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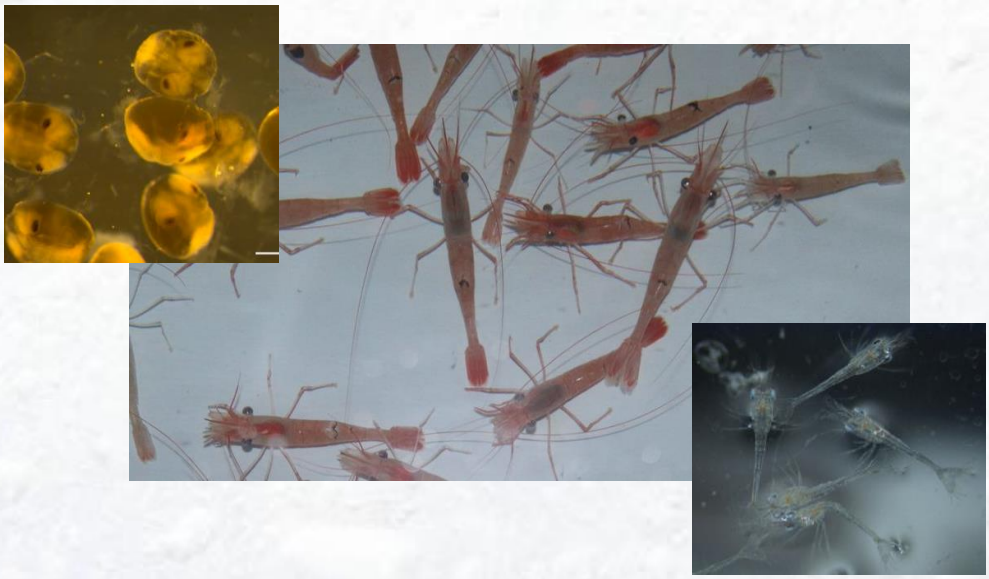
Rapport Report

Effects of hydrogen peroxide, azamethiphos and deltamethrin on egg-carrying shrimp (*Pandalus borealis*)



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Effects of hydrogen peroxide, azamethiphos and deltamethrin on egg-carrying shrimp (*Pandalus borealis*)

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Summary
The current study was designed to investigate effects of hydrogen peroxide, azamethiphos and deltamethrin in highly diluted concentrations on the non-target species, deep water shrimp (*Pandalus borealis).* Both mortality and sub-lethal effects (behaviour, embryo development, and reproductive output) were studied for each chemical alone and in different sequential combinations. Generally, our experiments show that the deep water shrimp is sensitive to delousing agents, as some of the bath treatments kills deep water shrimp at environmentally realistic highly diluted treatment concentrations. The most severe effect was observed for deltamethrin where 2 h exposure to 330 times diluted treatment dose (alone and in sequential use with hydrogen peroxide and azamethiphos) induced ~100% mortality within few days after exposure. Similar effects were not observed for hydrogen peroxide and azamethiphos. However, sequential treatment with hydrogen peroxide and azamethiphos (2 h exposure to each treatment chemical; 500 times diluted treatment dose) resulted in >50% mortality during the first week following treatment. No sub-lethal effects or loss of eggs in female shrimp could be related to exposure to the bath treatments.

Project manager

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Preface

In this project, impacts of three chemotherapeutants, used in bath treatments against sea lice, have been tested on egg-carrying deep water shrimp (*Pandalus borealis*).
The work has focused on ecotoxicological experiments and has produced important data for risk assessment of bath treatments. Three chemotherapeutants, hydrogen peroxide (H_2O_2), azametiphos (AZA), and deltametrin (DEL) have been included in the project.

Bjørn Munro Jenssen, Norwegian University of Science and Technology (NTNU), Kjetil Hylland, University of Oslo, and Anders Goksøyr, University of Bergen (UiB) has constituted a reference group for the project. We wish to thank the reference group for valuable input to the project.

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Trondheim 29.04.2019

Gro Harlaug Refseth
Project manager

1 Summary

Hydrogen peroxide, azamethiphos and deltamethrin are chemicals currently used in the Norwegian aquaculture industry to control sea lie on farmed salmon, and the chemical-laden treatment water is released to the surrounding marine environment by the end of the treatment time.

Several recent studies have shown that exposure to bath-treatment chemicals can cause increased mortality also in non-target species. The current study was designed to investigate effects of the bath treatments at highly diluted concentrations on the non-target species, deep water shrimp (*Pandalus borealis).* Both mortality and sub-lethal effects (behaviour, embryo development, and reproductive output) were studied for each chemical alone and in different sequential combinations. Three experiments, where egg-bearing shrimp was exposed to bath-treatments, were performed.

Generally, our experiments show that the deep water shrimp is sensitive to delousing agents, as some of the bath treatments kills deep water shrimp at environmentally realistic highly diluted treatment concentrations. However, the sensitivity varied between treatment chemicals. The most severe effect was observed for deltamethrin where 2 h exposure to 330 times diluted treatment dose (alone and in sequential use with hydrogen peroxide and azamethiphos) induced ~100% mortality within few days after exposure. Similar effects were not observed for hydrogen peroxide and azamethiphos. However, sequential treatment with hydrogen peroxide and azamethiphos (2 h exposure to each treatment chemical; 500 times diluted treatment dose) resulted in >50% mortality during the first week following treatment. No sub-lethal effects or loss of eggs in female shrimp could be related to exposure to the bath treatments.

Our results indicate that delousing routines in the aquaculture industry using deltamethrin probably will result in higher mortality of female deep water shrimp compared to hydrogen peroxide and azamethiphos. Different combinations of chemicals also result in higher mortality than use of single compounds. Non of the sub-lethal enpoints that were studied in this project could be related to treatment, but future studies should investigate potential sub-lethal effects at exposure concentrations close to the no effect concentration.

2 Sammendrag

Hydrogenperoksid, azametifos og deltametrin er kjemikalier som benyttes i norsk havbruksnæring som behandling mot lakselus. Midlene brukes som bademidler (tilsettes i vann) og etter avlusning slippes behandlingsvannet ut til marint miljø.

Flere studier har vist at bademidlene kan føre til økt dødelighet også hos andre marine arter enn lakselus. I prosjektet som rapporteres her var målsetningen å undersøke effekter av lave konsentrasjoner av de ovenfor nevnte bademidler på dypvannreke (*Pandalus borealis*). Både dødelighet og sublethale effekter (bl.a. oppførsel, fekunditet og embryoutvikling) ble studert for hvert kjemikalie alene og i forskjellige sekvensielle kombinasjoner. Tre forsøk, hvor eggbærende reker ble eksponert for ulike konsentrasjoner av bademidler, ble gjennomført.

Resultatene viser at dypvannrekene er følsomme overfor bademidler, men at toksisiteten varierer mellom de ulike kjemikaliene. Noen av badebehandlingene førte til høy dødelighet ved sterkt fortynnede behandlingskonsentrasjoner, dvs. ved konsentrasjoner som kan gjenfinnes i miljøet etter utslipp fra behandlingsmerder. Den mest alvorlige effekten ble observert for deltametrin, hvor en 2 timers eksponering til 330 ganger fortynnet behandlingsdose (alene og i sekvensiell bruk med hydrogenperoksid og azametifos) induserte ~ 100% dødelighet innen få dager etter eksponering. Lignende effekter ble ikke observert for hydrogenperoksid eller azametiphos. Imidlertid resulterte sekvensiell behandling med hydrogenperoksid og azametifos (2 timers eksponering for hver behandlingskjemikalie, 500 ganger fortynnet behandlingsdose) i > 50% dødelighet i løpet av den første uka etter behandling. Ingen sub-letale effekter eller tap av egg hos reker kunne relateres til eksponering av bademidler.

Våre resultater indikerer at bruk av deltamethrin til avlusning trolig vil føre til høyere dødelighet hos hunnreker enn bruk av hydrogenperoksid og azametiphos. Ulike kombinasjonsmetoder gir også høyere dødelighet enn bruk av enkelt-kjemikalier. Ingen av de sub-lethale endepunktene som ble vurdert kunne i våre forsøk relateres til behandling med bademidler, men ytterligere studier av potensielle sub-lethale effekter ved eksponeringskonsentrasjoner nær NEC (no effect concentration) bør gjennomføres på dypvannsreke i ulike stadier og i ulike sesonger for å få et komplett bilde av sårbarhet.

3 Introduction

The continued growth of the aquaculture industry in Norway has led to environmental and production challenges, and one of the major challenges is related to the ectoparasitic salmon louse *(Lepeophtheirus salmonis)* (Torrisen et al., 2013). A method to control sea lice within farm cages is treatment by various pharmaceutical delousing agents (Lillicrap, 2015). In Norway, the delousing agents are used either as bath treatments or in-feed drugs. The four bath treatments used in Norway are cypermethrin, deltamethrin, azametiphos or hydrogen peroxide (H_2O_2). Diflubenzuron, teflubenzuron and emamectin benzoate are used in the fish pellets. After a bath treatment of salmon with delousing agents added to the water, in the pen or in a well boat, the treatment water is discharged to the environment. Hydrodynamic dispersion modelling indicates that 100 – 1000 times dilution of the chemicals could be present more than one kilometre away from the discharge after they have been released (Page et al., 2014, Brokke, 2015, Refseth et al., 2016). Both fishermen, scientists, different stakeholders and fish farmers has raised concern about possible environmental impacts associated with the use of delousing chemicals.

While alternative methods for delousing have resulted in a reduction of the use of delousing agents in Norway over the last three years, there are large regional variations in this reduction, with use in certain production areas remaining high (Remen and Sæther, 2018).

Delousing agents used against salmon lice are designed to be specifically toxic to a crustacean parasite. Although the delousing agents are approved for use in aquaculture, the large amounts of medicated feed and the large volumes of bath chemicals used have raised concerns over the survival and wellbeing of populations of non-target crustaceans which represent key elements of many marine food chains (e.g. Langford et al., 2014). There is focus especially on the deep water shrimp (*Pandalus borealis*) which is one of the most important commercial crustacean species in Norway. Fishermen are reporting a decrease in shrimp fisheries, hence there is a need to assess whether realistic concentrations of bath treatments and combinations of bath treatment can impact the shrimp, either by direct mortality or sub-lethal effects.

H_2O_2 is considered the most environmentally friendly alternative to the chemicals used for salmon lice control because it rapidly breaks down into oxygen and water. Relatively large volumes of this chemical has therefore been used. However, recent research has shown that H_2O_2 can stay long enough in the environment, in areas from 0 – 1000 m from the release site, to induce mortality in shrimp after the treatment water is released. As H_2O_2 is heavier than seawater it will sink and when there is no stratification of the water column (during winter) it may reach the seabed a few minutes after release (Refseth et al., 2016). Sinking of H_2O_2 coincides with the time of year when deep water shrimp carry eggs. There is limited knowledge of the effects of H_2O_2 on egg-bearing shrimp.

