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Effects of exposing shrimp larvae (*Pandalus borealis*) to aquaculture pesticides at field relevant concentrations, with and without food limitation

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ABSTRACT

Anti-parasitic drugs used in the aquaculture industry are discharged to the sea after treatment of salmon. In this study, the effects of azamethiphos (AZA) in the Salmosan® formulation and deltamethrin (DEL) in the Alpha Max® formulation, have been assessed in Northern shrimp larvae (*Pandalus borealis)* when administered both separately and in combination. The exposure concentrations were 100 ng/L for AZA and 2 ng/L for DEL, each representing a 1000-fold dilution of the prescribed concentrations for salmon. These two chemicals were combined at these concentrations to give a third treatment (AZA + DEL). When larvae were exposed for two hours on the first, second and third days post hatch (dph), significantly increased mortality and reduced swimming activity were observed for larvae from the DEL and combined AZA + DEL treatments 4 dph, though not in larvae from the AZA treatment. A single pulse exposure, delivered on the first day post hatch, caused similar effects on mortality and swimming activity 4 dph as the three-pulse exposure. Mortality was driven by the presence of DEL in both experiments, with no amplification or reduction of effects observed when DEL and AZA were combined. Larvae were observed for 13 days following the single pulse exposure, with food limitation introduced as an additional stressor on day 4. In the DEL and AZA + DEL treatments mortality continued to increase regardless of food level, with no larvae completing development to stage II. The overriding toxicity of DEL masked any potential effects the reduced food ration may have exerted. Swimming activity was lower for AZA treated larvae than Control larvae 13 dph, when both groups were fed daily, though no other significant changes to mortality, development to stage II, feeding rate or gene expression were observed. Food limited Control and AZA larvae had lower swimming activity and feeding rate than daily fed Control larvae, with expression of pyruvate kinase and myosin genes also downregulated. However, there was no negative effect on survival or successful development to stage II in these treatments. In addition, mesencephalic astrocyte-derived neurotropic factor was downregulated in food limited Control larvae when compared with the daily fed Controls.

Results from this study together with reported estimates of dispersion plume concentrations of discharged pesticides indicate that toxic concentrations of deltamethrin could reach shrimp larvae several kilometers from a treated salmon farm.

1. Introduction

Chemicals are used to treat farmed salmon against the crustacean ectoparasitic salmon louse (*Lepeophtheirus salmonis*). These chemicals end up in the coastal marine environment (Langford et al., 2014; Samuelsen et al., 2015). Despite the increasing use of non-chemical methods to remove sea lice from farmed salmon, several chemicals are still used extensively (Aaen et al., 2015; Burridge et al., 2014;

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Folkehelseinsituttet, 2019; Lillicrap et al., 2015). Farmed salmon are treated several times with the same or different types of chemicals or with combinations of chemicals (Grefsrud et al., 2018; Johannessen, 2017). Hence, non-target marine organisms living in areas with salmon aquaculture activity may be exposed repeatedly to a combination of chemicals. Limited data are available on pulsed exposure of chemicals and reports on repeated pulse or fluctuating exposures are rare (Dennis et al., 2012). Repeated short term exposures are likely to occur in Norway due to the number and proximity of fish farms and additive toxicity effects cannot be ruled out (Burridge et al., 2000, 2008; Langford et al., 2014). It is also possible that in certain scenarios, where several fish farms are treating simultaneously with different medications, that mixture effects may be observed. Furthermore, azamethiphos (AZA) and deltamethrin (DEL) have been used as a combination treatment for delousing salmon (Grefsrud et al., 2018; Johannessen, 2017), potentially exposing non-target organisms to treatment effluent that is already a mixture of active ingredients. Data are available regarding the toxicity of AZA and DEL to non-target species when exposed to a single compound (Burridge et al., 2014; Burridge and Van Geest, 2014; Urbina et al., 2019). These studies provide estimates of lethal thresholds and show, not surprisingly, that exposures of short duration require higher concentrations of the chemicals to produce the same level of toxicity. There has been only one study, however, where the effects of these two pesticides, in combination, have been investigated is Brokke (2015). Synergistic effects of AZA and DEL were documented for chameleon shrimp *(Praunus flexuosus)* and grass prawns *(Palaemon elegans)* (Brokke, 2015). Burridge et al. (2014) showed that the American lobster was more sensitive to DEL than the shrimp species tested by Brokke (2015), but all three species were far less sensitive to AZA than to DEL under the same exposure scenario. These studies show that there are interspecies differences in sensitivity, and it is therefore important to study other relevant non-target crustaceans, and life-stages, to determine their sensitivity to AZA and DEL separately and in combination. Data from such experiments can be used for accurate assessment of risk. The northern shrimp, *Pandalus borealis*, has been selected as test organism. Northern shrimp is a keystone species in the marine ecosystem and an important commercial crustacean species (Bergström, 2000), with high sensitivity to anti-parasitic chemicals used in salmon farms (Bechmann et al., 2017, 2018, 2019).

AZA and DEL are both neurotoxic (Burridge et al., 2014). AZA is an acetylcholinesterase inhibitor that acts by causing the neurotransmitter, acetylcholine, to accumulate, resulting in paralysis. DEL disrupts the sodium ion channel and the result is also paralysis. Therefore, it is important to investigate the swimming activity of shrimp larvae and their ability to catch live prey after exposure to AZA and/or DEL. Reduced ability to swim and catch prey during early development could affect development time and survival. Furthermore, the gene expression of selected transcripts based on their known or proposed involvement in feeding, motor and neuronal activity was compared to understand better the effects on swimming and feeding activity in the post exposure period (Bechmann et al., 2018). In the shrimp larvae experiments post exposure effects on survival and sublethal endpoints were investigated.

Burridge and Van Geest (2014) investigated potential effects on anti-parasitic compounds, and in their discussion, they raised the question of whether or not chemicals could induce a generalized stress response independent of (or in addition to) stress caused by the specific mode of action of the compound. They suggest that this, potentially added, stress could affect non-target organisms. Feeding and swimming are common endpoints investigated in studies of generalized stress responses. Couillard and Burridge (2015) showed that lobsters exposed to very low concentrations of AZA for a long period (10 days) survived, however, a significant percentage of these lobsters did not survive a subsequent stress (simulated shipping) when compared against controls. In the shrimp larvae experiments some larvae were kept under low food conditions (starvation) after exposure to AZA and DEL to investigate if short term chemical exposure affected their tolerance to natural stress. Larvae from different shrimp mothers were exposed separately to investigate if there was high genetic variability in tolerance to chemical (AZA/DEL) and natural stressors (low food). Therefore, the experimental design can be useful for estimating population level effects of stressors.

