Effects of the sea lice bath treatment pharmaceuticals hydrogen peroxide, azamethiphos and deltamethrin on egg-carrying shrimp (*Pandalus borealis*)

Marianne Frantzen[a*], Jenny Bytingsvik[a], Luca Tassara[a], Helena Reinardy[b,c], Gro Harlaug Refseth[a], Ellie J. Watts[a], Anita Evenset[a, d]

[a]Akvaplan-niva, Fram Centre. 9296 Tromsø, Norway.
[b]The Scottish Association for Marine Science, Scottish Marine Institute, Oban, Argyll, PA37 1QA
[c]UNIS, the University Centre in Svalbard, Longyearbyen, 9171 Svalbard, Norway
[d]UiT, the Arctic University of Norway. Faculty of Biosciences, Fisheries and Economics. 9037 Tromsø, Norway

*corresponding author: mfr@akvaplan.niva.no

Declarations of interest: none

Abstract

This study investigated effects of sea lice pharmaceuticals on egg-bearing deep water shrimp (*Pandalus borealis).* Both mortality and sub-lethal effects (behavior, embryo development, and reproductive output) were studied for each of three pharmaceuticals alone and in different sequential combinations. The most severe effect was observed for deltamethrin where 2 h exposure to 330 times diluted treatment dose (alone and in sequential application with hydrogen peroxide and azamethiphos) induced almost 100% mortality within a few days after exposure. Similar effects were not observed for hydrogen peroxide or azamethiphos. However, sequential treatment of hydrogen peroxide and azamethiphos (2 h exposure to each pharmaceutical; 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. Future studies should investigate potential sub-lethal effects at exposure concentrations close to the no-effect concentration.

Keywords: Aquaculture**,** hydrogen peroxide, azametiphos, deltamethrin, *Pandalus borealis*, embryo

1. 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 et al., 2015). In Norway, the delousing agents are used either as bath treatments or in-feed drugs. The four bath treatments used are cypermethrin, deltamethrin (DEL), azametiphos (AZA) or hydrogen peroxide (H_2O_2). Diflubenzuron, teflubenzuron and emamectin benzoate are used in fish feed pellets. Due to development of resistance in sea-lice and implementation of alternative methods for lice control (mechanical and biological), the use of pharmaceuticals has decreased since 2015. The dynamic arms race of controlling sea lice levels in salmon aquaculture has seen some regional differences in bath pharmaceutical treatment regimes, and in many production areas (e.g. Northern-Norway) the total amounts of bath treatments applied is still high (Remen and Sæther, 2018). Due to resistance challenges (i.e. sea lice developing resistance towards the most commonly used delousing pharmaceuticals) different sequential combinations of bath treatment pharmaceuticals are also being used to improve efficiency.

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 pharmaceuticals used have raised concerns over the survival and wellbeing of populations of non-target crustaceans which represent key elements of many marine food webs (Langford et al., 2014). In Norway, there is a strong focus on the deep-water shrimp (*Pandalus borealis*), which is one of the most important commercial crustacean species. After a bath treatment of salmon with delousing agents added to the water, in the cage or in a well-boat, the treatment water is discharged to the environment as long as the discharge point is at least 500 m from any known shrimp field or fish spawning grounds. Hydrodynamic dispersion modelling indicates that 100 – 1000 times diluted concentrations of the pharmaceuticals can be present more than one kilometer away from the discharge point (Ernst et al., 2001, 2014; Page et al., 2014; Refseth et al., 2016).

H_2O_2 is considered the most environmentally friendly pharmaceutical used for salmon lice control because it rapidly breaks down to oxygen and water. Large volumes of this pharmaceutical have therefore been used by the Norwegian aquaculture industry (ca. 43 000 metric tonnes in 2015, ca. 9 000 metric tonnes in 2017 (Remen and Sæther, 2018). 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 (Refseth et al., 2016). 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. Sinking of H_2O_2 will coincide with the time of year when deep water shrimp carry eggs. It is known that H_2O_2 affect reproduction in salmon lice by reducing hatching success and development of early life stages (McAndrew et al., 1998; Toovey and Lyndon 2000; Aaen et al., 2014), and there is also concern that embryo development and hatching success of deep-water shrimp may be affected. There is limited knowledge of the effects of H_2O_2 on egg-bearing shrimp or on eggs and embryos.

It has been demonstrated that H_2O_2 can induce sub-lethal effects in non-target species, 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, and 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).

Additionally, other bath treatments, such as azamethiphos (AZA; trade name Salmosan/Azasure) and deltamethrin (DEL; trade name AlphaMax), can potentially impact survival and the reproductive cycle of shrimp. DEL is a pyrethroid and acts 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). It has a low water solubility, and the half-life of DEL in the water column is 2-4 hours (Muir et al., 1985). Laboratory and field studies have shown that DEL is toxic to crustaceans (e.g. Crane et al., 2011; Burridge and Van Geest, 2014; Urbina et al., 2019), with reported LC_{50}-values (24 h exposures) ranging from 0.0006 µg/L for stage ll larvae of American lobster (*Homarus americanus*) (Burridge et al., 2014) to > 9.4 µg/L for neonates of *Daphnia magna* (Toumi et al., 2013). The recommended user dose of DEL for control of sea lice of 2 µg/L is above the LC_{50}-values reported for most crustaceans (Urbina el al., 2019). AZA is a water-soluble organophosphate that 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 DEL (Burridge and Van Geest, 2014). Organophosphates are neurotoxic and inhibit acetylcholinesterase activity 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 DEL and AZA are known to affect locomotion capability and behavior 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 bath treatments and combinations of bath treatments in deep water shrimp. Since the delousing agents are rapidly diluted in the sea, it is important to study both lethal and relevant sub-lethal effects at low concentrations. The aim of our study was to examine acute and delayed lethal and sub-lethal effects on egg-carrying deep-water shrimp exposed to environmentally realistic concentrations of three bath treatments alone (H_2O_2, AZA, DEL), or in sequential use with each other (H_2O_2 and AZA, H_2O_2 and DEL, AZA and DEL).. All exposures lasted for two hours to simulate a realistic environmental exposure time at a given distance from the release point. Exposure concentrations were therefore defined as dilutions of recommended treatment doses of the different bath pharmaceuticals. Effects were assessed in terms of shrimp behavior and mortality, egg loss, embryo development and -mortality. Further, acute DNA-damage was assessed in shrimp tissue and eggs after exposure to a high (16 mg/L) H_2O_2 concentration.

2. Materials and Methods

2.1. Exposure scenarios

Three exposure experiments on egg-carrying shrimp were carried out in the periods January 25th – February 22nd 2018 (experiment one; Exp1), February 14th – March 14th 2018 (experiment two; Exp2) and February 21st – March 12th 2019 (experiment three; Exp3).

In Exp1, lethal and sub-lethal effects of exposure to 1000, 500 and 100-times dilutions of the recommended treatment dose of H_2O_2 (1600mg/L) were investigated (Table 1).

In Exp2, lethal and sub-lethal effects of the three different bath pharmaceuticals, H_2O_2, AZA (recommended treatment dose: 100μg/L) and DEL (recommended treatment dose: 2.0 μg/L), alone or in sequential treatments were investigated. Five hundred times diluted treatment doses for H_2O_2 and AZA, and 330[1] times diluted treatment dose for DEL were selected for this experiment (Table 1**Error! Reference source not found.**).