It has also been demonstrated that H_2O_2 can induce sub-lethal effects, e.g. through the production of reactive oxygen species, which can induce DNA damage, including base oxidation, and DNA strand breaks (El-Bibany et al., 2014; Valavanidis et al., 2006; Azqueta et al., 2009). Maintenance of DNA integrity is essential for proper cell and organismal function, prevention of disease, and mutations (Reinardy and Bodnar, 2015; Wurgler and Kramers, 1992). Unrepaired DNA damage may also be transferred to offspring via affected parents and lead to long-term effects in populations (Barber et al., 2006; Jha, 2004).

In addition, other bath treatments such as azamethiphos (AZA; trade name Salmosan) and deltamethrin (DEL; trade name Alpha Max) can potentially impact survival and the reproductive cycle of shrimp. Deltamethrin is a pyrethroid and act on nerve transmission by interfering with sodium channels (Miller and Adams, 1982), which results in the depolarization of motor neurons and repetitive discharges at nerve endings, leading to eventual paralysis and death (Crane et al., 2011; Haya et al., 2005). Deltamethrin has a low water solubility, and the half-life of deltamethrin in the water column is 2-4 hours (Muir et al., 1985). Laboratory and field studies have shown that deltamethrin is toxic to crustaceans (e.g. Crane et al., 2011; Burridge and Van Geest, 2014; Urbina et al., 2019). Azamethiphos is a water soluble organophosphate with a half-life in seawater of <5 days at 12 °C (Norwegian Medicines Agency, 2016). Azamethiphos can also have negative effects on e.g. crustaceans (Burridge et al., 2000; Ernst et al., 2014), but toxicity occurs at higher concentrations than for deltamethrin (Burridge and Van Geest, 2014). Organophosphates are neurotoxic and inhibit acetylcholinesterase activity (AChE) preventing the production of the enzyme responsible for hydrolyzing the acetylcholine neurotransmitter, which is released during the transmission of a nerve impulse (Intorre et al., 2004; Kaur et al., 2017). As such, both deltamethrin and azamethiphos are known to affect locomotion capability and behaviour in exposed individuals (Burridge and Van Geest, 2014; Urbina et al., 2019).

Given the sensitivity of crustaceans and the significant use of delousing agents in Norway, there is a need to assess effects of bah treatments and combinations of bath treatments in deep water shrimp. Since the delousing agents are diluted in the sea, it is important to study relevant sub-lethal effects at low concentrations.

The aim of our study was to examine delayed lethal and sub-lethal effects of environmentally realistic concentrations of H_2O_2, alone or in sequential use with AZA or DEL, on egg-carrying deep-water shrimp *Pandalus borealis*. Further, our study aimed at ranging the bath chemicals tested by the severity of any delayed effects observed after exposure to the chemicals alone (H_2O_2, AZA, DEL) or in sequential use with each other (H_2O_2-AZA, H_2O_2-DEL, AZA-DEL). All exposures lasted for two hours to simulate a relatively conservative environmental exposure time before natural dilution at a given distance from the release point. Exposure concentrations were defined as dilutions of recommended treatment doses of the different bath chemicals to simulate expected concentrations at specific distances from the release point. Effects were assessed in terms of mortality, behavioural changes, egg loss, embryo development and mortality, DNA-damage (experiment 1 only), and total lipid content and growth (experiment 3 only).

4 Material and Methods

Three exposure experiments were carried out in the periods January 25th – February 22nd 2018, February 14th – March 14th 2018 and February 21st – March 12th 2019. In addition, three pilot trials were carried out in the period January 23rd – February 12th 2019.

4.1 Shrimp collection and maintenance

Egg-carrying shrimp were collected by shrimp pots in the inner part of Porsangerfjorden during November 2017 and October 2018, and by trawl in Balsfjorden January 15th 2018 and in Malangen January 31st 2018, respectively. No fish farms exist in any of the fjords where the shrimp were caught. Collected shrimp were transported to Akvaplan-niva's marine station (FISK) where they were placed in 600 L tanks for acclimation and maintained until experiment start-ups (separate batches of shrimp were kept in separate holding tanks; acclimation times where minimum 10 days and holding times in the lab prior to experiments were maximum three months). Holding tanks were supplied with 60 µm filtered running seawater of ambient temperature and salinity (33–34‰) at a flow rate ensuring efficient self-rinsing of the tanks, and water O_2-saturation > 80% (> 10 mg/L). The water inlet is located at a depth of 60 m, ensuring a relatively stable water temperature between 3 and 9 °C throughout the year. Three times a week, the shrimp were fed in excess with frozen *Calanus sp*.

4.2 Baseline measurements

One to two days prior to all experiments, 10-20 shrimp from the stock-batch used for the respective experiment were sampled for documenting the natural variation in the batch in terms of shrimp size distribution, embryonic developmental stage, egg size, gonadosomatic index (GSI) and fecundity (number of eggs per g shrimp).

Shrimp length (± 1.0 mm) and total weight (± 0.001 g) was measured before all eggs were removed and weighted separately. Thereafter the eggs were fixed for 10-15 minutes in fix 1 (methanol, acetic acid and distilled water (dH_2O) at the ratio 1:1:1) and stored in fix 2 (37% formalin, glycerol, ethanol, acetic acid and dH_2O at the ratio 2:1:3:1:3) for later egg counting and embryo development staging. GSI was calculated according to the equation

$$GSI = (W_{egg} / W_{shrimp}) \times 100$$

where W_{egg} is the total egg weight (g) and W_{shrimp} is the total shrimp weight (g). Fixed eggs were counted and embryos development stage studied and photographed by stereomicroscopy (Leica MZ6 with integrated DFC camera and application software 2.8.1, Leica Microsystems (Switzerland) Ltd.). The photos of the eggs were used for accurate measurement of egg diameter and embryo eye diameter by ImageJ Processing and Analysis in Java.

4.3 Exposure experiments

An overview of the three exposure experiments conducted in this study is given in Table 1. The main objective of Exp1 was to study delayed lethal and sub-lethal effects of H_2O_2 on egg-

carrying shrimp, after 2 hours exposure to 1000, 500 and 100 times dilutions of the recommended treatment dose (i.e. 1600 mg/L) were selected as environmental realistic exposure scenarios.

In Exp2, the main objective was to compare potential delayed lethal and sub-lethal effects of the three different bath chemicals H_2O_2, azamethiphos and deltamethrin, and to study the effects of sequential treatment with these chemicals. The intermediate H_2O_2 scenario (i.e. 500 times diluted recommended treatment dose) was selected for this experiment. However, through personal communication with fish-health veterinarians prescribing bath chemicals to the fish farmers, we were informed that the deltamethrin dose most often used is 1.5 – 2 times higher than the recommended treatment dose (i.e. 2 µg/L). We therefore selected 500 times dilution of 1.5 times recommended treatment dose of deltamethrin for direct comparison with the other two bath chemicals. Recommended treatment dose of azamethiphos is 100 µg/L.

Due to high mortality in Exp2, it was decided to repeat this experiment with lower (sub-lethal) concentrations (Exp3). Three pilot trials were conducted to determine the limit between lethal and sub-lethal concentrations of the chemicals. Based on the pilot trial results (see section 3.3), 1000 times diluted recommended treatment dose was selected for the sequential treatment with H_2O_2 and azamethiphos. The pilot trials did however fail in determining the limit between lethal and sub-lethal deltamethrin concentrations, and a deltamethrin concentration range (10 000, 100 000 and 1000 000 times dilution of recommended treatment dose) was selected for Exp3 instead of sequential treatments with this chemical.

Table 1. Overview of exposure experiments. Exp1; experiment 1, Exp2; experiment 2, Exp3; experiment 3. H_2O_2; hydrogen peroxide, AZA; azamethiphos, DEL; deltamethrin.

Experiment	Treatment	Dilution of recommended treatment doze	Nominal concentration (mg/L)	Number of replicates	Number of shrimp per replicate
Exp1	Control	-	-	3	25-26
	H_2O_2 1000	1×10^3	1.6	3	25
	H_2O_2 500	5×10^2	3.2	3	24-25
	H_2O_2 100	1×10^2	16	3	24-26
Exp2	Control	-			
	AZA	5×10^2	2×10^{-4}	3	25
	AZA/DEL	$5 \times 10^2/3.3 \times 10^2$	$2 \times 10^{-4}/6 \times 10^{-6}$	3	25-27
	DEL	3.3×10^2	6×10^{-6}	3	25-27
	H_2O_2/AZA	$5 \times 10^2/3.3 \times 10^2$	$3.2/2 \times 10^{-4}$	3	25
	H_2O_2/DEL	$5 \times 10^2/3.3 \times 10^2$	$3.2/6 \times 10^{-6}$	3	25-26
Exp3	Control	-		3	5
	H_2O_2	1×10^3	1.6	3	5
	AZA	1×10^3	1×10^{-4}	3	5
	H_2O_2/AZA	$1 \times 10^3/1 \times 10^3$	$1.6/1 \times 10^{-4}$	3	5
	DEL1	1×10^4	2×10^{-7}	3	5
	DEL2	1×10^5	2×10^{-8}	3	5
	DEL3	1×10^6	2×10^{-9}	3	5

4.4 Experimental set-up and analysis

All exposure experiments were conducted in 60 L flow-through tanks rigged in two rows on top of each other (see Fig. 1). The water level in each tank was set to 45 L and the tanks were supplied with 60 µm-filtered seawater of ambient temperature and salinity (33–34‰) at a flow rate of 45 L/h. Shrimp were placed into the exposure tanks 48 h prior to exposure start for acclimation to the new conditions. To minimize stress during acclimation and exposure, no feeding was undertaken during the 48 h acclimation period and the following exposure day.
All exposures lasted for two hours each. For sequential exposures, a one hour break between exposure 1 and exposure 2 allowed for replacement of the water containing chemical 1 before chemical 2 was introduced (i.e. sequential exposures lasted for 2x2 h with a 1 h break in between). At the start of each exposure, the water flow was stopped and 5 L water removed from each exposure tank, and replaced with 5L bath chemical in seawater at a concentration 9 times higher than the wanted exposure concentration (i.e. diluted 9 times in the tank) to ensure the correct exposure concentration right from the start. The water flow was then restarted and, at the same time, two peristaltic multi-channel pumps were started, providing stock solution to each tank to ensure a constant concentration of the treatment chemicals throughout the exposure period. After 2 hours the peristaltic pumps were stopped and the stock solution tubes removed from the tanks (i.e. start of recovery). All control tanks were handled the same way as the exposure tanks (i.e. 5 L water was removed and replaced with 5 L clean water). Oxygen and temperature levels were measured at the beginning and end of the experiments, and at daily intervals throughout the post-exposure period. Hydrogen peroxide concentrations were measured at the start of the exposure (T0h) and at the end of the exposure period (T2h) in all replicates using Abcam's Hydrogen Peroxide Assay Kit (Https://www.chemetrics.com/index.php?route=product/product&product_id=497).

