooplankton, may be exposed. The toxicity of four pesticides to field-collected copepods was examined in 1-h exposures with lethality and feeding endpoints determined 5-h post-exposure using staining techniques. Copepods were immobilized within 1 h, at aquaculture treatment concentrations of deltamethrin (AlphaMax®), cypermethrin (Excis®), and hydrogen peroxide (Interox®Paramove™50). All organisms showed vital staining, indicating immobilized organisms were still alive, thus LC50s were not determined. Feeding on carmine particles was inhibited and EC50s ranged from 0.017 to 0.067 μg deltamethrin/L, 0.098–0.36 μg cypermethrin/L, and 2.6–10 mg hydrogen peroxide/L, representing 30- to 117-fold, 13- to 51-fold, and 120- to 460-fold dilutions of the respective aquaculture treatments. No effects were observed in copepods exposed to azamethiphos (Salmosan®) at 5-times the aquaculture treatment. Acute exposure to three of the four pesticides affected feeding and mobility of copepods at environmentally-realistic concentrations.

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1. Introduction

Sea lice are ectoparasites of many species of fish and are a serious problem in salmon aquaculture (Roth et al., 1993; MacKinnon, 1997). The species that infect cultured Atlantic salmon in Canada are parasitic copepods *Lepeophtheirus salmonis* and *Caligus elongatus*. Treatment of fish often involves immersion in a pesticide bath for up to 1 h by placing impervious skirts or tarps around aquaculture cages or pumping fish into specialized well boats. The pesticide is then released from the aquaculture cage or well boat and disperses as a plume, with the fate of the chemicals depending on water movement and chemical characteristics. Studies of pesticide dispersion from aquaculture sites have found that effluent plumes are detectable 2–5.5 h post-release at distances 0.9–3 km from the cage site, with pesticide concentrations that represent 1/1000–1/2000 of the pre-release concentrations (Ernst et al., 2001). Thus indigenous (or non-target) organisms may potentially be exposed to anti-sea lice pesticides released from aquaculture sites. In particular, pelagic organisms such as zooplankton may be entrained in effluent plumes and exposed for hours (Willis et al., 2005). Copepods are typically the most abundant organisms in the zooplankton of coastal ecosystems and an important component of marine and estuarine food webs (Verity and Smetacek, 1996; Gerber, 2000; Turner, 2004). Planktonic copepods have a similar life cycle as ectoparasitic copepods (sea lice) and also may be adversely affected by anti-sea lice pesticides (Willis et al., 2005).

Studies have examined the toxicity of the pyrethroids cypermethrin and deltamethrin to marine copepods, as these chemicals have been used in anti-sea lice pesticides. For example, eggs, nauplii, and adults of the species *Acartia tonsa* were exposed to cypermethrin for 96 h (Medina et al., 2002) or for 2–5 d (Barata et al., 2002a) and examined for lethality, clutch size, and feeding rates. Similarly, the toxicity of both cypermethrin and deltamethrin to another species *Tisbe battagliai* was examined in 4- to 6-d lethality tests with eggs, nauplii, and adult females, as well as 6-d tests examining feeding rates and clutch size in adults (Barata et al., 2002b). However, these studies used standard exposure durations of marine toxicity tests which do not reflect the very acute exposures (i.e., up to a few hours) expected to occur with

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aquaculture effluents. Using a more realistic exposure scenario, delayed mortality (up to 144 h post exposure) of adult *A. tonsa* was observed following 1- and 24-h pulse exposures to concentrations of cypermethrin close to the aquaculture treatment concentration (Medina et al., 2004). This suggests that effects can occur following short, pulsed exposures and that tests reflecting these conditions may be more appropriate than previous standard exposure durations.

Survival is a common endpoint in zooplankton studies such as those by Barata et al. (2002a) and Medina et al. (2002, 2004). Typically survival is evaluated by examining individuals under a dissecting scope for indications of movement, heartbeat, etc., which can be time consuming and limit the numbers of organisms that can be examined. Sublethal effects such as immobility and decreased feeding are also ecologically-relevant endpoints as they may impair survival, growth, and reproduction. These sublethal effects may occur well below lethal thresholds, particularly for acute exposures, and have sometimes been shown to be more sensitive than lethal endpoints for copepods (e.g., Barata et al., 2002a). Investigation of the potential effects of aquaculture effluents on copepods thus requires consideration of alternative experimental designs and use of techniques that will facilitate assessment of both lethal and sublethal endpoints. For example, staining techniques have been used to differentiate between alive and dead organisms in marine/estuarine copepod populations. A protocol for staining copepods with neutral red (a vital stain taken up into live tissues) was first described by Dressel et al. (1972) and has since been evaluated and developed into a protocol for field samples by Tang et al. (2006) and Elliot and Tang (2009). Filterable particles such as carmine in the digestive tract demonstrate feeding and have been used with copepods and other zooplankton as another indicator of relative organism health (e.g., Dressel et al., 1972; Jawecki et al., 2011). The use of such staining methods in laboratory-based toxicity tests would enable rapid and objective determination of lethality and feeding endpoints in a large number of zooplankton. This improves statistical power, which is particularly important for (sublethal) endpoints that may be highly variable or for populations with unknown "health" or exposure history (i.e., field samples).