Due to high mortality in some treatments of Exp2, a third experiment (Exp3) was set up to repeat Exp2, but with lower (sub-lethal) concentrations. Three pilot trials were conducted to determine the threshold between lethal and sub-lethal concentrations of the pharmaceuticals (see Supplemental Information Table S1 for pilot trial details and results). Based on the pilot trial results, 1000-times diluted treatment doses were selected for treatments including H_2O_2 and AZA. The pilot trials did however fail to determine the limit between lethal and sub-lethal DEL concentrations, and a DEL concentration range (10 000, 100 000 and 1 000 000 times dilution of recommended treatment dose) was selected for Exp3 (Table 1) instead of sequential treatments with this pharmaceutical.

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

Experiment	Treatment	Dilution of reccomended treatment dose	Nominal exposure concentration	Number of replicates	Number of shrimp per replicate	Weekly sub-sampling	Post-exposure period (days)
Exp1	Control	-	-	3	25-26	Yes	28
	H_2O_2 1000	1000	1.6 mg/L	3	25		
	H_2O_2 500	500	3.2 mg/L	3	24-25		
	H_2O_2 100	100	16 mg/L	3	24-26		
Exp2	Control	-	-	3*	25	Yes	29
	AZA	500	200 ng/L	3	25		
	AZA / DEL	500 / 330	200 ng/L / 6 ng/L	3	25-27		
	DEL	330	6 ng/L	3	25-27		
	H_2O_2 / AZA	500 / 500	3.2 mg/L / 200 ng/L	3	25		
	H_2O_2 / DEL	500 / 330	3.2 mg/L / 6 ng/L	3	25-26		
Exp3	Control	-	-	3	25	No	19
	H_2O_2	1000	1.6 mg/L	3	25		
	AZA	1000	100 ng/L	3	25		
	H_2O_2 / AZA	1000 / 1000	1.6 mg/L / 100 ng/L	3	25		
	DEL1	1 000 000	0.002 ng/L	3	25		
	DEL2	100 000	0.02 ng/L	3	25		
	DEL3	10 000	0.2 ng/L	3	25		

* Due to technical failure, one replicate control tank was lost 13 days post exposure (T13d).

[1] *Recommended treatment dose for deltamethrin is 2 μg/g. However, through personal communication with fish farmers, we learned that the deltamethrin dose most often used is 1.5 – 2 times higher than this, i.e. from 3 – 4 μg/l. We therefore selected 500 times dilution of 3 μg/g in the deltamethrin exposures in Exp2 that equals 330 times dilution of 2 μg/L.*

4

2.2. Shrimp collection and maintenance

Egg-carrying shrimp were collected in three different fjords in Northern Norway at four different occasions: By shrimp pots in the inner part of Porsangerfjorden during November 2017 and October 2018, and by trawl in Balsfjorden and Malangen in January 2018. 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 were minimum 10 days and holding times prior to experiments were maximum four months. Holding tanks were supplied with 60 μm filtered running seawater of ambient temperature (2.0 – 5.1 °C) 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 shrimp were fed in excess with frozen *Calanus* spp. three times a week.

2.3. Baseline measurements of experimental shrimp stock-batches

The shrimp used for Exp1 was a mix of Porsangerfjord 2017 and Balsfjord 2018 shrimp. The Malangen 2018 shrimp were used in Exp2, and the Exp3 shrimp were collected in Porsangerfjord in 2018. 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 individual shrimp size distribution, embryonic developmental stage, egg size, gonadosomatic index (GSI) and relative fecundity (number of eggs per g shrimp) (see Supplemental Information Table S2, Fig. S1 for results).

Shrimp total length (± 1.0 mm) and weight (± 0.001 g) were measured before all eggs were removed and the egg mass weighted separately. The eggs were fixed for 10-15 minutes in methanol, acetic acid and distilled water (dH_2O) at the ratio 1:1:1 (fix 1), and thereafter stored in 37% formalin, glycerol, ethanol, acetic acid and dH_2O at the ratio 2:1:3:1:3 (fix 2) for later egg counting and embryo development staging. Total number of eggs was counted and embryo developmental stage was 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. Linear regressions between total number of eggs and egg mass weight was modelled for each experimental shrimp stock-batch and the linear regression equations were used to calculate relative fecundity (eggs per g shrimp). GSI was calculated according to the equation

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

where W_{egg} is the egg mass weight (g) and W_{shrimp} is the shrimp weight (g).

2.4. Experimental set-up and analysis

All exposure experiments were conducted in 60 L flow-through tanks. The water level in each tank was set to 45 L and the tanks were supplied with 60 μm-filtered seawater of ambient temperature (2.0 – 5.1 °C) and salinity (33–34‰) at a flow rate of 45 L/h. Shrimp were placed into the exposure tanks 48 hours prior to exposure for acclimation to the new tanks. To minimize stress during acclimation and exposure, no feeding was undertaken during the acclimation period and the following exposure day.

All exposures lasted for two hours. For sequential exposures, a one-hour break between exposure 1 and exposure 2 allowed for replacement of the water containing pharmaceutical 1 before pharmaceutical 2 was introduced. At the start of each exposure, the water flow was stopped, and 5 L water removed from each exposure tank. This water was then replaced with 5L seawater containing bath pharmaceutical 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 pharmaceuticals 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 (Supplemental Information Fig. S2). H_2O_2 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 (CHEMetrics®, US) (Supplemental Information Table S3).

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 normal behavior (standing or swimming normally) or abnormal behavior (stress swimming (erratic panic swimming) and lying on the side (immobilized)). Feeding resumed the day following the exposure (T1d), and food (frozen *Calanus* spp.) was provided in excess throughout the post-exposure period. In Exp3, a mix of frozen *Calanus* spp. and fish pellets was provided as food.

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). Sampled shrimp were analyzed for total weight, embryonic developmental stage, relative fecundity (number of eggs per g shrimp) and percentage dead eggs (see section 2.3. for methodology). In Exp1, additional samples of controls and the high (H_2O_2 100) treatment shrimp were taken for DNA damage analysis at T0d: Approximately 0.2 g of the egg mass as well as 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, all shrimp included in the experiment were weighted prior to exposure start, and at the end of the experiment, and whole shrimp (exclusive of eggs) were stored at -20 °C for later analysis of total lipid.

2.5. DNA damage

The fast micromethod was followed (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 (USA Scientific Inc.), 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 out on ice, in the dark, and unwinding was initiated by increasing pH with the addition of alkaline unwinding solution (20 mM EDTA, 1M NaOH, pH 12.4 ± 0.1). Flourescence was detected (kinetic mode, excitation 480 nm, emission 520 nm, POLARstar Omega plate reader, BMG LABTECH) 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).

2.6. Statistical analyses

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.

3. Results

3.1. Shrimp behavior and mortality

In Exp1, there was no significant difference in behavior or mortality between treatments. Percentage shrimp performing normal behavior ranged between 90.0 – 100 % in the different tanks throughout the experiment. Overall, more shrimp were laying on their side in the period T1d – T5d (range 2.0 – 10.4 % per tank; average 6.8 ± 2.8 %) post-exposure compared to any other periods of the experiment (range 0 – 7.5 % per tank; average 1.1 ± 1.8 %). Overall, few incidences of stress swimming were observed throughout the experimental period (data not shown). Mortality ranged between 0 – 23 % in the different tanks, and most (91 %) of the shrimp that died during the experiment, died within the first week post exposure (T7d) (Fig. 1; left panels).