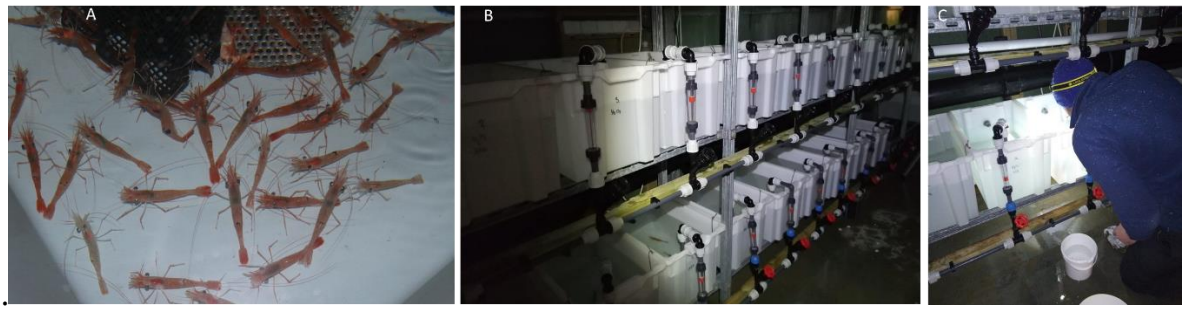

Figure 1. A: Shrimp in 600 L holding tanks. B: Rig with 60 L exposure tanks. C: Daily water quality monitoring and shrimp behaviour registration of all tanks. Pictures by Luca Tassara.

Shrimp behavior and mortality were monitored throughout the exposure day (T0d) and at daily intervals throughout the post-exposure period (T1d – T28d, T1d – T29d and T1d – T19d for Exp1, Exp2 and Exp3, respectively). Behavior was categorized as standing (normal behavior), normal swimming, stress swimming (abnormal behavior) or lying on side (abnormal behavior). Feeding was resumed the day following the exposure (T1d), and food (frozen *Calanus sp*.) was provided in excess throughout the post-exposure period. In Exp3, a mix of frozen *Calanus sp*. and fish pellets were provided as food, and feeding behavior was monitored for one hour three times a week after providing fresh food-supplies.

In Exp1 and Exp2, five shrimp per replicate were subsampled after the exposure, and thereafter at weekly intervals throughout the post-exposure period (T0d, T7d, T14d, T21d and T28d for Exp1 and T1d, T8d, T15d, T22d and T29d for Exp2). In Exp3, no shrimp were sampled until the end of the post-exposure period (T19d). All sampled shrimp were analyzed identical to the baseline shrimp regarding total weight, embryonic developmental stage, egg size, gonadosomatic index (GSI) and fecundity (number of eggs per g shrimp) (see section 2.2 for methodology). In Exp1, additional samples were taken for DNA-damage analysis: Approximately 0.2 g of the eggs and internal organs covered by the carapace (including pyloric stomach, heart, ovary and hepatopancreas) were snap-frozen in liquid N_2 and stored at -80 °C for later DNA-damage analysis. In Exp3, the whole shrimp (exclusive of eggs) were stored at -20 °C for later total lipid analysis.

4.4.1 DNA damage

DNA was extracted from a total of 30 carapace internal organ samples and 30 egg mass samples from the same individuals (n=5 individual shrimp per tank, n=3 tanks per treatment, n=2 treatments, control and high H_2O_2 exposure concentration (H_2O_2 100) of Exp1).

The fast micromethod was followed according to Schröder et al. (2006), with adaptations for DNA extracted from marine organisms according to Reinardy et al. (2016). In brief, 50 mg internal organs or egg mass tissue was extracted following the DNAzol ES ® Reagent protocol (MRC, USA) and quantified by nanodrop. For the fast micromethod, 100 ng of DNA was loaded into black-walled 96-well microplates, with triplicate wells per sample, with the addition of lysis solution (9 M urea, 0.01% SDS, and 0.2M EDTA) containing 1:49 Picogreen fluorescent dye (Life Technologies). Lysis was carried on ice, in the dark, and unwinding was then initiated by increasing pH with the addition of alkaline unwinding solution (20 mM EDTA, 1M NaOH). Flourescense was detected (kinetic mode, excitation 480 nm, emission 520 nm) from immediately after initiation of unwinding and quantified every 5 minutes for a 30 minute period. Strand scission factor (SSF) was calculated according to Schröder et al. (2006) using the following equation:

$$SSF = \log (\%\ dsDNA_{sample} / \%\ dsDNA_{control}) \times (-1)$$

where $dsDNA_{sample}$ are the exposed samples and $dsDNA_{control}$ are the unexposed samples, and percentages are calculated from relative fluorescent units (RFU) after 20-min unwinding compared with initial (0 min unwinding) RFU, after subtracting respective blank RFU values (distilled water was used as blanks).

4.4.2 Lipid analysis

The extraction of lipids from shrimp is based on the method of Folch (1957). In short, each whole shrimp was cut into fragments, weighed and transferred to test tubes. Samples were extracted with Methanol-Dichloromethane (1:2 by volume; a total of 85-90 ml of Methanol-Dichloromethane solvent was needed for each sample) for 16-48 h at -20 °C, then stirred with a REAX 200 shaker for 10 seconds. The extract was filtered through paper filters that had been prewashed with the same solvent. Thereafter, a fresh volume of Methanol-Dichloromethane was added to the shrimp sample, shaken on the REAX 2000, and filtered through the same paper filter, combining the two extracts. A volume of 0,88% KCl (aq), equal to ¼ of the total organic extract volume, was added to the combined extract, and mixed thoroughly before kept at -20 °C for 1 hour. After centrifuging the sample, the water phase (top) was removed, and the

remaining organic phase was reduced through evaporation under Nitrogen. The organic extract was then transferred to a pre-weighed sample vial, and further reduced in a vacuum desiccator until stable sample weight.

4.5 Pilot trials

Pilot trials were conducted at static conditions in bucket filled with 20 L seawater. Five shrimp from the holding tank were added to each bucket and left for some hours for acclimation. Thereafter 5 L seawater was removed from each bucket and replaced with 5L bath chemical in seawater at a concentration 4 times higher than the target exposure concentration (i.e. diluted 4 times in the bucket). After two hours of exposure in the buckets, the shrimp were moved to a flow-through tank supplied with clean running seawater and observed for one week regarding behaviour and mortality.

4.6 Statistics

Statistical analyses were performed with Statistica 13.3 or Stagraphics Centurion (XVII – X64). When requirements of normality and homogeneity of variances were met, a one-way ANOVA or a generalized linear model (GLM) factorial ANOVA with treatment and date as independent factors were used to test for differences between replicates and treatments. When a significant treatment effect was found, the Tukey HSD post hoc test or the Unequal N HSD post hoc test was applied to distinguish differences among treatment levels. When requirements for normality were not met, the nonparametric Kruskal-Wallis test, followed by Dunn's multiple comparison test was used. Correlations among variables were evaluated by the Pearson product-moment correlation. A probability level of $p<0.05$ was applied as the significance level for all analyses. All means are presented as average ± standard deviation unless otherwise noted.

5 Results and Discussions

5.1 Water chemistry and exposure conditions

Measured H_2O_2 concentrations in the exposure tanks was in agreement with nominal concentrations (see Fig. 2). The measured concentrations ranged between 73.5 – 92.2 % of the nominal concentration (average 82.9 %) for all three experiments. Control tanks and exposure tanks that did not include H_2O_2 did not show H_2O_2 concentrations above detection limit. In this study, we did not have the possibility to analyse the water concentrations of AZA and DEL in the exposure tanks. However, the same two multi-channel peristaltic pumps delivering H_2O_2 stock solution to the H_2O_2 treatments were synchronously delivering AZA and DEL to these treatments. We therefore find it reasonable to assume the same agreement between nominal and actual water concentrations for AZA and DEL as for H_2O_2.

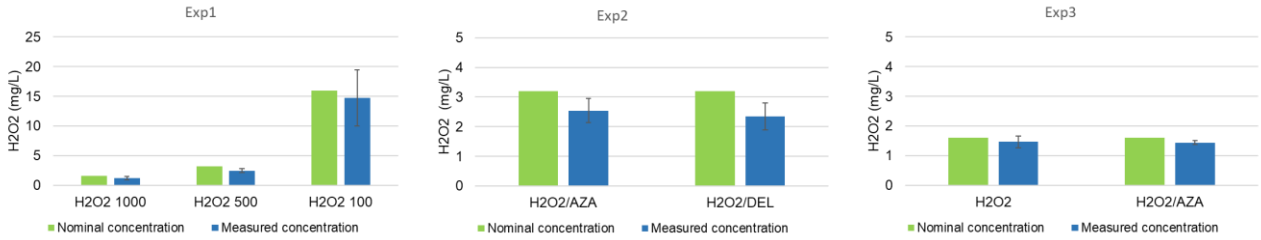

Figure 2. Nominal and measured H_2O_2 concentrations in treatment waters during exposure. Measured concentrations represent average of three replicate tanks ± standard deviation (error bars).

Average water temperature measured in one control tank of Exp1, Exp2 and Exp3 throughout the experiments was 3.5 (range 2.9 – 4.1), 2.9 (range 2.0 – 3.9) and 4.0 (range 3.0 – 5.1) °C, respectively (Fig. 3). Average oxygen saturation was 87.2 (range 83 – 91), 105.4 (range (97 – 113) and 101.0 (range 97 – 104), respectively (Fig. 3). Both temperature and O_2 saturation in all tree experiments are in agreement with expected environmental conditions at the habitats where the shrimp were collected from.

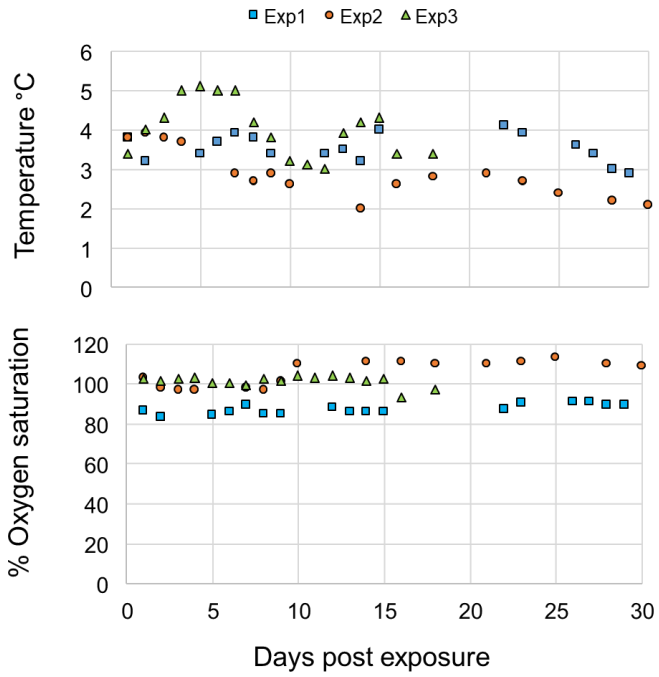

Figure 3. Control tank water temperature and oxygen saturation throughout Exp1 (square symbols), Exp2 (circular symbols) and Exp3 (triangular symbols).