The purpose of the present study was to determine the effects of four anti-sea lice pesticides on copepods from local zooplankton communities in southwest New Brunswick (NB), Canada; this is an area where salmon aquaculture and treatment with anti-sea lice pesticides are prevalent. The goal was to develop a laboratory test method representative of potential environmental exposures, and with easy-to-assess, robust, and relevant endpoints that relied on staining techniques. The present study addresses some of the data gaps in the literature by examining.

- Acute exposures that are environmentally-relevant;
- Potential for delayed toxicity;
- Lethal and sublethal effects;
- Relative toxicity of several chemicals used to control sea lice; and
- Actual anti-sea lice pesticide formulations instead of technical grade chemicals.

2. Materials and methods

2.1. Selection of anti-sea lice pesticides

Four anti-sea lice pesticide formulations were selected for this study because they are either currently or have been used to combat infestations of sea lice in eastern Canada. AlphaMax® was used under an emergency registration from Health Canada's Pest Management Regulatory Agency (HC-PMRA) in 2009 and 2010 (HC-PMRA, 2010). Excis® was applied experimentally in southwest New Brunswick in the mid 1990's, but is used extensively in other jurisdictions (Chang and McLelland, 1996, 1997). Interox®Paramove™50 and Salmosan® have been given emergency registration status and are being used to combat sea lice infestations in southwest New Brunswick (HC-PMRA, 2013a,b).

The anti-sea lice formulations AlphaMax® and Excis® are emulsifiable concentrates containing 1% of the synthetic pyrethroids deltamethrin or cypermethrin as the active ingredients, respectively. Pyrethroids affect nerve transmission by interfering with sodium (Na^+) channels resulting in depolarization and repetitive firing of the nerve ending, leading to eventual paralysis and death (Miller and Adams, 1982; Crane et al., 2011; Haya et al., 2005). Both pesticides are effective against all attached stages of sea lice including adults (Haya et al., 2005; Burridge et al., 2010). Treatment of salmon is a 40 min bath with AlphaMax® at a target concentration of 2.0 μg deltamethrin/L (SEPA, 2008) or a 1-h bath with Excis® at a target concentration of 5.0 μg cypermethrin/L in tarped cages (SEPA, 1998), herein referred to as "aquaculture treatment concentrations".

Interox®Paramove™50 is a liquid formulation made up of 50% hydrogen peroxide. The suggested mechanisms of action of hydrogen peroxide are mechanical paralysis, peroxidation of lipid and cellular organelle membranes by hydroxyl radicals, and inactivation of enzymes and DNA replication (Cotran et al., 1989). Most evidence supports toxicity via mechanical paralysis, as bubbles form in the gut and haemolymph and cause sea lice to release and float to the surface (Bruno and Raynard, 1994). The formulation is not effective against larval sea lice and its effectiveness against pre-adult and adult stages has been inconsistent (Mitchell and Collins, 1997). It is applied in a bath treatment at 1200–1800 mg hydrogen peroxide/L for 40 min, but the effectiveness is temperature dependent, and it has been suggested that treatment with these concentrations may not be effective below 10 °C (Treasurer et al., 2000).

Salmosan® is a wettable powder formulation consisting of 47.5% azamethiphos, an organophosphate pesticide that inhibits acetylcholinesterase (AChE) activity and causes repetitive firing of nerves (Baillie, 1985). The formulation is effective against pre-adult and adult stages of the sea louse, but it does not affect larval stages (SEPA, 2005). It is applied as a bath treatment at 100 μg azamethiphos/L for 30–60 min in well boats and tarps and at 150 μg/L in skirt treatments.

2.2. Organism collection and toxicity tests

Zooplankton samples composed almost completely of copepods (see SI Table 1) were collected monthly between January and May, 2013, from Passamaquoddy Bay, NB. Zooplankton samples were collected using vertical tows (max. 50 m depth) with a 75-cm diameter, 150-μm mesh plankton net. Contents of tows were transferred to glass jars with seawater, and stored in a cooler in the dark during transit to the laboratory (DFO – St. Andrews Biological Station, St. Andrews, NB). Zooplankton were distributed among large beakers which were topped up with sand-filtered (0.2 μm) seawater (source Pasmaquoddy Bay; ~30 parts per thousand) to reduce crowding and held overnight (max. 2 nights) in darkness and in a temperature-controlled room (at 9 °C) prior to use in toxicity studies. Toxicity tests were conducted at the same temperature under fluorescent lighting of ~250 lux.