In Exp2, significantly more shrimp in the DEL, AZA/DEL and H_2O_2/DEL treatments were performing abnormal behavior in the period T1d - T3d compared to controls, AZA and H_2O_2/AZA treatments (Fig. 2). In the period T1h – T5h, stress swimming ranged between 1.3 and 18.6 % in DEL, AZA/DEL and H_2O_2/DEL compared to 1.3 – 4.0 % in controls, AZA and H_2O_2/AZA treatments, In the period T1d – T3d, 45.0 - 100 % of the shrimp in DEL, AZA/DEL and H_2O_2/DEL were laying on their side compared 0 – 32.8 % in controls, AZA and H_2O_2/AZA treatments. From T6d and onward, all surviving shrimp were generally observed standing (normal behavior). Mortality ranged between 0 – 81 % in the different tanks (Fig. 1; middle panels), 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 by T3d (DEL; 93.6 %, AZA/DEL; 93.4 %, and H_2O_2/DEL; 87.1 %). At T9d, 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 were dead or sampled at this time point.

Similar to Exp1, there were no significant difference in behavior or 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 by T7d. Mortality ranged between 0 – 40 % in the different tanks (Fig.1; right panel). Most shrimp were observed standing throughout the experiment, except one shrimp in one of the H_2O_2-replicates that lay on the side throughout the experiment. Except for this shrimp, 1-2 shrimp were observed laying on the side randomly in 1 to 4 of the 21 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 were observed, but with no connection to exposure time or treatment (data not shown).

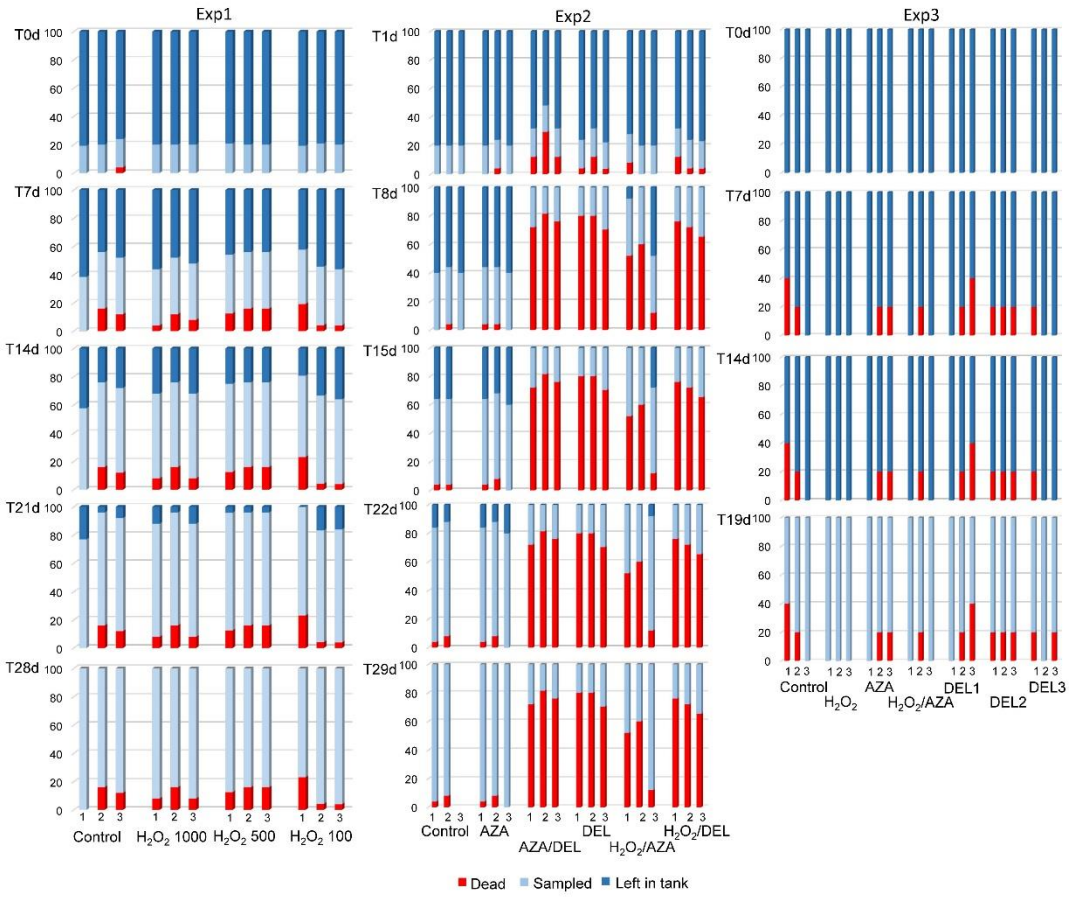

Figure 1. Percent cumulative mortality and sampling of shrimp in all experiments. Left panels: Experiment 1 (Exp1), T0d; n = 24 - 26 shrimp per tank. Middle panels: Experiment 2 (Exp2), T0d; n = 25 - 27 shrimp per tank. Right panels: Experiment 3 (Exp.3), T0d; n = 5 shrimp per tank. See Table 1. for designation of treatment acronyms- and concentrations.

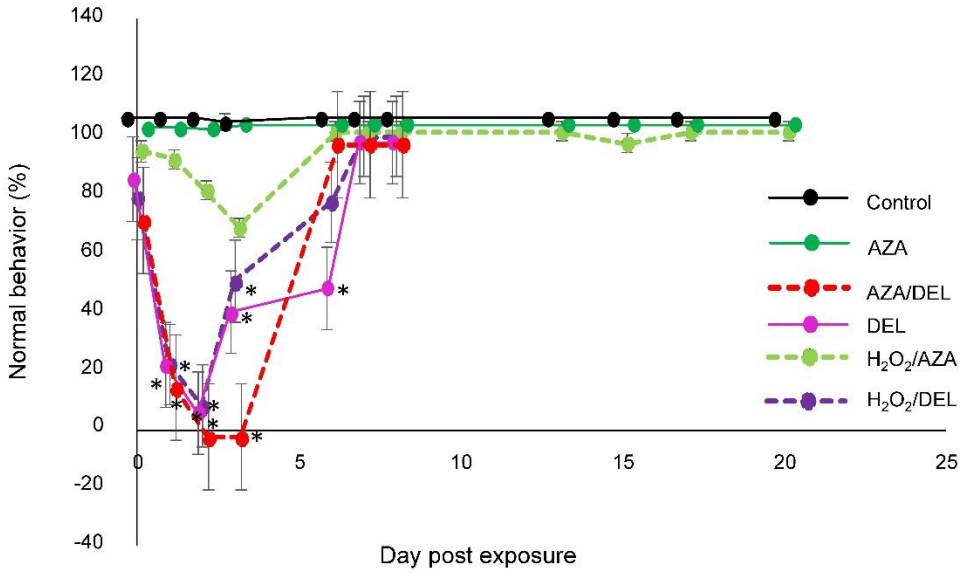

Figure 2. Percentage shrimp performing normal behavior in Exp2 throughout the experimental period. N=3 tanks per treatment. T0d, n = 25 - 27 shrimp per tank. Number of shrimp per tank decreased throughout the post exposure period due to subsampling and mortality (see Supplemental Information Table S4 for overview). *: Significant different from control. See Table 1. for designation of treatment acronyms- and concentrations.