5.2 Baseline measurements

The baseline (BL) measurements revealed that the shrimp batch used for Exp2 contained significantly smaller shrimp than those in Exp1 and Exp3 (Table 2.). The Exp2 shrimp were collected in Malangen on January 31st 2018 whereas the shrimp used for Exp1 was a mix of shrimp collected in Porsangerfjord in November 2017 and in Balsfjord in January 15th 2018, and the shrimp used for Exp3 were all collected in Porsangerfjord during November 2018. The GSI was similar for Exp1 and Exp2 baseline shrimp, whereas the GSI of the Porsangerfjord shrimp used for Exp3 was significantly lower. Proportion of dead eggs was <10 % in all three baseline shrimp batches, however significantly higher in the Exp2 Malangen-shrimp compared to the Exp1 mixed-origin baseline shrimp. Egg size and embryo developmental stages were significantly different between all three baseline shrimp batches: Shrimp used for Exp3 had the biggest eggs and the most developed embryos at the start of the experiment whereas Exp2 baseline shrimp had smaller eggs but more developed embryos than Exp1 baseline shrimp (Table 2, Fig. 4). The differences observed between the three baseline shrimp batches most probably reflects both temporal (i.e. timing of experiments, between year differences) and spatial (i.e. latitude, individual fjords) population differences.

Table 2. Overview of baseline shrimp weight, gonadosomatic index (GSI), proportion of dead eggs, egg size, embryo developmental stage and linear regressions between counted eggs and egg weight. Linear regression equations for each experiment were used to calculate relative fecundity (eggs per g shrimp) in main experiment shrimp.

	Shrimp weight (g)	GSI	% dead eggs	Egg diametre (mm)	Embryo eye diametre (mm)	Relative fecundity	*Linear regresion; egg weight - counted eggs*	R^2
BL Exp1 (n=20)	11.45 ± 2.64[a]	13.43 ± 1.63[a]	2.20 ± 1.48[a]	0.95 ± 0.05[a]	0.04 ± 0.03[a]	170 ± 23[a]	*y = 1083.3x + 15.9*	*0.64*
BL Exp2 (n=20)	7.27 ± 1.25[b]	13.85 ± 2.01[a]	8.33 ± 7.03[b]	0,89 ± 0,03[b]	0.09 ± 0.03[b]	134 ± 18[b]	*y = 659.6x + 164.9*	*0.76*
BL Exp3 (n=10)	11.63 ± 1.69[a]	5.46 ± 2.05[b]	4.23 ± 2.82[ab]	1.15 ± 0.04[c]	0.20 ± 0.01[c]	36 ± 16[c]	*y = 686.0x - 42.3*	*0.96*

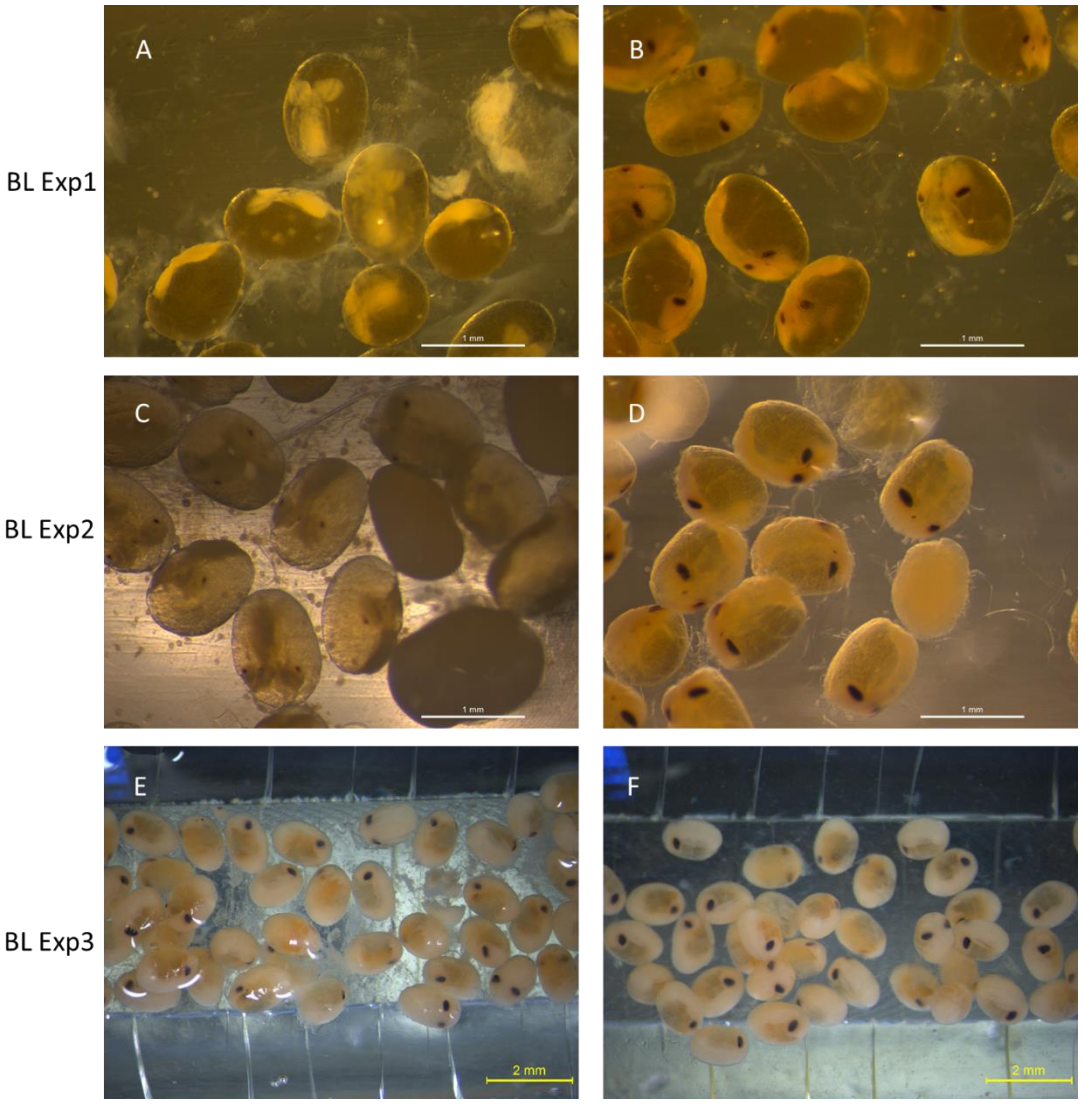

Figure 4. Range of eye pigmentation stages in baseline shrimp sampled prior to Exp1 (A, B; range 0.00 – 0.08 mm), Exp2 (C, D; range 0.03 – 0.12 mm) and Exp3 (E, F; range 0.18 – 0.22).

5.3 Mortality

In Exp1, there were no significant difference in mortality between treatments. Mortality ranged between 0 – 23 % in all replicates (Fig. 5; left panel), and between 9.3 ± 8.3 and 14.8 ± 2.0 % in all treatments. Most (91 %) of the shrimp that died during the experiment, died within the first week post exposure (T7d).

In Exp2, mortality ranged between 0 – 81 % in all replicates (Fig. 5; middle panel), being significantly higher in AZA/DEL (76.5 ± 4.8 %), DEL (76.8 ± 4.8 %), H_2O_2/AZA (41.3 ± 25.7 %) and H_2O_2/DEL (71.1 ± 5.4 %) compared to the control (6.0 ± 2.8 %) and the AZA (4.0 ± 4.0 %) treatments. Most shrimp that died in the treatments containing DEL, were dead within T3d (DEL; 93.6 %, AZA/DEL; 93.4 %, and H_2O_2/DEL; 87.1 %). At T7d, 98.5 % of all shrimp that died in all treatments during the experiment were already dead, and all shrimp in DEL, AZA/DEL, and H_2O_2/DEL was dead or sampled at this time point.

Similar to Exp1, there were no significant difference in mortality between the treatments in Exp3, and similar to both Exp1 and Exp2, most (93 %) of the shrimp that died during Exp3 were already dead within T7d. Mortality ranged between 0 – 40 % in all replicates (Fig. 5; right panel), and between 0 ± 0 and 20.0 ± 20.0 % in all treatments.

Overall, our experiments showed no delayed mortality in egg-carrying shrimp exposed to H_2O_2 at the concentration range 1.6 – 16 mg/L, to azamethiphos at the concentration range 100 - 200 ng/L and to deltamethrin at the concentration range 0.002 – 0.2 ng/L. A deltamethrin concentration of 6 ng/L was however highly toxic and did cause acute (i.e. < 96h) mortality in almost all exposed shrimp. Although no delayed mortality were seen in shrimp exposed to either 3.2 mg/L H_2O_2 or 200 ng/L azamethiphos (i.e. 500 times diluted recommended treatment doses), a sequential treatment with these concentrations of the two bath chemicals induced some few days delayed mortality in the exposed shrimp, indicating an synergistic effect of the chemicals.

Our H_2O_2 results (Exp1 and Exp3) are in agreement with previous studies in crustaceans showing that the concentration necessary to achieve 50 % mortality (LC_{50}) range between 1.9 and 1152.6 mg/L in exposures lasting for 24-96 hours, and between 937 - >3750 in exposures lasting for one hour (reviewed by Urbina et al., 2019). Our results are however in contrast to Bechmann et al. (unpublished data) who found that a few hours exposure to 1000 times diluted treatment solution (i.e. 1.6 mg/L H_2O_2) induced mortality in mature *P borealis*.

Short term exposures (0.5 - 6 h) to azamethiphos have shown LC_{50} values ranging between 2.8 – 37.7 µg/L (reviewed by Urbina et al., 2019). These concentrations are 3 - 35 times lower than recommended treatment dose, however order(s) of magnitude higher than the concentrations tested in our study.

Crustacean deltamethrin LC_{50} in exposures lasting for 1 hour range between 3.4 – 142 ng/L, with American lobster (*Homarus americanus*) larvae being most sensitive and the *Crangon septemspinosa* shrimp being least sensitive (reviewed by Urbina et al., 2019). Bechmann et al. (unpublished data) found increased mortality in *P borealis* larvae when exposed to 2 ng/L DEL.

The synergistic effect of sequential treatment with H_2O_2 and AZA seen in Exp1 is in contrast to findings by Burridge and Van Geest (2014) that did not find any additive effect of exposing shrimp for 1 hour to H_2O_2 and thereafter to AZA. A synergistic mortality effect of sequential treatment to AZA and DEL has been shown in the shrimps *P. flexuosus* and *P. elegans* (Brokke, 2015).