In this study the effects of pulsed exposures of planktonic larvae of *P. borealis* to AZA and DEL on mortality, swimming activity, developmental rate, feeding rate and gene expression are reported. Experiments were designed to address four questions:

1) What are the consequences of exposure of northern shrimp to either AZA or DEL in anti-louse formulations Salmosan® or Alpha Max® under our experimental conditions?
2) What are the immediate and delayed effects of exposure to a combination of AZA and DEL, compared to exposure to individual compounds?
3) Are there differences in sensitivity to chemical stressors between larvae from different females (genetic variability)?
4) Will a period of starvation (natural stressor) after exposure to AZA/DEL (anthropogenic stressors) cause added effects on shrimp larvae?

2. Materials and methods

2.1. Collection of shrimp

Northern shrimp (*Pandalus borealis)* were collected using a shrimp bottom trawl set at 100 m depth from Hillefjord, Norway (59° 04′ 00″ N, 5° 45′ 00′ E) in January 2017. To minimise damage to the shrimp, the net was modified to include a barrel (1 m x 1 m) at the cod end. Ovigerous shrimp were selected by hand from the catch on board the vessel and transferred to 50 L oxygenated seawater tanks, then transported to the laboratory within 2 hours (h) of capture. In the laboratory, shrimp were maintained in 500 L tanks fed with a continuous flow of sand filtered seawater pumped from a depth of 75 m from the adjacent fjord (Byfjord). A heat-exchanger maintained seawater temperature at 7 °C throughout the holding and experimental periods, with salinity remaining stable around 34‰. The ovigerous shrimp were held until larvae started hatching in March, with the larvae collected immediately after hatching as described below. Shrimp were fed 3 mm pellets of fish feed (Spirit supreme, Skretting, Norway) *ad libitum* throughout the holding period. All experimental procedures used were approved by the Norwegian Animal Research Authority (FOTS).

2.2. Experimental design

Shrimp larvae were exposed to short pulses of Alpha Max® and Salmosan® separately and in combination, followed by a post exposure period in clean seawater. The exposure concentrations were 2 ng/L deltamethrin (DEL) in the Alpha Max® formulation and 100 ng/L azamethiphos (AZA) in the Salmosan® formulation representing a 1000-fold dilution of the prescribed concentrations used to treat infestations of sea lice on Atlantic salmon[3] . The choice of concentration was based on the sensitivity of adult shrimp to AZA and DEL in experiments performed in our laboratory (Bamber et al., *in prep*.) and literature data on the effects of AZA and DEL to other crustacean species (Burridge and Van Geest, 2014; Urbina et al., 2019). The same dilution of Alpha Max® and Salmosan® was tested to simulate the treatment water discharged after combination treatment of salmon with these formulations.

Two experiments were performed, a three-pulse experiment, followed by a one-pulse experiment (Table 1). In the three-pulse experiment larvae were exposed to AZA, DEL and AZA + DEL for 2 hours (h)

[3] Summary of Product Characteristics (SPC) for Salmosan®: http://salmosan.net/no/preparatomtale/; and SPC for Alpha Max®: https://www.legemiddelsok.no/_layouts/15/Preparatomtaler/Spc/1999-08073.pdf

Table 1
Overview of exposure, feeding and effect parameters in the three-pulse and one-pulse experiments with shrimp larvae.

Day of experiment = Age of larvae	Exposure to AZA and/or DEL		Feeding		Effect parameters				
			Normal	Food Limited	Survival	Swimming activity	Stage I/II larvae	Gene expression	Feeding rate
The three-pulse experiment									
0	2 hours		x	No food limitation					
1	2 hours		x						
2	2 hours		x						
3	No exposure		x						
4			x		x	x			
The one-pulse experiment									
0	2 hours		x	x					
1	No exposure		x	x					
2			x	x					
3			x	x					
4	No exposure	No exposure and limited food	x	No food	x	x			
5			x						
6			x						
7			x						
8			x	x					
9			x	No food	x		x		
10			x						
11			x						
12			x						
13			No food		x	x	x	x	
14			Feeding test						x

on the first, second and third days post hatch (dph), with the experiment ended 2 days post exposure. Four replicate aquaria were used in the Control, AZA, DEL and AZA + DEL treatment. In the one-pulse experiment larvae were exposed to one 2 h pulse of the same treatments. Six replicate aquaria were used in the Control, AZA, DEL and AZA + DEL treatment. A 13 day post exposure period was included in the one-pulse experiment to investigate possible delayed effects (Weis, 2014).

Two weeks before the experiments started, ovigerous shrimp with eggs were placed in individual glass aquaria (volume 12 L, flow 140 ml/min, 7 °C. All aquaria were checked daily for larvae and when a sufficient number of larvae had hatched within a 24 h period, the experiments were started. All replicate tanks in both experiments were started with 70 shrimp larvae less than 24 h old. Larvae from individual females were used to provide one replicate for each of the 4 treatments (Supplementary Fig. 1). The shrimp larvae were fed freshly hatched *Artemia salina* nauplii and algae *(Thalassiosira weisslogi* 1200™, Microalgae, Vigra, Norway) as described in Arnberg et al. (2013). Food limitation was introduced as an add-on stressor 4 days post exposure in the one-pulse experiment. The aim was to investigate if the larvae that survived one pulse of AZA and/or DEL were less able to tolerate a natural stressor than control larvae (Pieters et al., 2006). Surviving larvae in each aquarium were divided between two aquaria; one aquarium was fed every day and the other was fed once (day 8) in the period from day 4–13 (Supplementary Fig. 1). From day 4 there were 8 treatments: Control, Food Limited Control, AZA, Food Limited AZA, DEL, Food Limited DEL, AZA + DEL and Food Limited AZA + DEL (Table 1, Supplementary Fig. 1).

2.3. Effect parameters

In the three-pulse experiment survival and swimming activity (Section 2.3.1) were documented for stage I larvae, 4 dph. In the one-pulse experiment, survival and developmental stage was determined at 4, 9 and 13 dph. All the larvae were siphoned out of each aquarium and into a glass bowl when determining survival and developmental stage. The surviving larvae were scored as active (normal) or weak. Active larvae were swimming when transferred to the glass bowl, while weak larvae were lying on the bottom of the glass bowl, only moving if disturbed by the flow of water from a pipette. Shrimp larvae reach stage II around day 9 at 7 °C (Bechmann et al., 2018). Stage I larvae have sessile eyes and stage II have stalked eyes (Rasmussen and Aschan, 2011). Swimming activity was investigated for stage I larvae 4 dph and for stage II larvae 13 dph. Feeding tests for stage II larvae were performed 14 dph after larvae had been starved for one day. Number of *Artemia* nauplii (prey) consumed per individual shrimp larvae per hour, was investigated as described in Arnberg et al. (2013). Gene expression was investigated for larvae exposed to AZA at optimal and low food levels and compared to corresponding controls (Table 1). The larvae used for analysis of gene expression were sampled 13 dph, snap frozen in liquid nitrogen and stored at −80 °C until they were sent to the University of Leicester, UK, for analysis (Section 2.3.2).