Following initial method development and range finding studies, a testing protocol was established with a defined concentration series for each pesticide. Toxicity tests with each of the four

pesticides were repeated with each zooplankton sampling event. On each test date, a stock solution of pesticide was prepared in seawater, test solutions were prepared by serial dilution, and 400 ml of each dilution was split between two 250-ml beakers to create a duplicate series of pesticide concentrations ($n = 1$ for each pesticide concentration per endpoint per date; considered acceptable in standard quantal tests, Environment Canada, 2005). Live copepods were attracted to the top of holding beakers with a hand-held fluorescent light and concentrated into a smaller volume of seawater. A 1–2 ml aliquot of the concentrated organisms was added to each 250-ml beaker, for a target density of ≥100 organisms/beaker. A subsample of organisms from each sampling date was also collected for identification.

Copepods were exposed to the anti-sea lice pesticides AlphaMax®, Excis®, Interox®Paramove™50, or Salmosan® for 1 h and then transferred to clean water for 5 h. This reflects the acute exposures that may occur in the field and considers the potential for delayed affects after such an acute exposure. Stock solutions of carmine particles (2 g/L) and neutral red (5 g/L) were prepared in seawater and sonicated (~10 min) to break up clumps of particles. Carmine particles were added to one replicate at a final concentration of 0.06 mg/ml to examine ingestion by copepods during the final 2 h. Neutral red, a vital stain, was added at a final concentration of 0.05 mg/ml to the other replicate for the last 0.5 h to determine the number of living copepods. At 5 h post-exposure, copepods were rinsed to remove particles or dye and transferred to scintillation vials. Samples were fixed with 40% formaldehyde (final concentration 2% v/v) and refrigerated at 4 °C until examined under a microscope. In addition, general observations regarding copepod mobility and distribution in the water column were made at 15 min intervals throughout the 1-h exposure and at ~5 h post-exposure.

Preserved samples were transferred to a black Petri plate marked with one cm squares to best observe staining and facilitate counting using an Olympus® (SZX16) stereomicroscope. Neutral red samples were acidified with a few drops of dilute hydrochloric acid to a pH < 7 to help develop the colour of the stain. Copepods alive at the time of neutral red staining appeared bright red, while dead organisms appeared unstained, cloudy white, or light pink (Fig. 1A). Copepods containing carmine particles (bright red) within their digestive tract were considered to have been feeding in the post-exposure period (Fig. 1B). For the purposes of counting stained and unstained organisms in this study, copepods included all life stages (adults, copepodites, and nauplii). False positive staining was considered negligible, based on method development in which organisms were killed with heat as a positive control (based on Elliot and Tang, 2009).

2.3. Chemical analyses

Water samples for analysis of deltamethrin, cypermethrin, or azamethiphos were collected (~400 ml volume) into IChem 200 amber bottles from the highest test concentration at the beginning of each test, preserved with dichloromethane (DCM; ~5% v/v in sample), shaken, and then stored at 4 °C until chemical analysis. Samples for hydrogen peroxide analysis were collected from the highest test concentration at the beginning of each test and analysed immediately by titration into cerium sulphate (IV) and sulphuric acid as prescribed by Solvay, Inc., the distributor of Interox®Paramove™50.

Samples were analysed for deltamethrin and cypermethrin at the University of New Brunswick (Saint John, NB, Canada) following standard methods. A liquid–liquid extraction was completed using DCM (US EPA, 1996). PCB 30 and PCB 204 were added to samples as surrogates prior to extraction. Each sample was extracted two times with DCM, which was then collected and combined for analysis. The extract was concentrated using a Büchi Rotavapor R200 and further concentrated with an N-Evap™ 112 nitrogen evaporator to a final volume in isooctane. PCB 103 and PCB 198 were added as internal standards to final extracts just prior to analysis using gas chromatography with electron capture detection (GC-ECD) and quantification using an internal standard calibration, based on standard methods (US EPA, 1995; Hladik et al., 2009). Quality assurance/quality control procedures performed on each set of 10 samples included: surrogates, calibration checks, method blank, and a method spike. The method detection limits were 0.005 µg/L for both deltamethrin and cypermethrin.

For analysis of azamethiphos, a 5 ml aliquot of the 20 ml of DCM from each bottle was transferred to a corresponding sample vial and blown down to dryness under nitrogen. After being exchanged into acetonitrile (ACN), the residue was transferred to a 10 ml volumetric flask and brought to volume. A 500 µl aliquot of each of the resulting solutions was transferred to its respective sample vial and brought to 1 ml with more ACN. Each sample was analysed twice by high-performance liquid chromatography with UV–visible spectrometry (HPLC/UV–vis) and the results averaged. A series of calibration standards was analysed before and after the samples and the resulting calibration curve was based on both sets of standards. The method detection limit was 42 µg/L.