Due to the high mortality in all treatments containing DEL and in the H_2O_2/AZA treatment of Exp2, three pilot trials were run for one week each to determine the limit between lethal and

sub-lethal concentrations of these chemicals (see Table 3 for summary of results). Regarding H_2O_2/AZA, none of the tested doses (1000, 5000 and 25 000 times diluted treatment doses) initiated any delayed mortality effect and the highest trial dose (i.e. 1000 times dilution of recommended treatment dose) was selected for Exp3 and confirmed no delayed mortality result of this concentration (1.6 mg/L H_2O_2 /100 ng/L AZA). The high, but inconsistent mortality rate seen in all three DEL pilot trials made it impossible to select one concentration for sequential treatments, and a DEL concentration gradient (DEL1; 1 000 000 times dilution, DEL2; 100 000 times dilution and DEL3; 10 000 times dilution) was selected for Exp3. The Exp3 DEL results contradicted the results of the pilot trials and our study could only estimate a mortality limit, either alone or in sequential treatment, of DEL between 0.2 – 6.0 ng/L. However, *P. borealis* seems to be highly sensitive to DEL when compared to other crustaceans reviewed by Urbina et al. (2019). The reason for the high mortality effect of DEL seen in the pilot trials is unknown but may be due to static exposure conditions instead of flow-through conditions used for the main experiments.

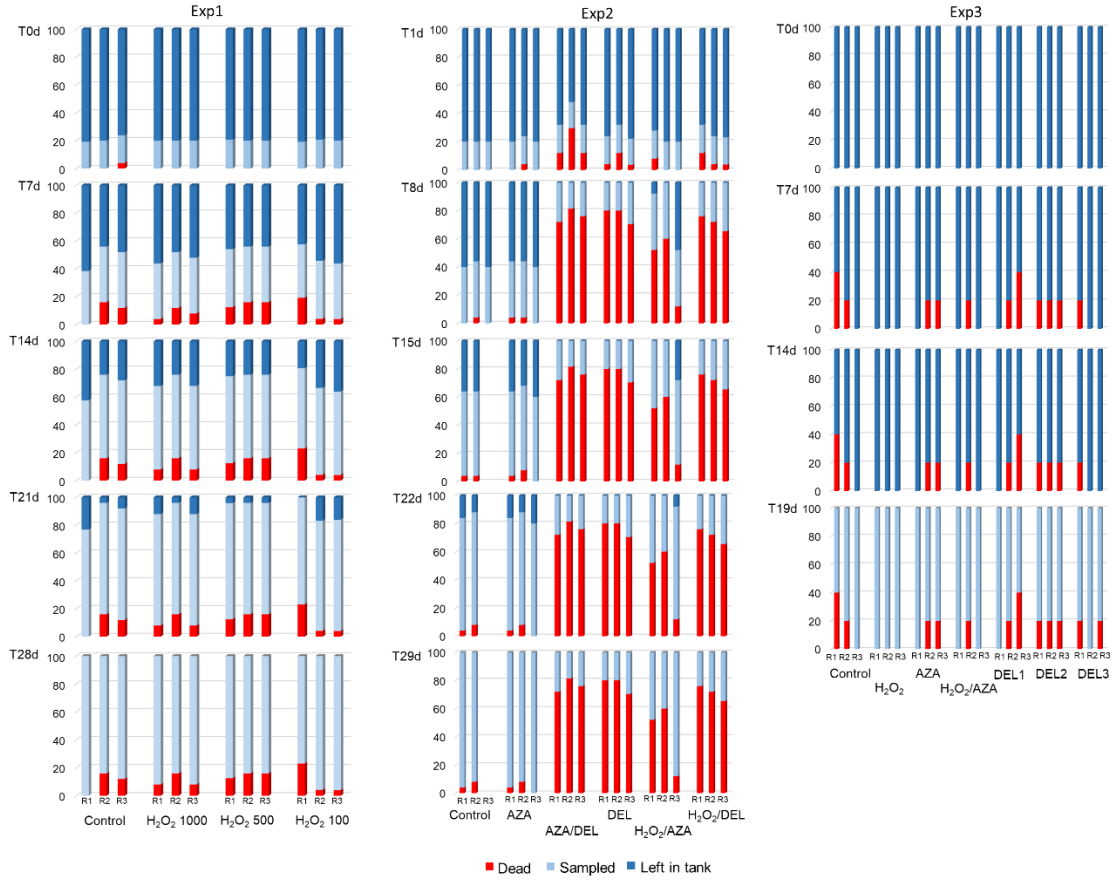

Figure 5. Percent cumulative mortality and sampling of shrimp in all experiments. Left panels; Exp1, n = 24 - 26 shrimp per tank. Middle panels; Exp2, n = 25 - 27 shrimp per tank. Right panels; Exp.3, n = 5 shrimp per tank.

Table 3. Overview of results from pilot trials with H_2O_2/AZA and DEL

Pilot trial	Treatment	Dilution of recommended treatment doze	Nominal concentration (mg/L)	Number of replicates	Number of shrimp per replicate	Number of dead shrimp	Time of death
I	Control	-	-	1	5	0	
	H_2O_2/AZA 1	1×10^3	1.6 / 0.0001	1	5	0	
	H_2O_2/AZA 2	5×10^3	0.32 / 0.00002	1	5	0	
	H_2O_2/AZA 3	25×10^3	0.16 / 0.00001	1	5	0	
	DEL 1	1×10^3	2×10^{-6}	1	5	5	T2d -T5d
	DEL 2	5×10^3	4×10^{-7}	1	5	1	T5d
	DEL 3	25×10^3	8×10^{-8}	1	5	1	T7d
II	Control	-	-	1	5	0	
	DEL 1	1×10^3	2×10^{-6}	1	5	4	T1d - T2d
	DEL 2	5×10^3	4×10^{-7}	1	5	5	T1d - T2d
	DEL 3	25×10^3	8×10^{-8}	1	5	5	T1d - T2d
III	Control	-	-	1	5	0	
	DEL 4	1×10^6	2×10^{-9}	1	5	5	T1d - T2d
	DEL 5	5×10^6	4×10^{-10}	1	5	5	T1d - T2d
	DEL 6	25×10^6	8×10^{-11}	1	5	5	T1d

5.4 Behaviour

In Exp1 there were no significant behavioural difference between treatments in terms of percentage shrimp standing, swimming normally, stress swimming or laying on their side throughout the experimental period. Percentage shrimp standing ranged between 74.4 – 100 % per tank (average 94.0 ± 8.5 %) whereas percentage shrimp swimming normally, stress swimming, or laying on their side ranged between 0 – 25.5 % (average 3.2 ± 7.4 %), 0 – 10.4 % (average 0.4 ± 2.2 %) and 0 – 10.3 % (average 2.3 ± 4.0 %), respectively. Overall, more shrimp were laying on their side in the period T1d – T5d post-exposure compared to any other periods of the experiment. Significantly more shrimp performed normal swimming behaviour towards the end of the experimental period compared to early in the period, resulting in fewer shrimp standing in the same period. Overall, few incidences of stress swimming were observed throughout the experimental period. Our finding is in contrast to findings by Van Geest et al. (2014) who showed total paralysis of adult, nauplii and copepodites of different species of copepods when exposed to 10 mg/L H_2O_2.

In Exp2, significantly more shrimp in the DEL, AZA/DEL and H_2O_2/DEL treatments were stress swimming in the period T1h – T5h (1.3 – 18.6 %), and laying on their side in the period T5h – T3d (45 – 100 %) (Fig. 6) compared to controls, AZA and H_2O_2/AZA treatments (1.3 – 4.0 % stress swimming and 0 – 32.8 % laying on their side in the corresponding periods). Except for a significantly higher proportion of shrimp in the AZA treatments performing normal swimming behaviour in the period T1d – T2d (16 %) compared to all other treatments (0 – 13%), no behavioural differences were observed between the control, AZA, and H_2O_2/AZA treatments throughout the experiment and all surviving shrimp were generally observed standing. Reduced swimming speed have been shown in the shrimp *Palaemon serratus* at concentrations equal to or higher than 0.6 ng/L of DEL after 96 h exposure at 18 °C (Oliveira et al., 2012). Also, *M. edwardsii* larvae exposed to concentrations between 0.1 and 0.5 µg /L DEL for 40 min at 15 °C showed no swimming capacity and only weak appendage movements (Gebauer et al., 2017). Azamethiphos at concentrations of 5-10 µg/L have been shown to agitate

American lobster (Burridge et al., 2000), and lobsters exposed to 12 or 57 µg /L AZA for 1 h under static conditions showed changes in behaviour and animals moribund (non-responsive but respiring) or dead (Burridge and Van Geest, 2014).

In Exp3, most shrimp were observed standing throughout the experiment except one shrimp in one of the replicate H_2O_2-tanks that laid on the side throughout the experiment. Except for this individual H_2O_2-exposed shrimp, 1-2 shrimp were observed laying on the side randomly in one to four of the twenty-one exposure tanks, but with no significant difference between treatments and no correlation between deaths of shrimp in the individual tanks. Only a few incidences of stress swimming was observed but with no connection to exposure time or treatment.

During the exposure and the following days, the shrimp from Exp3 were observed to clean themselves by brushing eyes, legs and bodies, and to flush their eggs with help of the pleopods. At T0d, the egg flushing was significantly higher in DEL1, DEL2 and DEL3 treatments compare to any of the other treatments, and the cleaning activity in the H_2O_2/AZA and the DEL treatments were higher than in the controls and the H_2O_2 and AZA treatments throughout the period T1d-T5d (data not shown).

When food was introduced to the exposure tanks at T1d, the responsiveness to food was significantly lower in H_2O_2/AZA and DEL3 compared to controls (Fig. 7). No treatment effect regarding feeding behaviour were however seen on T5d or any later time point during the experiment (data not shown). In general, the interest towards food was characterised as low in all tanks as most shrimp lost interest and dropped the food pellets within 10 minutes after feeding, and only some random feeding on *Calanus sp*. was observed. Change in feeding behaviour was shown in adult, nauplii and copepodites of different species of copepods after exposure to 5 mg/L H_2O_2 (Van Geest et al., 2014). Also, reduced feed intake has been observed in *P. borealis* exposed to 2 ng/L DEL (Bechmann et al., unpublished data).

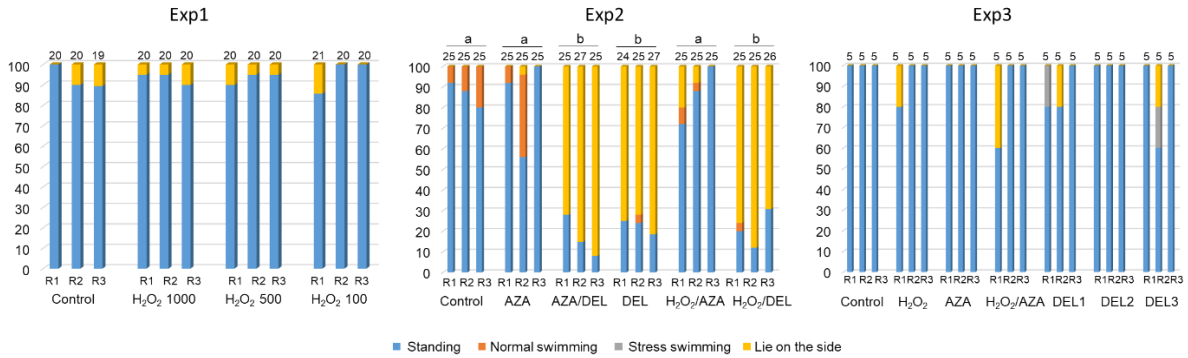

Figure 6. Shrimp behaviour registered in Exp1, Exp2 and Exp3 one day post exposure (T1d). Number of shrimp in each tank at the registration time point is indicated above bars. Different letters above bars indicate significant difference between treatments.