2.3.1. Swimming activity

Stage I larvae used in the swimming tests were sampled 4 dph in both experiments. Tests were performed with 2 × 5 larvae from each replicate in the three-pulse experiment and with 5 larvae from each replicate in the one-pulse experiment when sufficient numbers of live larvae were available. In addition, 5 stage II larvae from each aquarium were tested at the end of the post exposure period in the one-pulse experiment (13 dph).

Early larval stages of shrimp show positive phototaxy and this response to light is important in nature to place them close to their food in surface waters. The ability of larvae to swim towards light following their exposure to the various chemical treatments was assessed using a behavioural assay that continuously logged their movements in close proximity to a white light source. Swimming activity sensing units consisted of a pair of infrared light emitting diodes (LED) set at a right angle to one another and aligned with matching phototransistors within a drilled plastic block. For each assay 20 ml of seawater from the relevant treatment tank was added to a clear glass tube (16 cm length, 15 mm diam). 5 larvae from the treatment were then added. The top 20 mm of the glass tube was inserted through the centre of the sensor block at 90° to the path of the beams, with the lower portion of the tube suspended within a 5 L container filled with 7 °C seawater to buffer against temperature change during the one-hour duration of the test. A white light LED set within a drilled plastic block was positioned directly above the top of the glass tube. All tests were conducted in a constant low ambient light environment. A reduction in voltage output from the phototransistors signified breakage of the light beam by a swimming larva. A data logger (NI USB – 6009, National Instruments, Austin, TX, USA) was used to record all voltage changes, with these data processed using Microsoft Excel. Swimming responses in larvae following the various treatments are presented as mean total beam breaks per hour.

2.3.2. Gene expression

At the end of the post exposure period (13 dph), stage II larvae from all treatment groups (Supplementary Fig. 1) in the one-pulse experiment were sampled for analysis of gene expression. The expression of six transcripts selected from the annotated shrimp transcriptome (Bechmann et al., 2018) based on their known or proposed involvement in feeding, motor and neuronal activity was compared between the Control group and AZA, AZA Food limited and Control Food limited stage II larvae treatments using quantitative PCR (qPCR) as described in (Bechmann et al., 2018) and Supplementary Methods. These transcripts had significant sequence homology to genes encoding for the following proteins: pyruvate kinase, a glycolytic enzyme (Kayne and Price, 1973); cysteine-rich intestinal protein, an intestinal intracellular zinc transport protein (Hempe and Cousins, 1992); myosin regulatory light chain 2, part of the myosin complex involved in muscle contraction (Poetter et al., 1996), troponin C, part of the troponin complex essential for contraction in skeletal and cardiac muscle (Schreier et al., 1990); preproneuropeptide F1, a protein precursor of the neurotransmitter, neuropeptide F1 (Thongrod et al., 2017); and mesencephalic astrocyte-derived neurotropic factor, a protein involved in neuron maintenance (Palgi et al., 2009).

2.4. Chemical exposure

Stock solutions of DEL and AZA were prepared in 2 L Schott bottles using the commercial formulations Alpha Max® and Salmosan® and distilled water (Supplementary Fig. 2). The stock solutions were placed on magnetic stirrers. In the Alpha Max® stock solution the nominal concentration was 280 ng/L DEL, and in the Salmosan® stock solution the nominal concentration was 14 µg/L AZA. During the 2 h exposure pulse the stock solution was pumped through Teflon tubing into the seawater inlet to each exposure aquarium by a peristaltic pump with a multi channelling system (model 520, Watson and Marlow, Cornwall, UK). Seawater was gravity fed to each aquarium from a header tank. The mean measured flow of seawater and stock solution of AZA and DEL into each aquarium was 140 mL/min and 1 mL/min, respectively. Water samples from the aquaria were frozen and stored until chemical analysis of DEL and AZA was performed. In addition, freshly prepared samples of DEL solution from the header flasks were analysed due to very low levels of DEL detected in the water samples from the aquaria.

2.5. Analytical chemistry

2.5.1. Reagents and chemicals

Standards of azamethiphos, d5-atrazine and deltamethrin as well as HPLC grade acetonitrile, formic acid, acetic acid, ammonium acetate, sodium sulphate, sodium acetate, zinc chloride, Supelclean PSA sorbent and florisil (SPE-FL) column were purchased from Sigma-Aldrich (Steinheim Germany). Cyclohexane, ultra rezi- analysed, were obtained from J.T.Baker (Avantor, Poland). HPLC grade diethylether, iso-hexane, dichloromethane (DCM), 2-propanol and acetone were obtained from Rathburn Chemicals (Walkerburn Scotland). The d6-cyfluthrin was obtained from LGC Standards (Wesel, Germany), Costar nylon Spin-X filters were obtained from Corning (Salt Lake City USA). Standard stock solutions were prepared in acetone and diluted further to appropriate concentrations with acetonitrile or cyclohexane. All standard solutions were kept in the dark at +4 °C.

2.5.2. Chemical analysis of deltamethrin

Internal standard, d6-cyfluthrin, was added to 150–200 mL seawater samples and extracted with 30 mL of dichloromethane, for one hour under magnetic stirring. Sodium sulphate was added to the extracts to remove water and the extracts were then concentrated using nitrogen flush and transferred to 0.25 mL cyclohexane prior to the gas chromatographic analysis. Three blank samples and one spiked sample were analysed alongside the seawater samples as part of the quality assurance. The analysis was performed using an Agilent 7890BN GC system equipped with 30 m DB-5MS columns, i.d. 0.25 mm and 0.25 µm film thickness and Electron Capture detector. The identification and quantification were performed using external and internal standards. The recovery of the spiked seawater sample (2.5 ng/L) was 81 %. The limit of detection was 0.1 ng/L for the water samples.

2.5.3. Chemical analysis of azamethiphos

Internal standard, d5-atrazine, was added to 200 mL seawater samples and extracted with 30 mL of dichloromethane, for one hour under magnetic stirring. Sodium sulphate was added to the extracts to remove water and the extracts were then concentrated using nitrogen flush and transferred to 0.5 mL 40 % ACN in water.