3.2. Egg loss, embryo development and reproductive output

3.2.1. 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 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 treatments (data not shown). 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 % (19 out of 266) of the shrimp sampled when alive 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 Exp1.

In Exp2, all shrimp treated with DEL alone 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. 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 (data not shown). Ten of the shrimp that lost all their eggs died during the experiment (T1d –T17d) whereas 9 of the remaining 10 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 eggs from the remaining stock batch shrimp did not hatch until early April (approximately 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 (data not shown). Four of the shrimp without eggs died during the experiment whereas 14 were sampled at the end of the experiment (T19d). In Exp3 hatching started 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 was 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 was no significant difference in number of hatched larvae between the treatments of Exp3.

3.2.2. Embryo development and reproductive output

Overall, no significant sub-lethal effects on embryo development or reproductive output of any of the tested bath pharmaceuticals were revealed.

In Exp1, there were no significant differences in total shrimp weight, relative fecundity or embryo eye diameter between any treatments at any sampling point throughout the experiment (Table 2, Supplemental Information Fig. S3). Embryo eye diameters were bigger in all treatments compared to the baseline shrimp sampled prior to Exp1, indicating progressive embryo development throughout the experimental period in all treatments. Percentage dead eggs was significantly higher in controls than in the H_2O_2 treatments at T21d (Table 2). DNA damage in controls and the high H_2O_2 treatments (H_2O_2 100; 16 mg/L) shrimp sampled at T0d showed high variability in DNA damage between individuals (Fig. 3). 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.

Table 2. Overview of shrimp weight (g), relative fecundity (number of eggs per g shrimp), % dead eggs, and embryo eye diameter (average ± standard deviation; all three replicates per treatment combined) measured at the different sampling point of Exp1 (T0d, T7d, T14d, T21d and T28d). Embryo eye diameters; average of 10 embryos per shrimp. Different letters behind values indicate significant difference between treatments. See Table 1. for designation of treatment concentrations.

Days post exposure	Treatment	Shrimp weight (g)	*n*	Relative fecundity	*n*	% dead eggs	*n*	Embryo eye diameter (mm)	*n*
0	**Control**	10.75 ± 1.22	*15*	134 ± 55	*15*	0.7 ± 0.9	*10*		
0	**H_2O_2 1000**	9.79 ± 2.20	*15*	153 ± 74	*15*	1.5 ± 2.0	*15*		
0	**H_2O_2 500**	10.28 ± 1.57	*15*	111 ± 61	*15*	1.4 ± 1.8	*14*		
0	**H_2O_2 100**	10.19 ± 1.63	*15*	143 ± 45	*15*	1.4 ± 1.0	*14*		
7	**Control**	9.56 ± 2.04	*15*	155 ± 52	*15*	1.0 ± 0.8	*9*	0.15 ± 0.09	*5*
7	**H_2O_2 1000**	10.83 ± 2.31	*15*	144 ± 60	*15*	2.0 ± 1.9	*14*	0.10 ± 0.02	*3*
7	**H_2O_2 500**	10.76 ± 2.04	*15*	135 ± 57	*15*	0.9 ± 0.8	*12*	0.13 ± 0.12	*3*
7	**H_2O_2 100**	10.44 ± 1.87	*15*	108 ± 65	*15*	1.3 ± 1.0	*12*	0.13 ± 0.07	*4*
14	**Control**	9.82 ± 1.48	*15*	141 ± 50	*15*	3.5 ± 2.4	*15*		
14	**H_2O_2 1000**	9.91 ± 1.65	*15*	146 ± 60	*14*	4.8 ± 3.4	*14*		
14	**H_2O_2 500**	10.67 ± 2.79	*15*	172 ± 37	*15*	5.0 ± 4.1	*14*		
14	**H_2O_2 100**	10.05 ± 1.54	*15*	158 ± 34	*15*	5.6 ± 4.1	*15*		
21	**Control**	10.61 ± 1.04	*15*	180 ± 26	*10*	4.8 ± 2.2[a]	*8*	0.10 ± 0.02	*7*
21	**H_2O_2 1000**	9.79 ± 2.52	*15*	159 ± 127	*14*	1.4 ± 1.7[b]	*12*	0.10 ± 0.04	*11*
21	**H_2O_2 500**	9.97 ± 1.31	*15*	158 ± 43	*12*	1.5 ± 0.9[b]	*12*	0.10 ± 0.03	*12*
21	**H_2O_2 100**	9.76 ± 1.87	*14*	141 ± 47	*11*	1.7 ± 2.3[b]	*11*	0.13 ± 0.07	*10*
28	**Control**	9.59 ± 2.97	*9*	150 ± 22	*7*	2.1 ± 1.2	*7*	0.11 ± 0.02	*7*
28	**H_2O_2 1000**	10.65 ± 1.69	*7*	184 ± 15	*5*	2.5 ± 1.3	*4*	0.14 ± 0.02	*5*
28	**H_2O_2 500**	9.26 ± 1.05	*3*	185 ± 22	*3*	3.6 ± 2.3	*3*	0.15 ± 0.04	*3*
28	**H_2O_2 100**	10.35 ± 1.67	*8*	172 ± 16	*7*	2.4 ± 1.7	*7*	0.13 ± 0.06	*7*

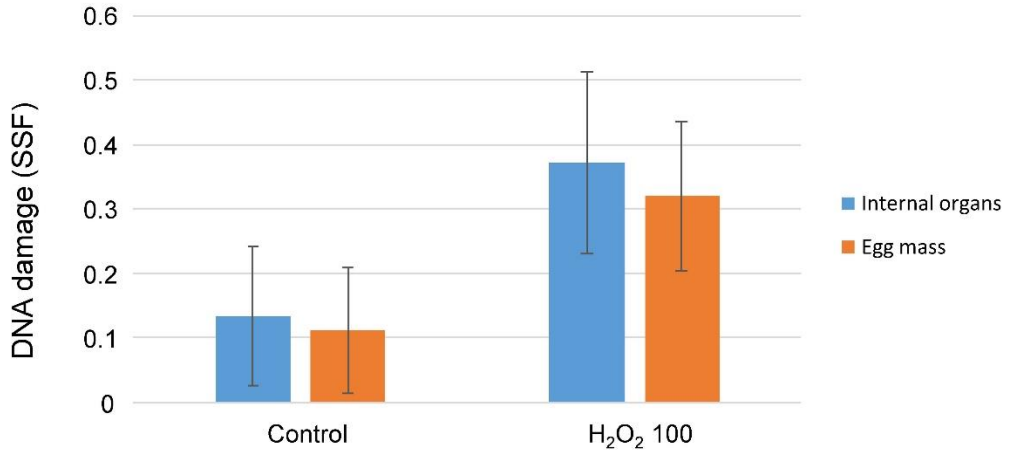

Figure 3. DNA damage (strand scission factor) in shrimp internal organs and egg mass tissues after exposure to 16 mg/L H_2O_2 (T0d). Data are average ± standard error of mean, n=15 shrimp per treatment. See Table 1. for designation of treatment acronyms- and concentrations.