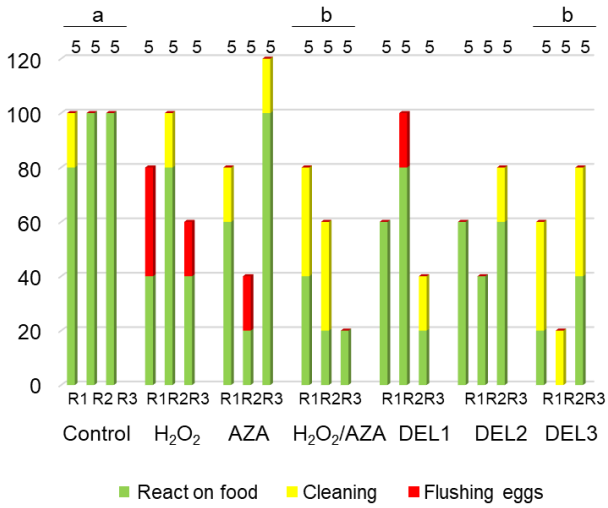

Figure 7. Feeding, cleaning and egg flushing behaviour of Exp3 shrimp one day post exposure (T1d). Number of shrimp in each tank at the registration time point is indicated above bars. Different letters above bars indicate significant difference between treatments regarding responsiveness to food.

5.5 Egg loss, embryo development and reproductive output

5.5.1 Egg loss

Partial egg loss did occur in all experiments as a few eggs were occasionally observed at the bottom of the tanks (especially just after handling). Partial egg loss was, however, not quantifiable since the number of eggs per shrimp at the start of the experiment was unknown.

In all experiments, some shrimp lost all of their eggs during the experimental period. In Exp1, between 0 and 29 % (0-6 shrimp per tank; 29 shrimp in total) lost all their eggs, but with no significant difference between the treatments (Fig. 8). In total, 32 shrimp died during Exp1 (T4d-T8d) and 10 of the shrimp that died (31 % of all dead shrimp) had lost all their eggs. In contrast, only 7 % of the shrimp sampled when alive (19 out of 266) had lost all their eggs, and 6.5 % of these were sampled towards the end of the experiment (T21d and T28d). No hatching of eggs occurred during the experiment.

In Exp2, all shrimp treated with deltamethrin alone (DEL) or sequentially (AZA/DEL, H_2O_2/DEL) were sampled (58 shrimp) or died (173 shrimp) within T8d, and none of these shrimp had lost all their eggs (Fig. 8). In controls and in the AZA and H_2O_2/AZA treatments, between 0 and 16 % (0-4 shrimp per tank; 20 shrimp in total) lost all their eggs with no significant difference between these treatments. Ten of the shrimp that lost all their eggs died during the experiment (T1d –T17d) whereas nine of the remaining ten shrimp that had lost all their eggs were sampled at the end of the experiment (T29d). No hatching of eggs occurred during the experiment, and remaining shrimp of the batch used for Exp2 did not hatch until early April (~three weeks after the end of the experiment).

In Exp3, between 0 and 60 % (0-3 shrimp per tank; 18 shrimp in total) lost all their eggs during the experiment, with no significant difference between the treatments (Fig. 8). Four of the shrimp without eggs died during the experiment whereas 14 were sampled at the end of the experiment (T19d). In Exp3 hatching was initiated before the end of the experiment (the first larvae were observed at T13d; some hatching was observed in all tanks in the period T13d-

T19d) and it is unsure whether the lack of eggs in some shrimp were due to hatching or release of the eggs prior to hatch. There were, however, no significant correlation between number of hatched larvae and shrimp with no eggs between tanks (data not shown). There were no significant difference in number of hatched larvae between the treatments of Exp3 (Fig. 9).

Overall, our results did not indicate any egg loss due to the bath treatment chemicals. However, a higher percentage of egg loss in shrimp dying during the experiments may indicate that stress and a general bad health condition may trigger the shrimp to release their eggs.

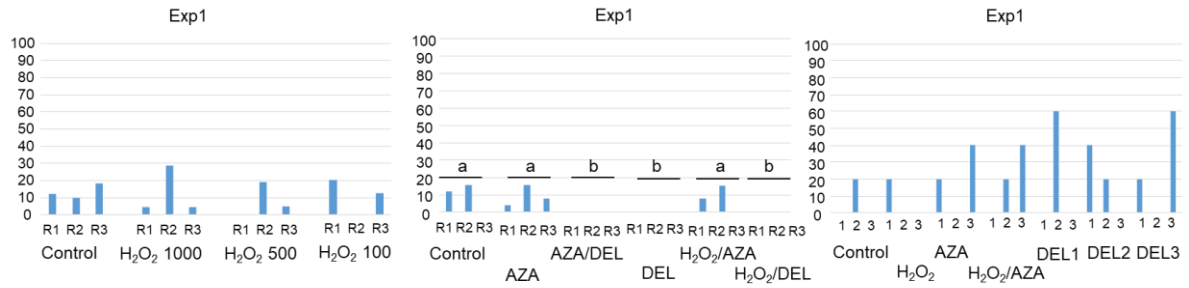

Figure 8. Percent shrimp that lost all eggs during Exp1, Exp2 and Exp3. Bars represent average of replicate tanks ± standard deviation (error bars). Exp1, n = 24 - 26 shrimp per tank. Exp2, n = 25 - 27 shrimp per tank. Exp.3, n = 5 shrimp per tank. Different letters above bars indicate significant difference between treatments.

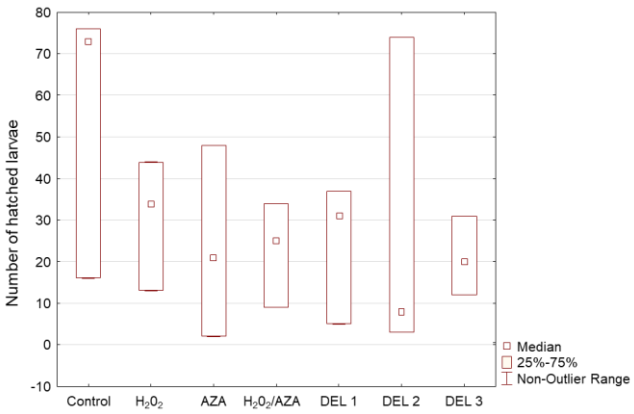

Figure 9. Number of hatched larvae in the different treatments of Exp3. For each treatment, 11-14 shrimp were still left during the period of hatching (3-5 shrimp per tank).

5.5.2 Embryo development and reproductive output

In all experiments, there were no significant differences between replicates within treatments for any of the investigated endpoint. Therefore, the replicates per treatment were combined in statistical analyses. Generally, no significant sub-lethal effects on embryo development and reproductive output of any of the tested bath chemicals were revealed.

In Exp1, there were no significant difference in total shrimp weight, relative fecundity or embryo eye diameter between any treatments at any sampling points throughout the experiment (Table 4, Fig. 10). Embryos eye diameter was bigger in all treatments compared to the baseline shrimp sampled prior to Exp1 due to fewer embryos with no eye pigmentation, indicating progressive embryo development throughout the experimental period in all treatments. At T7d, the egg diameter was significantly larger in controls than in the low H_2O_2 treatment (H_2O_2 1000). However, at T21d, the control eggs were significantly smaller than in all H_2O_2 treatments and further, percentage dead eggs were also significantly higher in controls than in the H_2O_2 treatments at this time point. At T28d, GSI was lower in controls than in the H_2O_2 1000 treatment. Overall, none of the observed differences between the treatments indicates any negative effect of H_2O_2 in the concentration range 1.6 - 16 mg/L on shrimp embryo development and reproductive output.

Table 4. Overview of shrimp weight (g), gonadosomatic index (GSI), relative fecundity (number of eggs per g shrimp), egg diameter and embryo eye diameter (average ± standard deviation; all replicates per treatment combined) measured at the different sampling point of Exp1 (T0d, T7d, T14d, T21d and T28d). Egg/embryo eye diameters; average of 10 eggs/embryos per shrimp. Different letters behind values indicate significant difference between treatments.

Days post exposure	Treatment	Shrimp weight (g)	*n*	GSI	*n*	Relative fecundity	*n*	% dead eggs	*n*	Egg diameter (mm)	*n*	Embryo eye diameter (mm)	*n*
0	**Control**	10.75 ± 1.22	*15*	10.7 ± 4.2	*15*	134 ± 55	*15*	0.7 ± 0.9	*10*				
0	**H_2O_2 1000**	9.79 ± 2.20	*15*	11.9 ± 4.9	*15*	153 ± 74	*15*	1.5 ± 2.0	*15*				
0	**H_2O_2 500**	10.28 ± 1.57	*15*	8.9 ± 4.7	*15*	111 ± 61	*15*	1.4 ± 1.8	*14*				
0	**H_2O_2 100**	10.19 ± 1.63	*15*	11.4 ± 3.3	*15*	143 ± 45	*15*	1.4 ± 1.0	*14*				
7	**Control**	9.56 ± 2.04	*15*	12.2 ± 3.9	*15*	155 ± 52	*15*	1.0 ± 0.8	*9*	1.16 ± 0.13^a	*6*	0.15 ± 0.09	*5*
7	**H_2O_2 1000**	10.83 ± 2.31	*15*	11.4 ± 4.6	*15*	144 ± 60	*15*	2.0 ± 1.9	*14*	1.03 ± 0.03^b	*6*	0.10 ± 0.02	*3*
7	**H_2O_2 500**	10.76 ± 2.04	*15*	10.7 ± 4.3	*15*	135 ± 57	*15*	0.9 ± 0.8	*12*	1.12 ± 0.11	*4*	0.13 ± 0.12	*3*
7	**H_2O_2 100**	10.44 ± 1.87	*15*	8.7 ± 5.0	*15*	108 ± 65	*15*	1.3 ± 1.0	*12*	1.11 ± 0.06	*4*	0.13 ± 0.07	*4*
14	**Control**	9.82 ± 1.48	*15*	11.2 ± 3.8	*15*	141 ± 50	*15*	3.5 ± 2.4	*15*				
14	**H_2O_2 1000**	9.91 ± 1.65	*15*	11.5 ± 4.7	*14*	146 ± 60	*14*	4.8 ± 3.4	*14*				
14	**H_2O_2 500**	10.67 ± 2.79	*15*	13.5 ± 2.7	*15*	172 ± 37	*15*	5.0 ± 4.1	*14*				
14	**H_2O_2 100**	10.05 ± 1.54	*15*	12.5 ± 2.4	*15*	158 ± 34	*15*	5.6 ± 4.1	*15*				
21	**Control**	10.61 ± 1.04	*15*	14.0 ± 1.8	*10*	180 ± 26	*10*	4.8 ± 2.2^a	*8*	0.97 ± 0.03^a	*9*	0.10 ± 0.02	*7*
21	**H_2O_2 1000**	9.79 ± 2.52	*15*	11.9 ± 7.7	*14*	159 ± 127	*14*	1.4 ± 1.7^b	*12*	1.12 ± 0.08^b	*13*	0.10 ± 0.04	*11*
21	**H_2O_2 500**	9.97 ± 1.31	*15*	12.5 ± 3.2	*12*	158 ± 43	*12*	1.5 ± 0.9^b	*12*	1.06 ± 0.04^b	*12*	0.10 ± 0.03	*12*
21	**H_2O_2 100**	9.76 ± 1.87	*14*	11.3 ± 3.5	*11*	141 ± 47	*11*	1.7 ± 2.3^b	*11*	1.10 ± 0.05^b	*11*	0.13 ± 0.07	*10*
28	**Control**	9.59 ± 2.97	*9*	12.0 ± 1.6^a	*7*	150 ± 22	*7*	2.1 ± 1.2	*7*	0.99 ± 0.03	*7*	0.11 ± 0.02	*7*
28	**H_2O_2 1000**	10.65 ± 1.69	*7*	14.4 ± 1.0^b	*5*	184 ± 15	*5*	2.5 ± 1.3	*4*	1.02 ± 0.02	*5*	0.14 ± 0.02	*5*
28	**H_2O_2 500**	9.26 ± 1.05	*3*	14.4 ± 1.5	*3*	185 ± 22	*3*	3.6 ± 2.3	*3*	1.05 ± 0.06	*3*	0.15 ± 0.04	*3*
28	**H_2O_2 100**	10.35 ± 1.67	*8*	13.5 ± 1.1	*7*	172 ± 16	*7*	2.4 ± 1.7	*7*	1.01 ± 0.03	*7*	0.13 ± 0.06	*7*