2.4. Data analysis and test endpoints

Percent mortality was determined as the fraction of unstained organisms in the neutral red samples. The percentage of live organisms not feeding in the carmine samples was determined by the

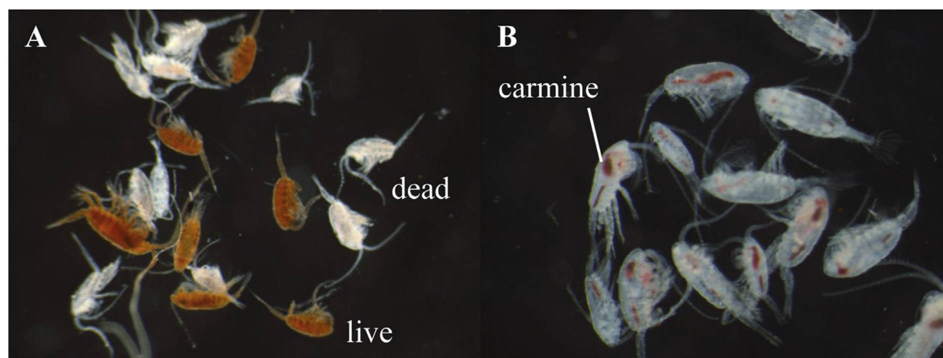

Fig. 1. Appearance of copepods treated with A) neutral red or B) carmine particles under a stereomicroscope (Olympus® SZX16). Copepods that were alive during staining appeared bright red; dead copepods were unstained, white, or light pink. Bright red carmine particles in the digestive tracts of copepods indicate feeding. Pictures taken with an Olympus® Q-Color5 digital camera. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

following equation, which corrects for non-feeding attributable to mortality (Equation (1)).

$$\% \text{ of live copepods not feeding} = \left[1 - \left(\frac{C_{\text{stained}}}{C_{\text{total}} \times f_{\text{alive}}}\right)\right] \times 100 \quad (1)$$

where $C_{stained}$ is the number of stained copepods in the carmine sample, C_{total} is the total number of copepods in carmine sample, f_{alive} is the fraction of live (i.e., stained) copepods in the neutral red sample ($=NR_{stained}/NR_{total}$) of the corresponding pesticide concentration. Feeding inhibition was calculated as the % of non-feeding copepods (shown above) corrected for the control response in that test (Equation (2)).

$$\% \text{ feeding inhibition} = \left(\frac{\% \text{ treatment}_{\text{non-feeding}} - \% \text{ control}_{\text{non-feeding}}}{100 - \% \text{ control}_{\text{non-feeding}}}\right) \times 100 \quad (2)$$

Chemical concentrations were based on the active ingredient of the pesticide formulation measured in the highest test concentration on each date. Chemical concentrations in the lower test concentrations were assumed to reflect the appropriate dilution of the measured concentrations in each test. Loss of active ingredient was not accounted for because it has been found to be minimal in 1-h exposures (Burridge et al., 2014; Van Geest unpublished data).

Estimates of the concentration of chemical causing 50% mortality in organisms (LC50) or inhibiting feeding in 50% of organisms (EC50; corrected for mortality and control response) with 95% Confidence Intervals (C.I.) were determined for each test. These endpoints were determined according to Stephan (1977), using the computer program Toxstats and Probit or Spearman-Karber methods where appropriate, and were based on the measured and assumed chemical concentrations determined for each test. For a given pesticide, thresholds were considered to be significantly different if C.I.s did not overlap (Environment Canada, 2005).

3. Results

Zooplankton samples from Passamaquoddy Bay were typically dominated by adult copepods or copepodites of the species *Acartia hudsonica* (identification by R. Milne, Atlantic Reference Centre, St. Andrews, NB; based on Pollock, 1998) and contained varying proportions of nauplii that could not be readily identified, but were presumed to be the same species (Fig. 3, SI Table 1). Nauplii were not present in samples from January and February, but comprised 80% and 21–35% of the April and May samples, respectively.

In toxicity tests with AlphaMax®, Excis®, and Interox®Paramove™50, copepods exposed to the two or three highest test concentrations typically sank to the bottom of beakers and showed little or no movement by the end of the 1-h exposure. This immobilization occurred at concentrations ≥0.11 μg deltamethrin/L, ≥ 0.44 μg cypermethrin/L, and ≥10 mg hydrogen peroxide/L. In some beakers, immobilization occurred within 15 min of exposure to concentrations equal to or below the corresponding aquaculture treatment concentration of each pesticide (listed in Section 2.1). Some recovery and movement of copepods back into the water column was observed 3–5 h post-exposure (data not shown).