Due to the high mortality in some treatments of Exp2, only the first sampling point one day post exposure (T1d) represented a full dataset including all replicate tanks. There was no significant treatment effect on relative fecundity or % dead eggs at this time point (Supplemental Information Fig. S4), 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 and 6 ng/L DEL, 3.2 mg/L H_2O_2 and 6 ng/L DEL, or 3.2 mg/L H_2O_2 and 200 ng/L AZA. Shrimp weight and embryo eye diameter were similar in all treatments, confirming similar weight range and ranges of embryonic developmental stages in all shrimp at the start of the experiments.

Like Exp1, there were no significant differences in Exp3 between treatments in shrimp weight, relative fecundity, percentage dead eggs or embryo eye diameter at the end of the experiment (T19d) (Fig. 4). Further, hatching was initiated in all tanks with no significant difference in number of hatched larvae between treatments (Supplemental Information Fig. S5). No abnormalities were observed in any of the hatched larvae of any of the treatments (data not shown).

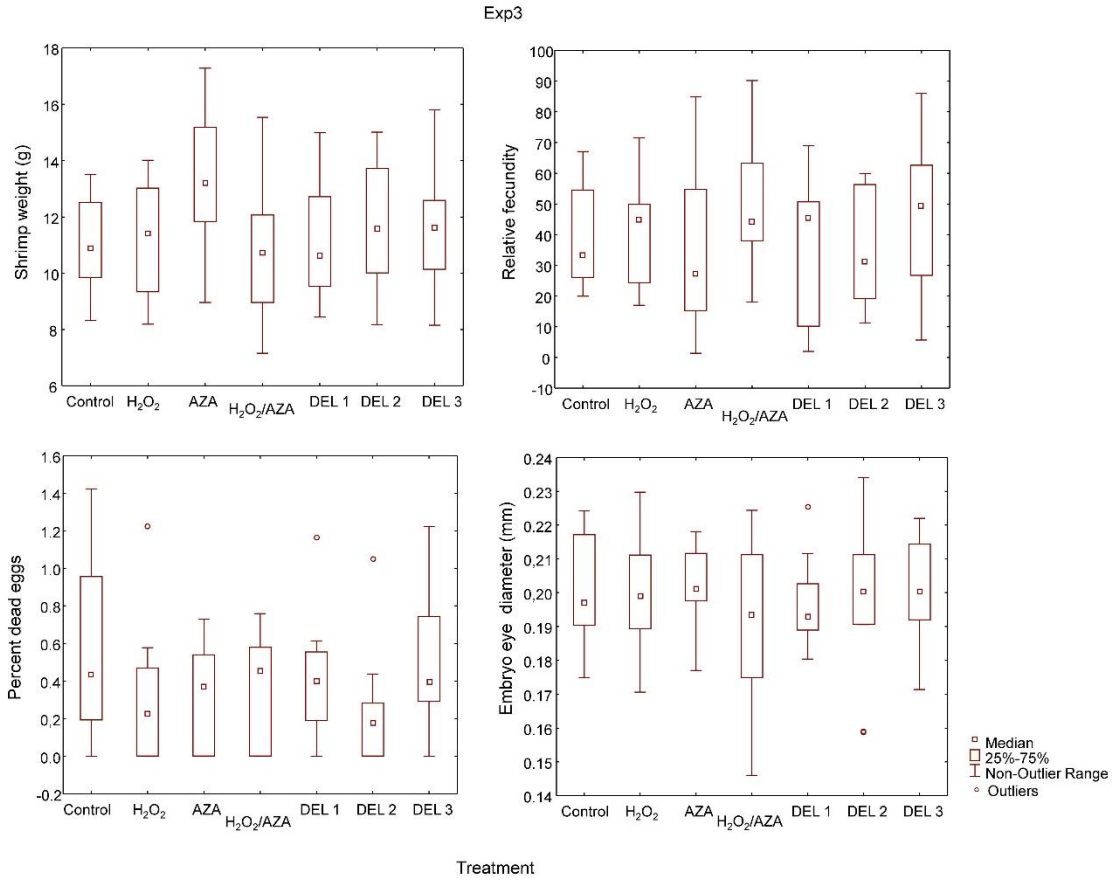

Figure 4. Shrimp weight (g), relative fecundity (number of eggs per g shrimp), % dead eggs, and embryo eye diameter measured at the end of Exp3 (T19d). N=12-15 shrimp per treatment. Egg/embryo eye diameters; average of 10 eggs/embryos per shrimp. No significant difference between treatments was revealed. See Table 1. for designation of treatment acronyms- and concentrations.

4. Discussion

4.1. Experimental conditions

In the present study *P. borealis* was exposed to H_2O_2, DEL and AZA in water in short (2 hours) single-pulse exposures to reflect the acute nature of effluent plumes from aquaculture sites. In addition, sequential exposures with H_2O_2 and AZA, H_2O_2 and DEL and AZA and DEL were performed. The concentrations selected represent environmentally realistic concentrations. Field studies of dispersion of AZA and DEL from aquaculture sites have found that effluent plumes are detectable 2 to 5.5 hours after release at distances 0.9 to 3 km from the cage site, with pharmaceutical concentrations equaling 1/1000 to 1/2000 of the pre-release concentrations (Ernst et al., 2001, 2014).

4.2. Shrimp mortality

Overall, our experiments showed no acute or delayed mortality in egg-carrying shrimp exposed to H_2O_2 at the concentration range 1.6 – 16 mg/L (100 – 1 000 times diluted treatment dose), to AZA at the concentration range 100 - 200 ng/L (500 – 1 000 times diluted treatment dose) or to DEL at the concentration range 0.002 – 0.2 ng/L (10 000 – 1000 000 times diluted treatment dose). A DEL concentration of 6 ng/L (330 times diluted treatment dose) was however highly toxic and did cause acute (i.e. < 96h) mortality in almost all exposed shrimp across single and sequential exposure groups.

Our H_2O_2 results from the 2 hours exposure (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 mg/L in exposures lasting for one hour (reviewed by Urbina et al., 2019). Our results are however in contrast to those reported by Bechmann et al. (2019) who found that a 2 hours exposure to 100 times diluted treatment solution (15 mg/L H_2O_2) or three executive pulses of 1.5 mg/L H_2O_2 (i.e. 1000 times dilution) at 6.8 °C (versus 3.5 °C in this study), induced mortality in maturing *P. borealis*. Variations in toxicity can possibly be explained by temperature differences, as the toxicity of H_2O_2 in general increases with increasing temperatures, or by seasonal variations in sensitivity (Urbina et al. 2019).

Short term exposures (0.5 - 6 hours) to AZA have shown LC_{50} values ranging between 2.8 – 37.7 µg/L in American lobster (*Homarus americanus*) (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. The results from our study confirm results from previous studies that show that the toxicity of AZA is much lower than that of DEL, and that concentrations that may have a negative impact on deep-water shrimp will only occur close to the discharge site (Ernst et al. 2014).