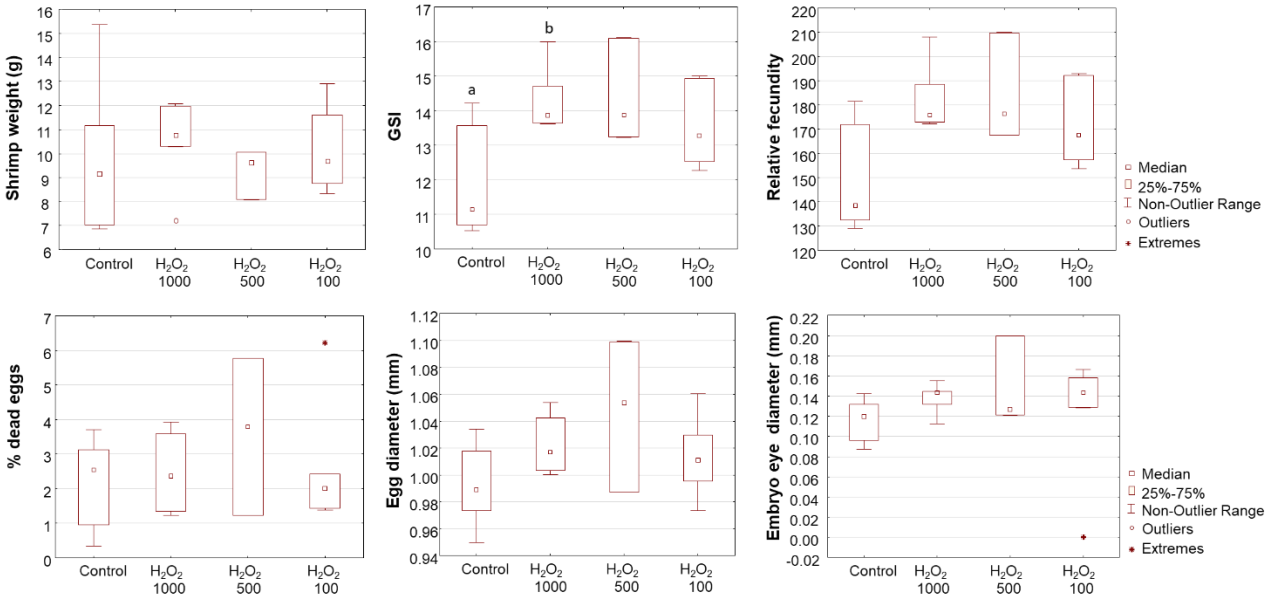

Figure 10. Shrimp weight (g), gonadosomatic index (GSI), relative fecundity (number of eggs per g shrimp), egg diameter and embryo eye diameter measured at the end of Exp1 (T28d). N =3-9 shrimp per treatment. Egg/embryo eye diameters: average of 10 eggs/embryos per shrimp. Different letters above bars indicate significant difference between treatments.

Due to the high mortality in some treatments of Exp2, only the first sampling point undertaken one day post exposure (T1d), represented a full dataset including all replicate tanks. Therefore, the Td1 dataset was analysed regarding embryo development and reproductive output of Exp2. There was no significant difference in total shrimp weight between any treatments and no significant treatment effect on GSI, relative fecundity or % dead eggs (Fig. 11), indicating no acute egg loss or embryo mortality in shrimp exposed to 200 ng/L AZA, 6 ng/L DEL or sequentially to 200 ng/L AZA/6 ng/L DEL, 3.2 mg/L H_2O_2/6 ng/L DEL or 3.2 mg/L H_2O_2/200 ng/L AZA. Embryo eye diameter were similar in all treatments, confirming similar ranges of embryonic developmental stages in all shrimp at the start of the experiments. The egg diameter in the AZA/DEL and H_2O_2/DEL treatments were however significantly smaller than in the controls (Fig. 11). Whether the difference in egg diameter was a background variability or an effect of treatments needs further investigation, the destiny of these embryos would however most probably be fatal due to death of the mother shrimp carrying them.

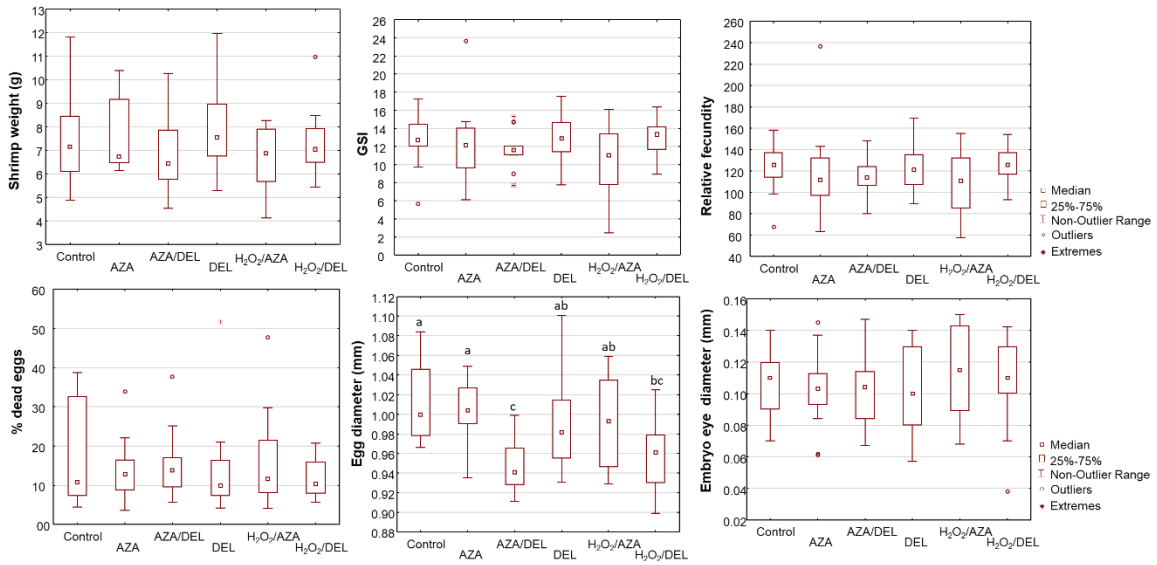

Figure 11. Shrimp weight (g), gonadosomatic index (GSI), relative fecundity (number of eggs per g shrimp), egg diameter and embryo eye diameter measured one day post exposure of Exp2 (T1d). N =15 shrimp per treatment. Egg/embryo eye diameters: average of 10 eggs/embryos per shrimp. Different letters above bars indicate significant difference between treatments.

In Exp3, there were no significant differences in shrimp weight, GSI, relative fecundity, percentage dead eggs, egg diameter or embryo eye diameter at the end of the experiment (T19d) (Fig. 12). Further, hatching was initiated in all tanks with no significant difference in number of hatched larvae between treatments (Fig. 9). No abnormalities were observed in any of the hatched larvae of any of the treatments (data not shown). This confirm the finding of Exp1, showing no sub-lethal effects of 1.6 ml/L H_2O_2 on embryo development and reproductive output. Further, the results confirm no sub-lethal effects of 100 ng/L azamethiphos or sequential treatment with 1.6 ml/L H_2O_2/100 ng/L azamethiphos or deltamethrin in the concertation range 0.002 – 0.2 ng/L. Burridge et al. (2008) have shown that repeated exposures to 10 µg/L AZA can have a negative effect on the survival and reproduction of the American lobster, but that such sensitivity is influenced by the season of the year. Malformations have been reported in neonates of two strains of *Daphnia magna* after 21 days of exposure to 0.080 and 0.15 µg /L DEL, respectively. The observed changes included general malformations, anthesis underdevelopment, curvature of carapace spines and abdomen, and changes in the percentage of males (Toumi et al., 2013).

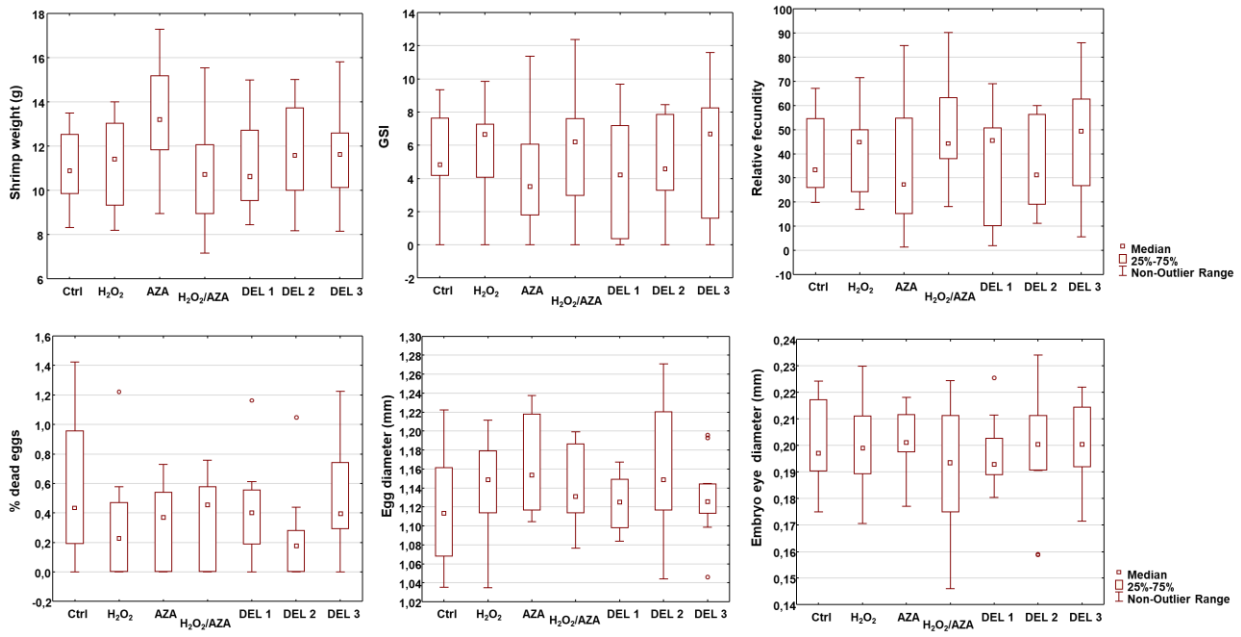

Figure 12. Shrimp weight (g), gonadosomatic index (GSI), relative fecundity (number of eggs per g shrimp), egg diameter and embryo eye diameter measured at the end of Exp3 (T19d). N =12-15 shrimp per treatment. Egg/embryo eye diameters; average of 10 eggs/embryos per shrimp.