After 5 h in clean water post-exposure, almost all organisms were stained with neutral red, including those immobilized at high test concentrations, indicating that they were still alive. When examined under a microscope, the immobilized organisms showed little to no movement other than occasional twitching of antennae. As a result, no concentration-response was observed with the vital stain and LC50s were not determined. The exception was the February test with Interox®Paramove™50 in which 22 and 69% of copepods exposed to 32 and 108 mg hydrogen peroxide/L, respectively (i.e., the two highest test concentrations), showed poor or no staining. An LC50 (95% C.I.) of 68 (58–82) mg hydrogen peroxide/L was calculated.

Although lethality typically was not observed, the animals that were immobilized at higher pesticide concentrations were not feeding. In tests with AlphaMax®, Excis®, and Interox®Paramove™50, the proportion of copepods with carmine particles in their digestive tracts decreased with increasing exposure concentrations (Fig. 2). A concentration-response in feeding inhibition was observed in each test with these pesticides and 80–100% inhibition was frequently observed in the highest test concentrations. For a given pesticide, the response curves generally had the same shape between test dates (Fig. 2). The EC50s for feeding inhibition ranged from 0.017 to 0.067 μg deltamethrin/L, 0.098–0.36 μg cypermethrin/L, and 2.6–10 mg hydrogen peroxide/L (Fig. 3, SI Table 1). Confidence intervals around EC50s were narrow in most cases, indicative of low variation due to adequate sample sizes, and the lack of overlap of C.I.s suggested statistical differences between some EC50s for a given pesticide (Fig. 3, SI Table 1).

In contrast to the other pesticides, copepods exposed to Salmosan® were not immobilized even at 5-times the aquaculture treatment concentration, and a consistent concentration-response for feeding inhibition was not observed in repeated tests. For this reason, an EC50 for Salmosan® could not be determined (data not shown).

4. Discussion

A certain degree of variability in test endpoints is expected in laboratory toxicity tests using field-collected organisms due to changes in the sampled population (e.g., species composition, age, size, condition) over time. In the present study, toxicity tests were conducted using copepods collected in the field and over several months. While these collections were consistently dominated by *A. hudsonica*, the proportion of different life stages (adults or copepodites vs. nauplii) and ocean temperatures varied across sampling dates, potentially affecting test results. *A. hudsonica* collected in February had the lowest EC50s for Interox®Paramove™50, AlphaMax®, and Excis®, and the only lethal response to a pesticide (Interox®Paramove™50). The surface temperature of Passamaquoddy Bay was lowest in February, and this may have increased organism sensitivity. Temperature is an important environmental variable for aquatic organisms as it can influence physiological mechanisms and affect toxicokinetic and toxicodynamic processes. The toxicity of pyrethroid pesticides has been shown to be temperature-dependent, with increased toxicity at lower temperature exposures (Coats et al., 1989; Harwood et al., 2009). The differences in organism sensitivity in the present study were not related to exposure temperature as tests were conducted at the same temperature across dates. However, Willming et al. (2013)