Although no acute or delayed mortality was seen in shrimp exposed to either 3.2 mg/L H_2O_2 or 200 ng/L AZA (i.e. 500 times diluted recommended treatment doses), a sequential treatment with these concentrations of the two bath pharmaceuticals induced delayed mortality (T4d-T8d post exposure) in the exposed shrimp, indicating an synergistic effect of the pharmaceuticals. This result contrasts with findings by Burridge and Van Geest (2014) that did not find any additive or synergistic effect of exposing mysid shrimp for 1 hour to H_2O_2 and thereafter to AZA (i.e. no changes in LC_{50}-values when compared to single - pulse exposure to the pharmaceuticals; exposure concentrations not reported). More research is needed to assess limits for effects and to understand mechanisms causing mortality when these two pharmaceuticals are used in combination treatment. In our study, no delayed mortality was seen in shrimp sequentially exposed to 1000 times diluted recommended treatment doses of H_2O_2 (1.6 mg/L) and AZA (100 ng/L) (Exp.3).

All exposures that included DEL in Exp2 (6 ng/L DEL, 200 ng/L AZA followed by 6 ng/L DEL, and 3,2 mg/L H_2O_2 followed by 6 ng/L DEL) induced high mortality. Due to the high mortality caused by DEL, our study failed to reveal potential synergistic effects by these sequential treatments (i.e. potential synergistic effects were camouflaged by DEL). A synergistic mortality effect of simultaneous treatment to AZA and DEL (1 and 24 hours exposure) has previously been shown for the shrimps *P. flexuosus* and *P. elegans* in terms of significantly decreased LC_{50}-values for both pharmaceuticals (Brokke, 2015).

Due to the high mortality in all treatments containing DEL of Exp2, three pilot trials were run for one week each to determine the threshold between lethal and sub-lethal concentrations of this pharmaceutical (see Supplemental Information for summary of results). A high but inconsistent mortality rate was seen in all pilot trials (Supplemental Information Table S1). These mortality results made it impossible to select one concentration for sequential treatments, and a DEL concentration gradient (10 000, 100 000 and 1 000 000-times dilution) was selected for Exp3. The Exp3 DEL results contradicted the results of the pilot trials and our study could only estimate that DEL induce mortality at a concentration somewhere between 0.2 – 6.0 ng/L. Although, *P. borealis* is highly sensitive to DEL, and comparable to of other sensitive crustaceans investigated; published crustacean DEL LC_{50} in exposures lasting for 1 hour range between 3.4 – 142 ng/L, with American lobster larvae being most sensitive and *Crangon septemspinosa* shrimp being least sensitive (reviewed by Urbina et al., 2019). Bechmann et al. (pers. comm.) found increased mortality in *P. borealis* larvae when exposed to 2 ng/L DEL. The lethal toxicity mechanism of DEL seen in the pilot trials is unknown.

4.3. Behavior

Several behavioral endpoints were studied, including swimming activity and immobility (lying on the side).. Behavioral endpoints, and especially those related to immobility are important, as in the field an immobilized organism is unable to feed, seek shelter, or avoid predation (van Geest et al. 2014).

In Exp1 there were no significant difference in behavioral endpoints between treatments (1.6 – 16 mg/g H_2O_2). However, more shrimp were laying on their side in the period T1d – T5d post-exposure compared to any other periods of the experiment. This may indicate a reduced ability to respond to stress, e.g. an approaching predator. Our finding contrasts with 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. When it comes to effects of H_2O_2 the size of the organisms is thought to be important, as it probably affects the surface of the organisms (i.e. the carapace) (Hansen et al. 2017), and the bigger size of the shrimp (volume:surface-ratio) compared to copepods could possibly explain differences in responses.

In Exp2, shrimp that were exposed to DEL (6 ng/L) alone or in sequential treatment were more frequently stress swimming or lying on the side than those exposed to H_2O_2 (3.2 mg/L) or AZA (200 ng/L) alone or in sequential treatment. 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 hours exposure at 18 °C (Oliveira et al., 2012). Also, larvae from the crab *Metacarcinus edwardsii* that were 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). These larvae all died within 24 hours after exposure. AZA 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 hour under static conditions showed changes in behavior and an increased numbers of moribund (non-responsive but respiring) or dead individuals (Burridge and Van Geest, 2014).

No significant effects on behavior was seen in Exp3, indicating that exposure concentrations were below the no-effect concentration (NEC). Although no significant behavior effects were seen in Exp.1 and Exp.3, immobilized shrimp observed in the three experiments generally reflected shrimps that died within a few days.

4.4. Egg loss, embryo development and reproductive output

Overall, our results did not indicate any egg loss or negative effects on embryo development and reproductive output due to the bath treatment pharmaceuticals.

A higher percentage of total egg loss was seen in shrimp dying during the experiments but with no significant difference between treatments. No negative effects of H_2O_2 in the concentration range 1.6 - 16 mg/L were observed for shrimp embryo development or reproductive output (Exp1 and 3). H_2O_2 has been used as a disinfectant for fish eggs, and eggs in rearing facilities has been shown to have a relatively low sensitivity to H_2O_2. This is also the case for eggs from Atlantic cod (*Gadus morhua*) that had a LC_{50}-value of 342 mg/L (Refseth et al. 2016). The chorion may have a protective effect, both in fish and shrimp, but it cannot be ruled out that the early larval stages are more sensitive.

No sub-lethal effects on embryo development of 100 ng/L AZA or sequential treatment with 1.6 ml/L H_2O_2/100 ng/L AZA or DEL in the concentration range 0.002 – 0.2 ng/L were observed. Burridge et al. (2008) showed that repeated exposures to 10 ng/L AZA can have a negative effect on the survival and reproduction of the American lobster, but that 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 80 and 150 ng/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).

To our knowledge, this is the first study of egg loss and embryo-development in deep-water shrimp exposed to pharmaceuticals used for de-lousing in aquaculture. The results from our study indicate that the survival of the adult shrimp is most critical to produce viable offspring. The tested pharmaceuticals seem to have no effects on embryo development at the concentrations tested. However, if the egg-carrying shrimp dies the embryos will probably also die before hatching. Thus, the result indicates that bath treatments will exert their effects on eggs/embryos via their effects on the egg-carrying females rather than directly by damaging eggs/embryos.

4.5. DNA-damage by H_2O_2

It has been demonstrated that H_2O_2 can induce sub-lethal effects in non-target species for instance through the production of reactive oxygen species, which in turn can induce DNA damage. No significant difference in acute DNA damage levels were found in adult tissue or egg mass embryos between control and individuals exposed to the highest (16 mg/L) H_2O_2 concentration. Embryos of grass shrimp (*Paleomonetes pugio*) are known to be sensitive to DNA strand breaks after exposure to H_2O_2 concentrations as low as 0.34 mg/L (Hook and Lee, 2004), and significantly increased levels of DNA damage was detected in immune cells of a range of echinoderm (sea urchins and a sea cucumber) at concentrations of H_2O_2 down to 3.4 mg/L (El-Bibany et al., 2014). The LC_{50} value for H_2O_2 is estimated at 20.4 mg/L for larvae of sea urchins (*Lytechinus variegatus*) (Reinardy and Bodnar 2015). Hansen et al. (2017) found that H_2O_2-concentrations close to the LC_{50}-value did not cause oxidative stress in *Calanus*

finmarchicus, but they did not study DNA-damage in the exposed individuals. In general, there is a lack of data of H_2O_2-induced genotoxicity in marine invertebrates.

5. Conclusions

DEL 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 pharmaceutical. The limit concentration for no lethal or sub-lethal effects of DEL on egg-carrying shrimp remains to be established. Further, the synergistic effect of H_2O_2 and AZA, inducing >40% mortality at a 500 times diluted treatment concentration, is of concern and emphasizes the need for more knowledge regarding the effects of combined/sequential treatments in general.