A 200 mL sample of seawater was spiked with 10 ng d5-atrazine and shaken with 50 mL DCM. The DCM extract was evaporated to dryness and resolved in 1 mL of 1 + 1 ACN and water, filtered and injected into the LC–MS as described. Azamethiphos was analysed on a Waters Acquity UPLC system connected to a Quattro Ultima triple quadrupole mass spectrometer. Separation was achieved with a Waters BEH C8 column (2.1 × 100 mm) column using a gradient elution with ACN and water (with 0.1 % formic acid). Azamethiphos and the internal standard d5-atrazine were detected in positive ESI mode with mass transitions 325-139, 325-183 and 221-179 respectively. The average recovery of three spiked seawater samples was 95 % with RSD (relative standard deviation) of 2.8 % for azamethiphos, while the average recovery of three tissue samples was 92 % with RSD of 3.1 %. Limit of detection was 1.0 ng/L and 1.0 ng/g (w.w.)

2.6. Statistical analysis

All data analyses were performed in v 23 SPSS® (IBM, Chicago, USA), using the Wilcoxon Rank sum test. Levels of effect observed after exposure to either of the formulations or both of the formulations were compared statistically to levels of effect observed to untreated controls handled in the same manner. The criterion for significance was set at $p < 0.05$. REST software (Pfaffl et al., 2002) was used for statistical analysis of relative mean gene expression ratios, which uses randomisation tests with a pair-wise reallocation that makes no assumptions about the distribution of observations in populations. The REST software was used to perform 2000 random allocations to determine if the results were due to chance or to the effects of the treatment, with differences considered to be significant at $p < 0.05$.

3. Results

3.1. Physical environment and exposure concentrations

Mean seawater temperature was 6.9 °C (SD: 0.1), with mean oxygen saturation at 91.6 % (SD: 0.6) throughout the experiments. The mean salinity in the inlet water was 34.3‰ (SD: 0.2). The mean measured concentrations of azamethiphos in water samples from the aquaria corresponded well to the target concentration of 100 ng/L. In the AZA aquaria the mean concentration was 87.9 ng/L (SD: 15.7, n = 6) and the mean concentration in the AZA + DEL aquaria was 131.5 ng/L (SD: 18.8, n = 6). The concentration of azamethiphos in the control was less than 0.1 ng/L. The target concentration of deltamethrin in the aquaria was 2 ng/L. The concentration of deltamethrin was below the detection limit (< 0.1 ng/L) in 7 of the 12 samples analysed from the aquaria receiving exposure to DEL or DEL + AZA, and less than 0.2 ng/L in the remaining 5 samples. The uncertainty associated with measuring low concentrations of DEL is discussed in Section 4.1. New samples of the header solution (Supplementary Fig. 2) were prepared and analysed to check the concentration in the water pumped into each aquarium during the pulsed exposure. The target concentration in the header was 280 ng/L deltamethrin, and the mean measured concentration in samples from the header was 194 ng/L (SD: 20 ng/L, n = 9).

3.2. Effects of one vs three pulses of exposure

One pulse and three pulses of exposure to AZA and/or DEL caused similar effects in shrimp larvae 4 dph (Fig. 1). The percentage mortality and the percentage weak surviving larvae 4 dph was significantly increased in the DEL and AZA + DEL treatments in both experiments (Fig. 1A, Wilcoxon, $p < 0.05$). The swimming activity (ability to respond to light) of larvae exposed to DEL and AZA + DEL was significantly reduced (Fig. 1B, Wilcoxon, $p < 0.05$). There was no significant effect on larvae exposed to AZA in any of the experiments (Fig. 1A, B, Wilcoxon, $p > 0.05$), although there was a tendency to negative effect in the three-pulse experiment. Larvae exposed to AZA in the three-pulse experiment had lower mortality and less weak larvae than DEL and AZA + DEL, but the difference was only significant for AZA *vs* DEL (Supplementary Table 2). In the one-pulse experiment there was significantly lower mortality and less weak larvae in AZA than in DEL and AZA + DEL (Supplementary Table 3). There was no significant difference in percentage mortality or percentage weak larvae between DEL and AZA + DEL in the three-pulse experiment (Supplementary Table 2). All the 6 replicate batches of larvae exposed to DEL and AZA + DEL in the one-pulse experiment had high mortality in the 13 d

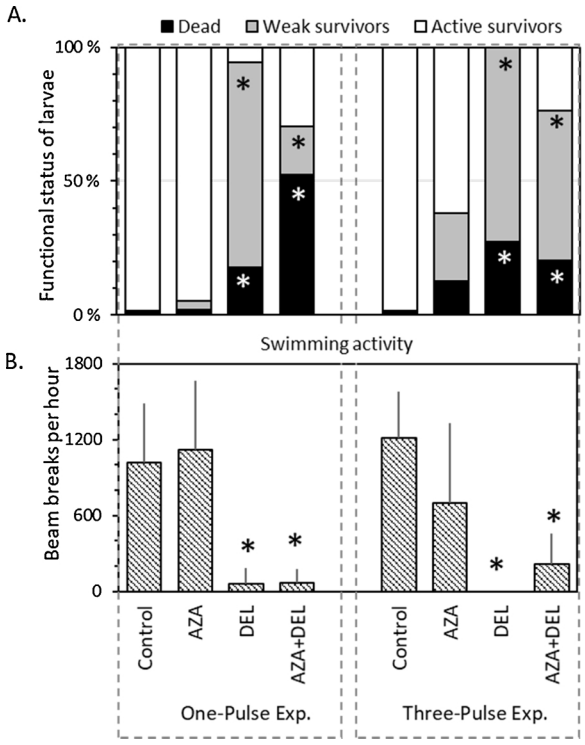

Fig. 1. Effects on survival and swimming activity of shrimp larvae exposed to AZA and/or DEL in the one-pulse and three-pulse experiment 4 dph. A: Percent dead, weak and active shrimp larvae. B: Swimming activity, beam breaks per hour (mean + SD). The * shows treatments that are significantly different from the corresponding control, Wilcoxon rank sum, $p < 0.05$.

post-exposure period, hence the higher percentage of weak larvae in DEL than in AZA + DEL day 4 was not relevant for long-term survival (Supplementary Table 3, Fig. 3).

3.3. Long term effect of one pulse of exposure

The three-pulse experiment was ended 4 dph, but the post exposure period in the one-pulse experiment was extended to 13 dph and food limitation was introduced as an add-on stressor (see Section 2.1 and Supplementary Fig. 1). Survival, swimming activity, development, feeding and gene expression were investigated.