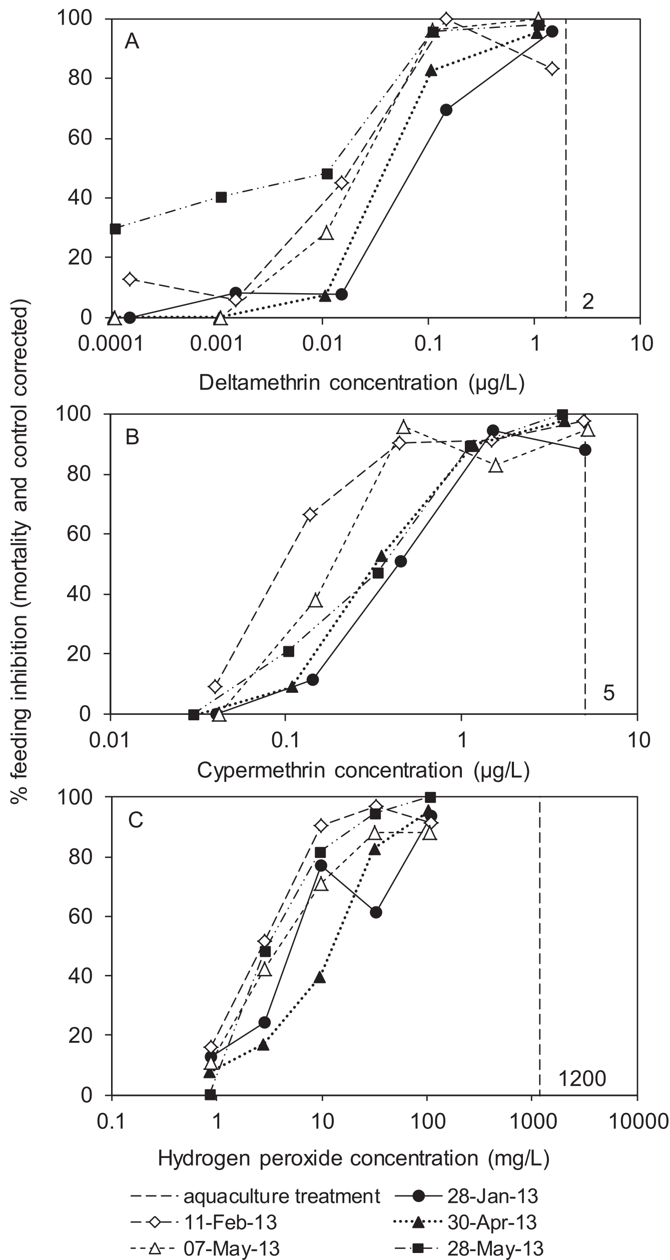

Fig. 2. Feeding inhibition of copepods *Acartia hudsonica* exposed to the anti-sea lice pesticides A) AlphaMax®, B) Excis®, and C) Interox®Paramove™50. Organisms were exposed to a pesticide for 1 h, and then transferred to clean water for 5 h, with carmine particles provided in final 2 h. Reported concentrations are based on dilution of the active ingredient measured in the highest test concentrations. Vertical dashed lines are corresponding aquaculture treatment concentrations.

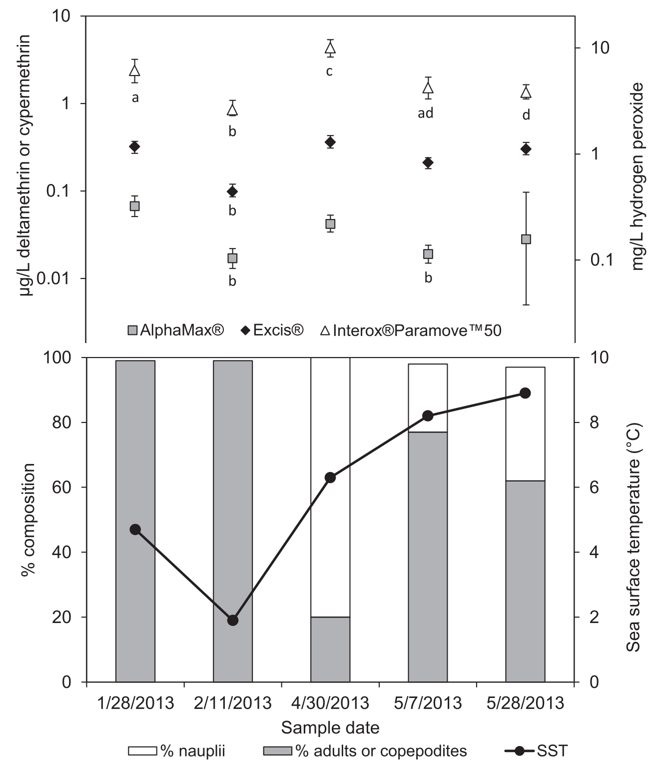

Fig. 3. Estimates of EC50s (feeding inhibition) for copepods *Acartia hudsonica* exposed to the anti-sea lice pesticides AlphaMax® (μg/l deltamethrin), Excis® (μg/L cypermethrin), and Interox®Paramove™50 (mg/L hydrogen peroxide). Organisms were exposed to a pesticide for 1 h, and then transferred to clean water for 5 h. For a given pesticide, EC50s with different letters do not have overlapping confidence intervals and are considered significantly different. Other sample parameters include % composition of copepod life stages and sea surface temperature of Passamaquoddy Bay, NB at time of zooplankton collections.

demonstrated that some aquatic invertebrates were more sensitive to pesticides when they experienced daily temperature variations (e.g., Δ of 10 °C) compared to a constant temperature exposure. The variation they observed in responses suggested that temperature acts as another stressor throughout daily cycles. In the present study, the increased sensitivity of copepods collected in February to pesticides may be due to stress associated with the temperature change from the field to the lab (Δ ~5 °C), as supported by the observations of Willming et al. (2013), or stress associated solely with the lower temperature which can affect physiological condition. Temperature did not appear to be an influencing factor in the other months as EC50s did not appear to follow a trend with sea surface temperatures at the time of collection (not shown).