5.6 DNA-damage

DNA-damage was analysed only in a sub-set of individuals from Exp1, i.e. controls and those exposed to 16 mg/L H_2O_2. In general, there was high variability in DNA damage between individuals, and no consistent trend towards individuals with high levels of DNA damage in both internal organs and eggs. There was a non-significant trend for higher levels of DNA damage in internal organs from exposed shrimp, but overall no statistical difference in DNA damage levels between control and exposed individuals. Reported LC_{50} values for H_2O_2 in sea urchin larvae approximated to 20.4 mg/L, with significantly higher levels of DNA damage after exposure to 3.4 mg/L (Reinardy and Bodnar, 2015), indicating that shrimp may be less sensitive to H_2O_2 exposure compared with sea urchin larvae.

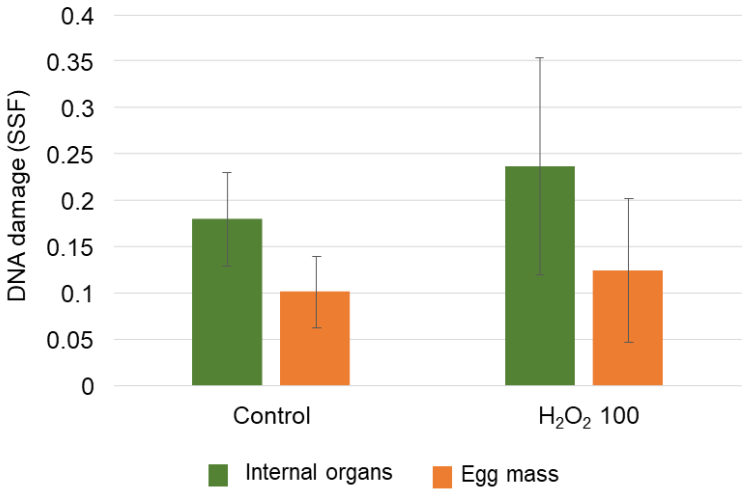

Figure 13. DNA damage (strand scission factor) in shrimp internal organs and egg mass tissues after exposure to 16 mg/L H_2O_2. Data are average ± standard error of mean, n=3 tanks per treatment and 5 shrimp per tank.

5.7 Growth and total lipid content

In Exp3 all shrimp were weighted before exposure, and at the end of the experiment (T19d) (or at the time of death). Average weight per tank at the start and at the end of the experiment is shown in Fig. 14. Overall, the shrimp weight decreased in all tanks during the experiment, but no significant differences were found, neither pre-exposure nor post-exposure. The reason for the shrimp weight loss during the experiment is unknown but may be a combination of weight loss due to egg loss/hatched larvae as well as a low food intake due to the limited interest for the food items (fish pellets and *Calanus* sp.) supplied to the tanks.

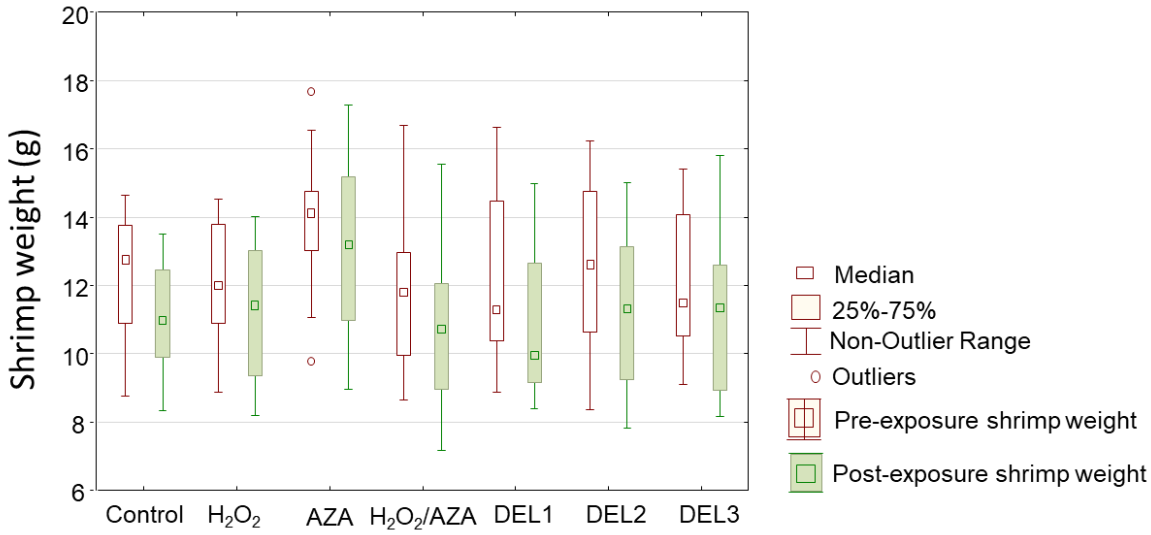

Figure 14. Total shrimp weight per treatment at the start of the experiment (open, red boxes) and at the end of the experiment (green boxes). N=15 shrimp per treatment.

Median total lipid content of shrimp from the different treatments at T19d ranged between 1.2 – 3.2 % (Fig. 15) which is in accordance to expected levels (1-11%; Hopkins et al., 1993). No treatments were significantly different from the control treatment, indicating no treatment effects. The total lipid content in the AZA treatment was however significantly lower than in the H_2O_2, DEL1, DEL2 and DEL3 treatments. The reason for this is unknown but may be partly explained by a non-significant difference in shrimp sizes between treatments as a significant negative correlation between shrimp size and total lipid content was revealed (Fig. 15). Bigger shrimp may have relatively heavier extraskeletons than smaller shrimp, or relatively more muscle mass compared to hepatopancreas tissue. Extraskeltons most probably contain less lipid than soft tissue parts, and hepatopancreas have been shown to be the main lipid storage organ in prawns whereas muscle mainly contained phospholipids (Muriana et al., 1993). This hypothesis needs further investigation.

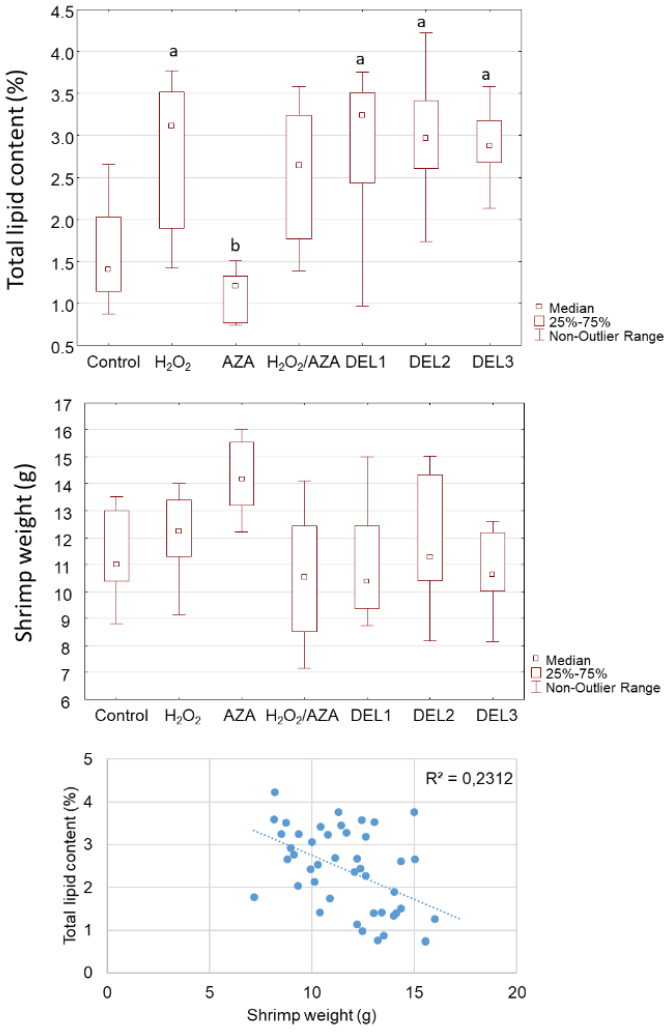

Figure 15. Total lipid content and total shrimp weight of shrimp selected for total lipid analysis (n=6 shrimp per treatment represented by two shrimp randomly selected from each replicate tank).

6 Conclusion

Deltamethrin was shown to be highly toxic to shrimp at 330 times dilution of recommended treatment dose (500 times dilution of a dose often used in practice), emphasising the need for specific precautions regarding the use of this bath chemical. The limit concentration for no lethal or sub-lethal effects of deltamethrin on egg-carrying shrimp remains to be established. Further, the synergistic effect of H_2O_2 and azamethiphos, inducing >50% mortality at a 500 times diluted treatment concentration, is of concern and emphasise the need for more knowledge regarding the effects of combined/sequential treatments in general. The total chemical load needs to be taken into consideration when evaluating potential effects on non-target organisms of the ecosystem in a recipient.

The results from the experiments conducted in this study did not reveal any negative sub-lethal effects on egg-carrying shrimp of exposure to highly diluted (H_2O_2; ≤100 times diluted treatment dose, azamethiphos; ≤500 times diluted treatment dose, deltamethrin; ≤10 000 times diluted treatment dose) bath chemicals. Exposed shrimp were monitored for five weeks and no sub-lethal effects were seen on, energy allocation in terms of total lipid content (Exp3 only), DNA damage (H_2O_2 only), behaviour, embryo development or reproductive output.

Our results may indicate that mortality is the most likely effect when exceeding the no effect concentration. However, potential sub-lethal effect should be further investigated at concentration closer to the no effect concentration.

Although no effects of the investigated bath chemicals, general stress and a bad health condition may induce partial or complete loss of eggs in egg-carrying shrimp.

The results from the present study constitute a valuable input to the knowledge base required for conducting environmental risk assessments for aquaculture industry areas.

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