3.3.1. Survival and development

Two hours exposure to AZA the first day after hatching and/or limited food conditions 4–13 dph did not significantly affect survival of larvae or development from stage I to II larvae (Table 2, Fig. 2 and 3). There was, however, a clear negative effect on survival and larval development in the DEL and AZA + DEL treatments. Survival was significantly lower than in the control, and none of the larvae developed to

Table 2
Percent survival in treatments with and without food limitation. Food limited larvae were fed *Artemia* only once in the period 4-13 dph.

Treatment	Percent survival (mean ± SD)			
	Fed daily		Food limited	
	4-9 dph	4-13 dph	4-9 dph	4-13 dph
Control	99 ± 2 n = 6	93 ± 6 n = 6	97 ± 5 n = 6	93 ± 6 n = 6
AZA	96 ± 6 n = 6	92 ± 5 n = 6	97 ± 4 n = 6	92 ± 9 n = 6
DEL	17 ± 21 n = 5[1)]	0 n = 1[1)]	15 ± 29 n = 5[1)]	36 n = 1[1)]
AZA+DEL	57 ± 18 n = 3[1)]	[1)]	55 ± 25 n = 3[1)]	[1)]

[1)]All treatments were started with 6 replicates. For animal welfare reasons replicates with high mortality were ended 4 and 9 dph.

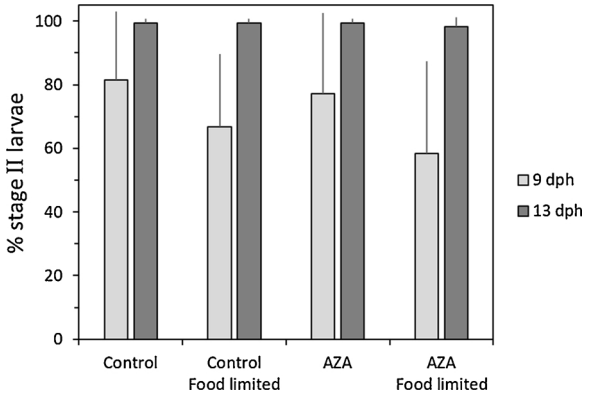

Fig. 2. Percentage stage II shrimp larvae 9 dph and 13 dph (mean + SD). None of the larvae in the DEL and AZA + DEL treatment reached stage II.

stage II in these treatments (Table 2, Fig. 3, $p < 0.05$, Wilcoxon). Since there was no significant difference in survival between aquaria with and without food limitation, survival data was combined in Fig. 3. One DEL replicate and three AZA + DEL replicates were ended 4 dph due to high mortality. Furthermore, four DEL replicates and the remaining AZA + DEL replicates were ended 9 dph for the same reason (Fig. 3). Comparison of survival 9 dph is based on the DEL and AZA + DEL replicates with highest survival 4 dph (Table 2). Fig. 3 also shows that offspring from the 6 females used to start the experiment had similar sensitivity to the chemicals (Fig. 3). At 9 dph the cumulative mortality had reached a minimum of 42 % in all DEL and AZA + DEL aquaria compared to a maximum of 12 % in the Control and AZA aquaria. Since mortality was high and none of the survivors reached stage II in the DEL and AZA + DEL treatments it was not possible to investigate swimming, feeding and gene expression in stage II larvae at 13 dph.

3.3.2. Swimming and feeding

The swimming activity test performed with stage II larvae 13 dph documented significantly lower swimming activity for food limited control larvae and for larvae from the AZA treatment with and without food limitation compared to control larvae that were fed every day (Fig. 4A, Wilcoxon, $p < 0.05$). The fed control larvae had approximately 60 % higher swimming activity than the other treatments.

Control and AZA larvae that had been fed daily had approximately twice as high feeding rate 14 dph as food limited larvae (Fig. 4B, Wilcoxon, $p < 0.05$). The AZA exposure on day zero did not affect feeding rate two weeks post exposure.

3.3.3. Gene expression

Quantitative PCR performed on 13 dph stage II larvae from the one-pulse experiment showed that the transcripts *pyruvate kinase* and *myosin regulatory light chain 2* were significantly downregulated in the food limited Control and AZA larvae relative to the Control larvae (Fig. 5). In addition, the transcript, *mesencephalic astrocyte-derived neurotropic factor*, was significantly downregulated 1.6 fold (Randomisation test, $p < 0.05$) in only the food limited Control larvae. AZA did not affect expression of the selected transcripts.

4. Discusssion

The experiments conducted in this study were designed to address several environmentally relevant questions associated with potential exposure of a non-target organism, the northern shrimp, to anti-parasitic pesticides. The experiments were conducted using the commercial formulations of these products, Alpha Max® for DEL and Salmosan® for AZA. A range of endpoints were investigated covering whole-organism responses as well as molecular endpoints such as gene expression. The results reported here show that shrimp larvae are very sensitive to a

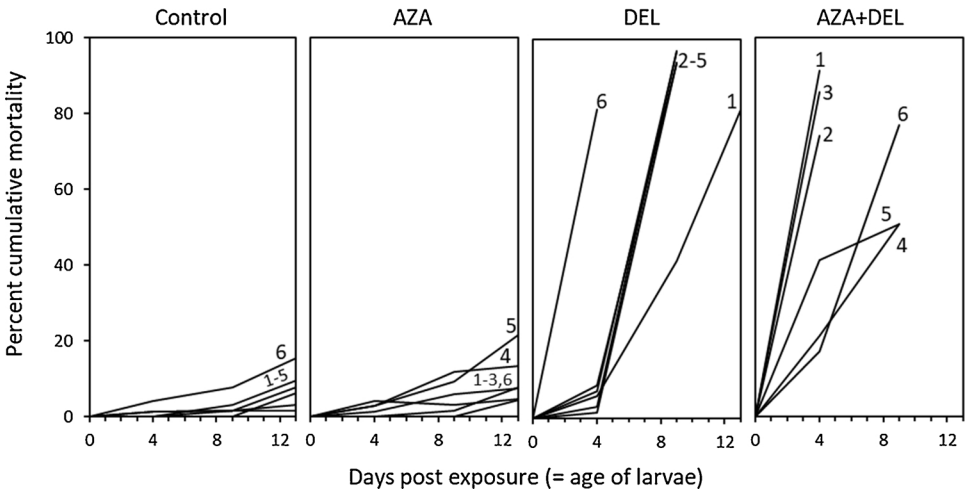

Fig. 3. Cumulative mortality post exposure for shrimp larvae in the 6 replicate aquaria in each treatment. The larvae were exposed for 2 h on day 0, followed by a 13 d post exposure observation period in clean seawater. The larvae in replicates with the same number next to the graph have the same mother. Data from aquaria with fed and food limited larvae are combined. For details see Section 2.2, Supplementary Fig. 1 and Table 2.