Life stage-related variation in sensitivity has been observed in copepods and is thought to be due to a number of factors including advanced development of detoxification mechanisms in adults, allometric differences (i.e., surface area to volume), and molting frequency (Medina et al., 2002). In the present study, the April zooplankton was dominated by nauplii and had the highest EC50 for Interox®Paramove™50, suggesting that nauplii may be less sensitive to hydrogen peroxide than adults and copepodites. This concurs with the observation that hydrogen peroxide is not effective against larval stages of sea lice (Mitchell and Collins, 1997). For AlphaMax® and Excis®, no life stage-related differences in feeding inhibition were apparent between April and other months (C.I.s overlapped with EC50s from January and late May). Barata et al. (2002b) reported that nauplii of the species *T. battagliai* were 1.5-times more sensitive to technical grade cypermethrin and the polycyclic aromatic hydrocarbon fluoranthene than gravid adult females, but were equally sensitive to deltamethrin, in 96- to 144-h lethality tests. Studies of other copepod species exposed to metals and other organic pesticides typically found nauplii to be 1.3- to 5-times more sensitive than adults in 48- and 96-h lethality tests (Verriopoulos and Moraittou-Apostolopoulou, 1982; O'Brien et al., 1988; Forget et al., 1998). In contrast, Medina et al. (2002) reported that nauplii were 28-times more sensitive to technical grade cypermethrin than adult *A. tonsa* in 96-h lethality tests. Although there were some significant differences between months in the present study, EC50s varied by less than a factor of 4 between the different tests for each of AlphaMax®, Excis®, and Interox®Paramove™50. In a general context, under the conditions of the

present study, there appears to be little difference in zooplankton sensitivity over time despite some changes in the relative abundance of different copepod life stages.

In the current study, immobilization of copepods occurred within 1-h exposure to Interox®Paramove™50 at concentrations ≥10 mg hydrogen peroxide/L. Bruno and Raynard (1994) observed that 33% and 98% of sea lice were immobilized after 20 min exposure to 500 and 2000 mg/L of stabilized hydrogen peroxide, respectively, but full or partial recovery occurred 2 h post-treatment. They observed gas bubbles in the gut and haemolymph of immobilized lice, which caused most to float to the water surface. In contrast, in the present study immobilized copepods sank to the bottom of test beakers. Recovery of these copepods (indirectly measured by feeding response) was not as evident as that reported for sea lice, despite the lower exposure concentrations of hydrogen peroxide, albeit over a longer exposure duration. These differences in response and recovery may be influenced by the formulation of Interox®Paramove™50 (discussed below), but also suggests greater sensitivity of planktonic copepods when compared to ectoparasitic sea lice.

In the present study, a 1-h pulse exposure to anti-sea lice pesticides typically did not result in lethality to copepods. Studies in which copepods were exposed to technical grade chemicals for longer, more conventional, durations (96–144 h) reported the following LC50s: 0.005 and 0.142 μg cypermethrin/L for nauplii and adult *A. tonsa*, respectively (Medina et al., 2002); 0.108 μg cypermethrin/L for adult *A. tonsa* (Barata et al., 2002a); and 0.011 μg deltamethrin/L and 0.052 μg cypermethrin/L for adult *T. battagliai* (Barata et al., 2002b). These 96- to 144-h LC50s are 1–3 orders of magnitude lower than the associated aquaculture treatment concentrations, which were the highest test concentrations of deltamethrin and cypermethrin in the present study. With a much shorter (1 h) exposure to similar pyrethroid concentrations, it is not surprising that lethality was not observed in the present study. Further work by Medina et al. (2004) investigated delayed toxicity in adult *A. tonsa* following 1- or 24-h pulse exposures to technical grade cypermethrin, which is more representative of the acute exposures associated with aquaculture effluent plumes. They observed high survival during the 1-h pulse, but delayed toxicity following treatment, with LC50s between 2 and 4 μg cypermethrin/L when assessed at 24–144 h post exposure. Copepods in the present study were exposed to concentrations of cypermethrin (in Excis® formulation) within this range, but were not held for as long and delayed mortality was not observed in as short as 5 h post exposure. The data of Medina et al. (2004) suggest that delayed mortality in copepods could occur following exposures as short as 1 h to concentrations close to the aquaculture treatment concentration of 5 μg cypermethrin/L. Of note, the present study used anti-sea lice pesticide formulations as opposed to the technical grade product used in these other studies. The presence of solubilizers and other components of the formulation may affect the fate of the active ingredient by keeping it in solution longer than would be expected based on chemical properties of the technical grade compound. This may change the exposure profile and contribute to differences in toxicity observed between studies. Unfortunately, the constituents of pesticide formulations are not publically available to comment further on this issue.

Feeding inhibition in copepods was observed following 1-h exposures to AlphaMax®, Excis®, and Interox®Paramove™50. Barata et al. (2002a,b) also assessed how some of these pesticides affected feeding in copepods using the changes in algal cell density over time. The 48-h EC50 (95% C.I.) for 8-d old *A. tonsa* copepodites was 0.065 (0.041–0.089) μg cypermethrin/L (Barata et al., 2002a) and the 144-h EC50s (95% C.I.) for adult *T. battagliai* were 0.028 (0.013–0.042) μg cypermethrin/L and 0.058 (0.041–0.076) μg deltamethrin/L (Barata et al., 2002b). In the present study with a much shorter exposure, EC50s for feeding were an order of magnitude greater for cypermethrin (0.098–0.32 μg/L), but of a similar magnitude for deltamethrin (0.017–0.042 μg/L). As suggested by Barata et al. (2002a) and shown in the present study, short term exposures can affect feeding endpoints in copepods at concentrations below those affecting other lethal and sublethal endpoints (e.g., egg production, egg and adult survival).