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; AZA: 500 times diluted treatment dose; DEL: ≥10 000 times diluted treatment dose) bath pharmaceuticals. Exposed shrimp were monitored for five weeks and no sub-lethal effects were seen on DNA damage (H_2O_2 only), behavior, 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 concentrations closer to the no effect concentration.

In this study shrimp were exposed to a short-term pulse of pharmaceuticals in concentrations that can be expected in the vicinity (10 – 1000 m) of aquaculture facilities after delousing in cages. However, in risk assessments the total pharmaceuticals load that non-target organisms can be exposed to needs to be taken into consideration. Further, it must be taken into consideration that often multiple cages are treated at one site within a short time frame (i.e. same day or subsequent days), and this could increase both the concentrations and types of pharmaceuticals, and the length of time over which non-target organisms are exposed to toxic concentrations. Balancing the risks to fish health through pharmaceutical delousing treatments against protection of the wider environment from treatment discharge, is a very active area of concern and research from both industry and regulatory perspectives. The results from the present study constitute a valuable input to the knowledge base required for conducting environmental risk assessments for aquaculture industry areas.

Ethics statement

Permission to carry out experiments was granted by the Norwegian Animal Welfare Authority in 2017 (ID 13353).

Acknowledgement

This study was funded by Norwegian Seafood Research fund (project no. 901425) and Akvaplan-niva AS. We thank the staff from the Akvaplan-niva FISK facility for support during the experiments, as well as personnel onboard RV Hyas from UiT, the Arctic University of Norway for providing shrimp. Perrine Geraudie and Lisa Torske are thanked for technical assistance with DNA extractions and lipid analyses.

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677 **Supplemental information**

678 **S1. Pilot trials**

679 Pilot trials were conducted at static conditions in buckets filled with 20 L seawater. Five shrimp from the
680 holding tank were added to each bucket and left for 24 h hours for acclimation. Thereafter 5 L seawater
681 was removed from each bucket and replaced with 5L bath pharmaceutical in seawater. After two hours
682 exposure, the shrimp were moved to a flow-through tank supplied with clean running seawater and
683 observed for one week for behavior and mortality (Table S1).
684

685 *Table S1. Overview of exposures and results from pilot trials with H_2O_2/AZA (trial I) and DEL (trial I-III).*

Pilot trial	Treatment	Dilution of recommended treatment dose	Nominal exposure concentration	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	25 000	0.064 mg/L / 4 ng/L	1	5	0	
	H_2O_2 / AZA 2	5 000	0.32 mg/L / 20 ng/L	1	5	0	
	H_2O_2 / AZA 3	1 000	1.6 mg/L / 100 ng/L	1	5	0	
	DEL 1	25 000	0.08 ng/L	1	5	5	T2d -T5d
	DEL 2	5 000	0.4 ng/L	1	5	1	T5d
	DEL 3	1 000	2 ng/L	1	5	1	T7d
II	Control	-	-	1	5	0	
	DEL 1	25 000	0.08 ng/L	1	5	4	T1d - T2d
	DEL 2	5 000	0.4 ng/L	1	5	5	T1d - T2d
	DEL 3	1 000	2 ng/L	1	5	5	T1d - T2d
III	Control	-	-	1	5	0	
	DEL 4	25 000 000	0.00008 ng/L	1	5	5	T1d - T2d
	DEL 5	5 000 000	0.0004 ng/L	1	5	5	T1d - T2d
	DEL 6	1 000 000	0.002 ng/L	1	5	5	T1d

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S2. 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 S2). GSI was similar for Exp1 and Exp2 shrimp, whereas the GSI of the Porsangerfjord 2018 shrimp used for Exp3 was significantly lower. Proportion of dead eggs was <10 % in all three shrimp batches, however significantly higher in the Exp2 Malangen shrimp compared to the Exp1 mixed-origin 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 S2, Fig. S1).

Table S2. Overview of baseline measurements conducted on the shrimp batches used for Exp1, Exp2 and, respectively. BL: base line; GS: gonadosomatic index; relative fecundity; number of eggs per g shrimp. Linear regressions lines between total number of counted eggs and egg weight was modelled for each shrimp batch, and the linear regression equation was used to calculate relative fecundity in the corresponding experiments. Different letters behind values indicate significant difference between shrimp batch-stocks.

	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*

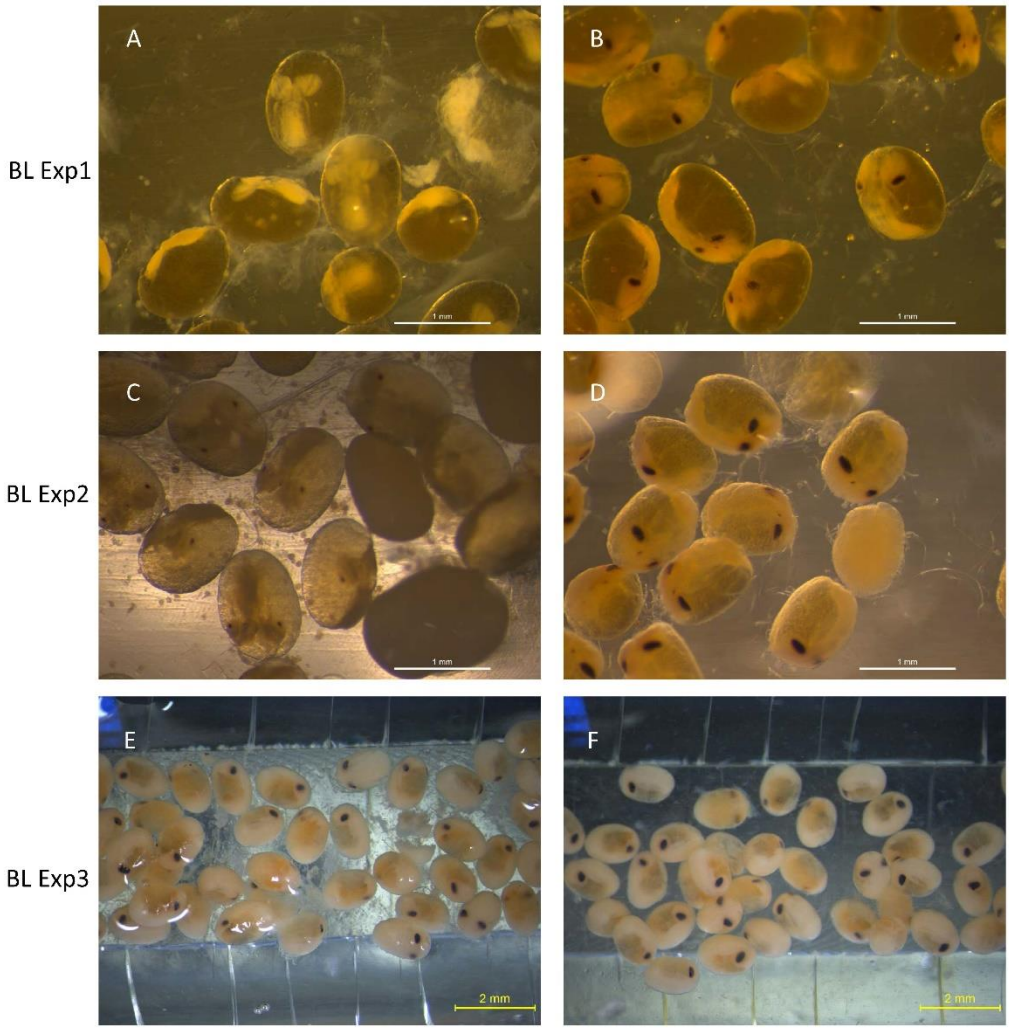

Figure S1. Minimum and maximum eye pigmentation stage in baseline shrimp sampled prior to the experiments. Exp1: A (minimum eye diameter; 0.00 mm) and B (maximum eye diameter; 0.08 mm), Exp2: C (minimum eye diameter; 0.03 mm) and D (maximum eye diameter; 0.12 mm) and Exp3: E (minimum eye diameter; 0.18 mm) and F (maximum eye diameter; 0.22 mm).