1000-fold dilution of the concentration of Alpha Max® used in the treatment of farmed salmon. No effect on survival was detected for shrimp larvae exposed to the same dilution of Salmosan®.

4.1. Exposure concentrations

AZA was recovered and measured at levels consistent with the estimated nominal concentration in the aquaria, but the concentration of DEL was below or close to the level of quantification in the frozen and stored samples from the aquaria. Analysis of DEL in freshly prepared samples of stock solution from header flasks documented that the concentration of DEL pumped into the aquaria during the exposure pulse was close to the nominal concentration. Fairchild et al. (2010) state that aged water samples showed a significant loss of deltamethrin after 48 h. The authors do not discuss how the samples were stored but this result may indicate what happened to the DEL samples in our study. The concentration of DEL measured in samples from header flasks and the fact that organisms were affected strongly indicate that DEL was present, however not detected in all samples. A more conservative interpretation would be that effects are being observed at concentrations of DEL that are below the level of quantification. Ernst et al. (2014) discuss problems they had in measuring DEL in seawater during field testing and lab-based studies. They consistently measured much lower concentrations of DEL than would be estimated during toxicant preparation. They had no explanation for this. These authors also filtered water samples to separate bound *vs* free DEL. Not surprisingly, they found more DEL on the filter than in filtered water indicating that DEL was being bound to organic material in the water.

4.2. Effects of DEL and AZA + DEL

Synergistic effects of AZA and DEL have been documented for chameleon shrimp and grass prawns (Brokke, 2015). In the current study, however, mortality was driven by the presence of DEL with no amplification or reduction of effects observed when DEL and AZA were combined. After one 2 h pulse of exposure mortality increased with time and reached 50–100 % in all the DEL and AZA + DEL replicates during the 13 d post exposure period. Furthermore, none of the surviving DEL or AZA + DEL larvae had reached stage II 13 dph, indicating that long term survival may be zero after 2 h exposure to DEL or AZA + DEL. Effects arising from food limitation were difficult to detect in DEL and AZA + DEL treated larvae, with toxicity of DEL overriding any potential chronic influence of food ration reduction.

When adult *P. borealis* were exposed for 2 h to 6 ng/L DEL almost

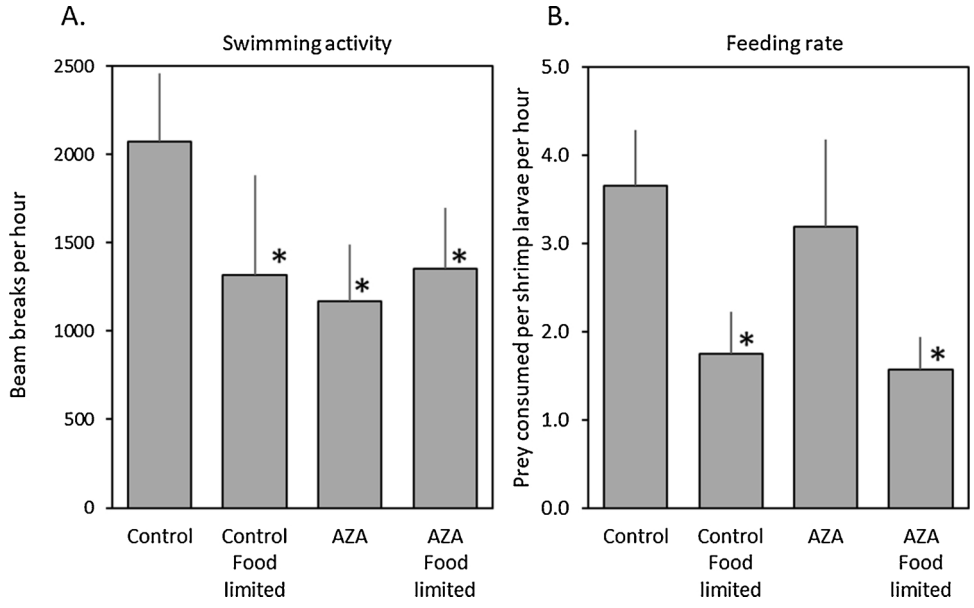

Fig. 4. Swimming activity and feeding rate for stage II larvae. A: Beam breaks per hour indicating swimming activity. B. Number of *Artemia* nauplii (prey) consumed per shrimp larvae per hour in the feeding test. Mean plus SD, n = 6, (*): significantly different from the corresponding control, Wilcoxon rank sum, $p < 0.05$.

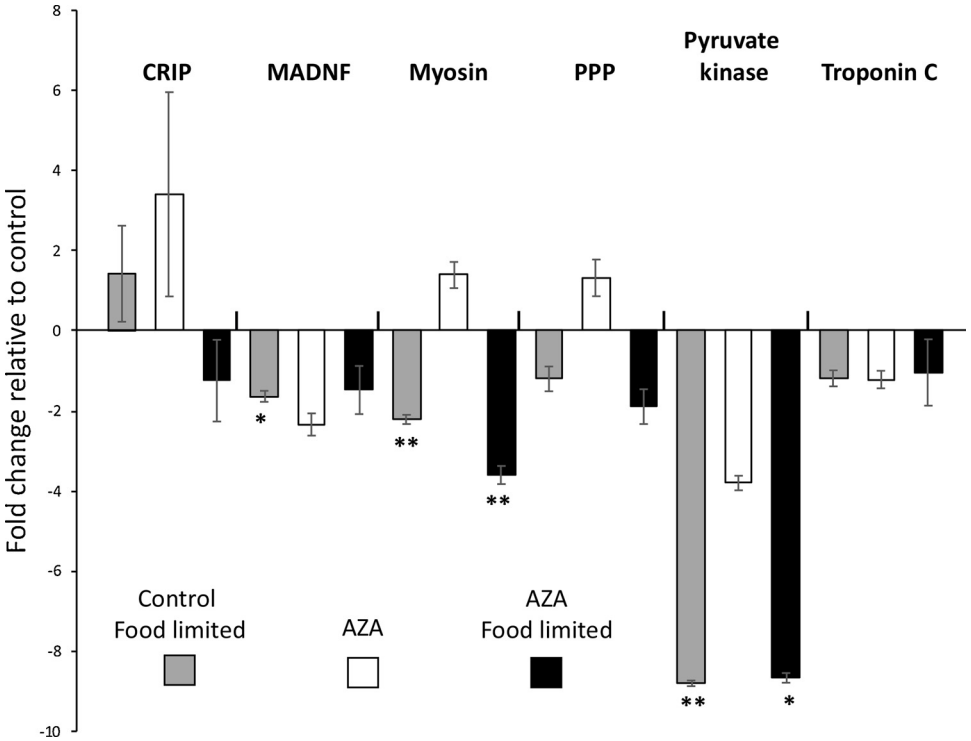

Fig. 5. Effect of azamethiphos on gene expression in shrimp larvae fed either low or high food relative to control shrimp larvae (unexposed/high food). CRIP: *cysteine-rich intestinal protein*; MADNF: *mesencephalic astrocyte-derived neurotrophic factor*; Myosin: *myosin regulatory light chain 2*; PPP: *preproneuropeptide F1*. Gene expression is represented as mean (n = 6) relative expression ratios (means ± SE). Significant differences are represented by asterisks: * = $p < 0.05$, ** = $p < 0.01$ (Randomisation test).