Copepods in the present study were not affected by 1-h exposures to Salmosan® at concentrations up to 620 μg azamethiphos/L, more than 5-times the aquaculture treatment concentration. Burridge et al. (2014) examined the toxicity of Salmosan® to other crustaceans (stage I and adult lobster, sand and mysid shrimp) from 1- and 24-h pulse exposures with monitoring up to 96 h post exposure. A 1-h LC50 could only be determined for adult lobster following exposures up to the aquaculture treatment concentration (100 μg/L), while 24-h LC50s were determined for all species and life stages tested. This suggests that acetylcholinesterase inhibition by azamethiphos during a 1-h exposure may not be severe enough to cause adverse effects in many of the crustacean species tested.

It is noted that, in principle, zooplankton are likely to be exposed to toxic levels of anti-sea lice pesticides only in a relatively small area around an aquaculture site and only for short periods after treatment events. However, uncertainty exists regarding the dispersion characteristics of effluent plumes, as pesticides can be difficult to track and detect at low concentrations (Willis et al., 2005). In the present study, immobilization of copepods occurred within as little as 15 min of exposure to pesticides at and below aquaculture treatment concentrations for AlphaMax®, Excis®, and Interox®Paramove™50. Immobilized organisms in the field would likely die because they cannot feed or avoid predation, and/or will settle out of the water column. Although there was some indication of recovery post exposure (based on visual observation) in the present study, feeding response in treatments was still inhibited relative to the controls. Feeding inhibition was the most sensitive response in the present study and the estimated EC50s represent approximately 30- to 117-fold, 13- to 51-fold, and 120- to 460-fold dilutions of the aquaculture treatment concentrations for AlphaMax®, Excis®, and Interox®Paramove™50, respectively. Models of effluent plumes from aquaculture sites (by Ernst et al., 2001; Page et al., DFO unpublished) suggest that dilutions of these magnitudes could occur between 20 min and 1.5 h and for as long as 6 h post release. This suggests that zooplankton in the field could be exposed to concentrations of pesticides and for sufficient duration to cause the biological effects observed herein.

The present study used pesticide formulations and zooplankton collected from an area where the two could potentially coincide, which provides a high degree of environmental relevance for this work. Aquaculture operations in southwest New Brunswick treat for sea lice in the spring and fall as part of their pest management programs. Monitoring for sea lice and treatment of salmon occurs once seawater temperatures are above 5 °C, which is typically April through December (Beattie, M. NB Dept. of Agriculture, Aquaculture and Fisheries, 2013, Personal Communication). While copepods collected in February appeared to be most sensitive, sea lice treatments are not expected at this time of year in New Brunswick. The toxicity data from later months are more relevant to potential environmental exposures since treatments are likely to occur at these times. While the pyrethroid-based pesticides AlphaMax® and Excis® are not currently applied in Canada, the results of the present study provide valuable data for any risk assessment should there be requests to register these products in Canada, or re-register these products in other jurisdictions where they are in use. Interox®Paramove™50 is presently used in southwest New Brunswick, and the present study suggests that zooplankton

exposed to aquaculture effluent plumes would be adversely affected. This issue is potentially made worse by treatment of multiple cages at a site in one day and the relative close proximity of multiple aquaculture sites within southwest New Brunswick.

5. Conclusions

The present study examined the effects of acute exposure to four anti-sea lice pesticides on local marine copepods, using laboratory toxicity tests. Immobilization of copepods was noted to occur rapidly at pesticide concentrations at and below corresponding aquaculture treatment concentrations. Lethality typically was not observed following a 1-h pulse exposure, when assessed at 5 h post-exposure. Inhibition of feeding was the most sensitive endpoint and EC50 thresholds occurred at concentrations representing 30- to 117-fold, 13- to 51-fold, and 120- to 460-fold dilutions of the aquaculture treatment concentrations for AlphaMax®, Excis®, and Interox®Paramove™50, respectively. The ranking of feeding inhibition thresholds is: AlphaMax® > Excis® > Interox®Paramove™50, consistent with toxicity rankings in other research. Copepods were not affected by Salmosan® in the present study. Therefore, for three of the four anti-sea lice pesticides studied, acute exposure affected feeding and mobility of zooplankton in laboratory tests at environmentally-realistic concentrations.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.marenvres.2014.09.011.

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