100 % mortality was observed a few days after the exposure (Frantzen et al., 2019). Experiments performed in our lab documented high mortality of adult *P. borealis* exposed to 2 ng/L DEL for 24 h (Bamber et al., *in prep*.). High sensitivity to Alpha Max® (DEL) has also been documented for other crustaceans (Brokke, 2015; Burridge et al., 2014; Fairchild et al., 2010; Gebauer et al., 2017; Van Geest et al., 2014a, b). Stage I lobster larvae *(Homarus americanus)* were particularly sensitive to DEL. Burridge et al. (2014) reported that LC50 was 3.4 ng/L after 1 h exposure and 95 h recovery. When chameleon shrimp and grass prawns were exposed to DEL, they were less sensitive than *P. borealis* and *H. americanus* (Brokke, 2015; Burridge et al., 2014). This could be due to species or life stage differences in sensitivity to DEL, or to the longer post exposure observation period included in the lobster experiment (1 h, 95 h recovery) and the current shrimp larvae experiment (2 h exposure, 13 d recovery) than in the chameleon shrimp and grass prawns experiments (1 h or 24 h exposure, 24 h recovery). Brokke (2015) describes increased mortality during the 24 h immediately post exposure and Burridge and Van Geest (2014) observed delayed mortality in Stage I lobster larvae exposed to DEL. They report an LT50 (time to 50 % mortality) and in all cases where 50 % mortality occurred the estimate was greater than 24 h indicating that a single exposure may have significant negative impacts. Delayed mortality after short-term DEL exposure was also observed in crab *Metacarcinus edwardsii* larvae (Gebauer et al., 2017) and the amphipod *Echinogammarus finmarchicuss* (Van Geest et al., 2014b). Furthermore, delayed effects have been reported for *P. borealis* exposed to the anti-parasitic chemical Paramove® with hydrogen peroxide as the active ingredient (Bechmann et al., 2019) and for *P. borealis* exposed to oil (Arnberg et al., 2019). These results emphasize the importance of observing the test organisms for several days post-exposure to detect delayed effects that are relevant for evaluating potential environmental effects of chemical discharges.

One or three pulses of DEL and AZA + DEL resulted in similar responses; reduced survival and swimming activity 4 dph, indicating that a single 2 h exposure to this concentration is more than shrimp larvae can tolerate. Burridge and van Geest (2014) reported preliminary results of repeated exposures of adult American lobsters to ~20 ng/L DEL. Interestingly, a higher number of exposures (n = 6) of a shorter duration (30 min) resulted in only 10.5 % cumulative mortality. These data suggest that lobsters may be able to tolerate repeated exposures to DEL at this concentration if the exposure is short. It is also possible that shrimp larvae may tolerate 30 min exposures better than the 2 h exposures tested in the current experiments.

Use of the aquaculture formulation of DEL (Alpha Max®) ensures relevance. However, it also raises the question of the effect, if any, of the formulation ingredients. The Alpha Max® solution was 5 million times diluted in the aquaria in the current experiments (10 mg/L DEL in Alpha Max®, 2 ng/L in the aquaria). It is unlikely that N-methyl-pyrrolidone or any of the other additives listed in the Summary of Product Characteristics (SPC) for Alpha Max® could have contributed to the toxicity observed in the shrimp experiments based on toxicity information available from The European Chemicals Agency (ECHA) and the scientific literature. (Fairchild et al., 2010) state that their estimates of lethal thresholds for Alpha Max® with shrimp or lobsters are comparable to thresholds for technical grade deltamethrin estimated by (McLeese et al., 1980) and (Zitko et al., 1979). These authors tested several pyrethroids in the technical grade form and found them to be very toxic, indicating that the toxicity of Alpha Max® is caused by deltamethrin.

It can be concluded that the no effect level for early life stages of northern shrimp is less than 2 ng/L DEL (nominal concentration). The measured concentration of DEL in the exposures was consistently near or below the level of quantification raising the question of whether the effects are the consequence of much lower than predicted concentrations or if there is an analytical problem with detecting and measuring DEL at low concentrations. Measured concentration of DEL in the stock delivery system was very close to predicted. A range of DEL concentrations would need to be tested to determine a no effect level.

4.3. Effects of AZA

Three 2 h pulses of 88 ng/L AZA did not significantly reduce survival or swimming activity of shrimp larvae. After one pulse of exposure to the same concentration there was no post-exposure effects on survival, swimming activity of stage I larvae, development to stage II

larvae, feeding rate for stage II larvae or gene expression of the selected genes. The swimming activity for stage II AZA larvae was, however, lower than in the Control. The feeding test documented that there was no reduction in the number of live prey caught by stage II Control and AZA larvae. Furthermore, the swimming activity for stage II AZA larvae was higher than for stage I Control larvae. Compared to the > 90 % reduction of swimming activity for DEL and AZA + DEL stage I larvae, the effect on stage II AZA larvae was moderate. It is unclear without further investigation why the stage II, but not the stage I larvae had reduced swimming activity in the AZA treatment. One possible explanation is that exposure to AZA induced a delayed effect on development, *e.g.* in skeletal form that caused decreased swimming ability. Negative effect on swimming activity has been reported for crab larvae after 30 min exposure to higher concentrations of AZA (≥ 10 µg/L) (Gebauer et al., 2017).

The results from the current experiments suggest that the no effect concentration for AZA is equal to or greater than 88 ng/L AZA (mean measured concentration in the aquaria). This is consistent with results reported by (Burridge et al., 2014). These authors exposed various larval stages plus adult lobsters *(H. americanus)* to a range of concentrations of AZA, including levels higher than reported in this study, for only an hour with insufficient deaths to calculate an LC50. One hour of exposure to AZA at concentrations as high as 85 µg/L, resulted in no mortality (Burridge et al., 2014). Copepods also appear to tolerate high concentrations of AZA. No effect on feeding rate, immobility or survival was recorded for copepods exposed to several orders of magnitude higher concentrations of AZA than tested in the current experiment (Van Geest et al., 2014a). On the contrary, survival of crab larvae (*Metacarcinus edwardsii*) was reduced after 30 min exposure to 62 ng/L AZA per day for 7 days (Gebauer et al., 2017). These results show that it is important to investigate the sensitivity of several species to evaluate the potential environmental effects of discharging chemicals used to treat salmon against lice.

Four days post exposure in the one-pulse experiment, food limitation was introduced as an add-on stressor. Larvae that survived one pulse of AZA responded in the same way to periods of starvation as control larvae: there was no significant effect on survival or development rate compared to the corresponding larvae fed daily. There was, however, a significant reduction of swimming activity and feeding rate in starved stage II Control and AZA larvae 13 dph compared to daily fed Control larvae. Two gene transcripts were also significantly down regulated in the food limited AZA and Control larvae relative to Control larvae (*pyruvate kinase* and *myosin regulatory light chain 2*) while *mesencephalic astrocyte-derived neurotrophic factor* was significantly down regulated only in food limited Control larvae. This investigated gene transcription, but post-transcriptional and post-translational modifications can also alter protein abundance. The effects of food limitation identified at the transcript level are, however, supported by the swimming and feeding observations. The *pyruvate kinase* transcript and *myosin regulatory light chain 2* transcript are involved in metabolism and motor activity respectively (Kayne and Price, 1973; Poetter et al., 1996), and were significantly down regulated in food limited Control and AZA larvae which agrees with the reduction in feeding and swimming in the 13 dph stage II larvae as described above. Palgi et al. (2009) found that in the fruit fly (*Drosophila melanogaster*), the gene *mesencephalic astrocyte-derived neurotrophic factor* was crucial for the maintenance of dopaminergic neurons and dopamine levels, and it is known that dopamine plays an important role in movement (Chinta and Andersen, 2005), so the down regulation of this gene in food-limited control larvae fit into the observations above.

It is likely that the larvae from the low food treatments were stressed and weak due to lack of food and thus not as active with respect to swimming and feeding when food was reintroduced.

Periods of starvation during early development may lead to long term negative effects on survival. It has been shown that starved shrimp larvae reach a "point of no return" after which, even though provided with adequate food, they will not survive (Shumway et al., 1985). On the contrary, feeding rate for *P. borealis* larvae starved for up to 6 days after hatching was not significantly affected when food was introduced, suggesting that *P. borealis* larvae were resistant to starvation (Nunes, 1984). In the "low food" treatments in the current experiment larvae received food the first 4 days after hatching but were fed only once in the period from 4 to 13 dph. Different timing and length of the starvation period may explain the difference in effect on feeding rate in the current experiment and (Nunes, 1984). AZA treated and control larvae showed similar responses to reduced food, with reduction in swimming activity and changes in gene expression when compared with daily fed Control shrimp. Both reduced food groups did however successfully develop to stage II without significant mortality.

Couillard and Burridge (2015) reported that long term exposure of adult lobsters to a low concentration of AZA (61 ng/L) did not result in significant mortality compared to controls. However, when treated lobsters were subjected to conditions simulating live transport a significant number of lobsters died. These results are not consistent with those reported for the larval shrimp in this experiment. This could be attributable to the nature of the "added" stressor or to the duration of the exposure. Feeding is a natural behaviour, and periods of lack of food may be normal. Simulated transport is definitely a man-made stress. In addition, adult lobsters have a fully developed nervous system that may be more susceptible to AChE inhibition than Stage II shrimp larvae.

4.4. Effects of DEL: relevance for the field

The concentrations of pesticides in the area around the salmon farm will depend on local hydrodynamic conditions and the degradation time for the chemical, in addition to the quantity of medicines used and the frequency of treatments (Page and Burridge, 2014). Results from field experiments with fluorescent dye added to the treatment water and dispersion modelling using different types of models indicate that the dispersion plume may contain 100–1000 times diluted concentrations of chemicals for several hours at distances several kilometers away from the discharge point (Brokke, 2015; Ernst et al., 2001; Page et al., 2015; Page and Burridge, 2014; Refseth and Nøst, 2018). These results together with the current experiments indicate that toxic concentrations of deltamethrin could reach *P. borealis* larvae living several kilometers away from a treated salmon farm.

In addition to overt mortality, the irreversible, delayed effects of 2 h DEL exposure are ecologically important. Surviving larvae that do not moult through subsequent larval stages are essentially dead from an ecological perspective. Similarly, reduced swimming ability may have negative consequences. Any organism trapped within the effluent plume can be expected to be exposed constantly as the plume moves away from the treatment cage. Stationary organisms or those that are able to swim out of the plume will be exposed for significantly shorter periods of time, depending on current speeds.

Large volumes of salmon treatment water containing pesticides are discharged to the coastal marine environment in Norway each year (Supplementary Table 4). Although several orders of magnitude more hydrogen peroxide than DEL has been used in Norway over the last 10 years, the volumes of DEL treatment water discharged to the environment has been twice as high as that recorded for hydrogen peroxide (Supplementary Table 4)(< opt_COMMENT > Author has rejected a copyediting change here. "." is retained..Folkehelseinstituttet, 2019). The reason is that the dose needed to treat salmon with H_2O_2 is 750 000 times higher than the treatment concentration of DEL (link to the SPCs for Alpha Max® and Salmosan® in Section 2.2).

Many salmon farms in Norway are placed close to shrimp fishing areas according to maps available on the web page of the Directorate of Fisheries (https://kart.fiskeridir.no/). Furthermore, it has been documented that *P. borealis* consume spilled salmon feed (Olsen et al., 2012). Hence the high sensitivity of shrimp to both DEL (current experiments), H_2O_2 (Bechmann et al., 2019) and medicated feed (Bechmann et al.,

2017, 2018) indicate that there is a potential for negative consequences for shrimp populations in areas with frequent use of chemical de-lousing.

Contributors

RKB designed the study, participated in the practical work in the lab and is the main writer of this manuscript with the help of **MA** who participated in the design, practical work, analyses of the data and writing. **SB** designed and performed the swimming tests and gave input to the manuscript. **SW** was responsible for the exposure system and read and commented on the manuscript. **EL** contributed to the daily practical work with the lab experiments and read and commented on the manuscript. **JTR** and **AK** performed the AZA and DEL analysis and gave input to the manuscript. **PS** analysed gene expression in the shrimp larvae, including data treatment and gave input about the results to the manuscript. **LB** contributed to writing the manuscript. **LB** is scientific advisor in the PestPuls#267746/E40 project funded by the Research Council of Norway, and **RKB** is project leader for PestPuls.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aquatox.2020.105453.

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