S3. Exposure conditions

Measured H_2O_2 concentration for all three experiments ranged between 75 – 93 % of the nominal concentration (Table S3). No control tanks, or exposure tanks not including H_2O_2, showed H_2O_2 concentrations above detection limit. Water concentrations of AZA and DEL in the exposure tanks were not measured. 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, assuming the same agreement between nominal and actual water concentrations for AZA and DEL as for H_2O_2.

Table S3. Nominal and measured H_2O_2 concentrations in treatments including H_2O_2 of experiment one (Exp1), experiment two (Exp2) and experiment three (Exp3). Measured concentrations represent average T0h (exposure start) and T2h (end of exposure) concentrations of three replicate tanks ± standard deviation (n=6 measurements per treatment).

Experiment	Treatment	Nominal H_2O_2 concentration (mg/L)	Measured H_2O_2 concentration (mg/L)
Exp1	H_2O_2 1000	1.6	1.2 ± 0.3
	H_2O_2 500	3.2	2.5 ± 0.3
	H_2O_2 100	16	14.8 ± 4.7
Exp2	H_2O_2 / AZA	3.2	2.5 ± 0.4
	H_2O_2 / DEL	3.2	2.4 ± 0.5
Exp3	H_2O_2	1.6	1.2 ± 0.3
	H_2O_2 / AZA	1.6	1.3 ± 0.2

Average water temperature measured in one control tank of Exp1, Exp2 and Exp3 throughout the experiments was 3.5 ± 0.3 (min.-max. range 2.9 – 4.1), 2.9 ± 0.6 (min.-max. range 2.0 – 3.9) and 4.0 ± 0.7 (min.-max. range 3.0 – 5.1) °C, respectively (Fig. S2). Average percentage oxygen saturation was 87.2 ± 2.4 (range 83 – 91), 105.4 ± 6.3 (range 97 – 113) and 101.0 ± 2.7 (range 97 – 104), respectively (Fig. S2).

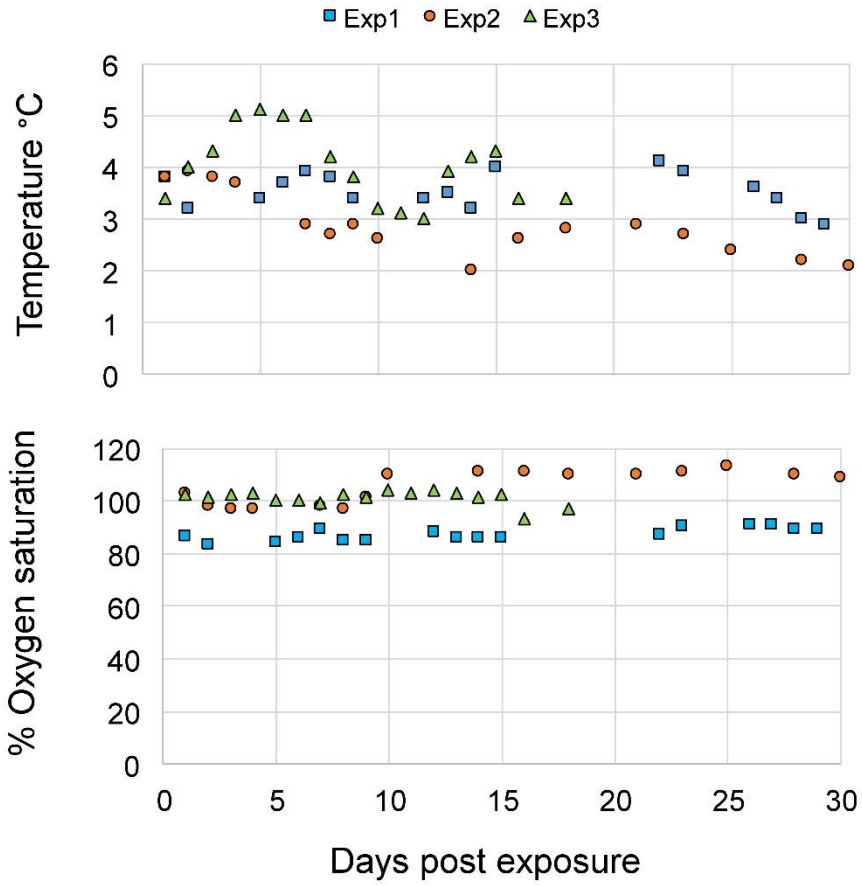

Figure S2. Control tank water temperature and oxygen saturation throughout Exp1 (blue square symbols), Exp2 (orange circular symbols) and Exp3 (green triangular symbols).

S4. Behavior observations

Table S4. Number of shrimp per tank during behavior observations of Exp2. R1-R3; replicate tank 1-3. s/d; no shrimp left because all shrimp were previously sampled (s) or dead (d).

	Control			AZA			AZA/DEL			DEL			H_2O_2/AZA			H_2O_2/DEL		
Day post exposure	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3
0	25	25	25	25	25	25	25	27	25	24	25	27	25	25	25	25	25	26
1	25	25	25	25	25	25	25	27	25	24	25	27	25	25	25	25	25	26
2	20	19	20	20	19	20	14	6	13	15	12	18	18	18	20	12	16	17
3	20	19	20	20	19	20	6	*s/d*	2	2	*s/d*	9	12	16	20	1	11	7
6	20	19	20	20	19	20	2	*s/d*	1	*s/d*	*s/d*	4	7	5	18	1	3	4
7	20	19	20	19	19	20	2	*s/d*	1	*s/d*	*s/d*	3	7	5	17	1	2	4
8	20	19	20	19	19	20	2	*s/d*	1	*s/d*	*s/d*	3	7	5	17	1	2	4
13	14	14		14	13	15	*s/d*	*s/d*	*s/d*	*s/d*	*s/d*	*s/d*	2	*s/d*	12	*s/d*	*s/d*	*s/d*
15	14	14		14	13	15	*s/d*	*s/d*	*s/d*	*s/d*	*s/d*	*s/d*	2	*s/d*	12	*s/d*	*s/d*	*s/d*
17	9	8		9	8	10	*s/d*	*s/d*	*s/d*	*s/d*	*s/d*	*s/d*	*s/d*	*s/d*	7	*s/d*	*s/d*	*s/d*
20	9	8		9	8	10	*s/d*	*s/d*	*s/d*	*s/d*	*s/d*	*s/d*	*s/d*	*s/d*	7	*s/d*	*s/d*	*s/d*

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803 **S5. Embryo development and reproductive output**

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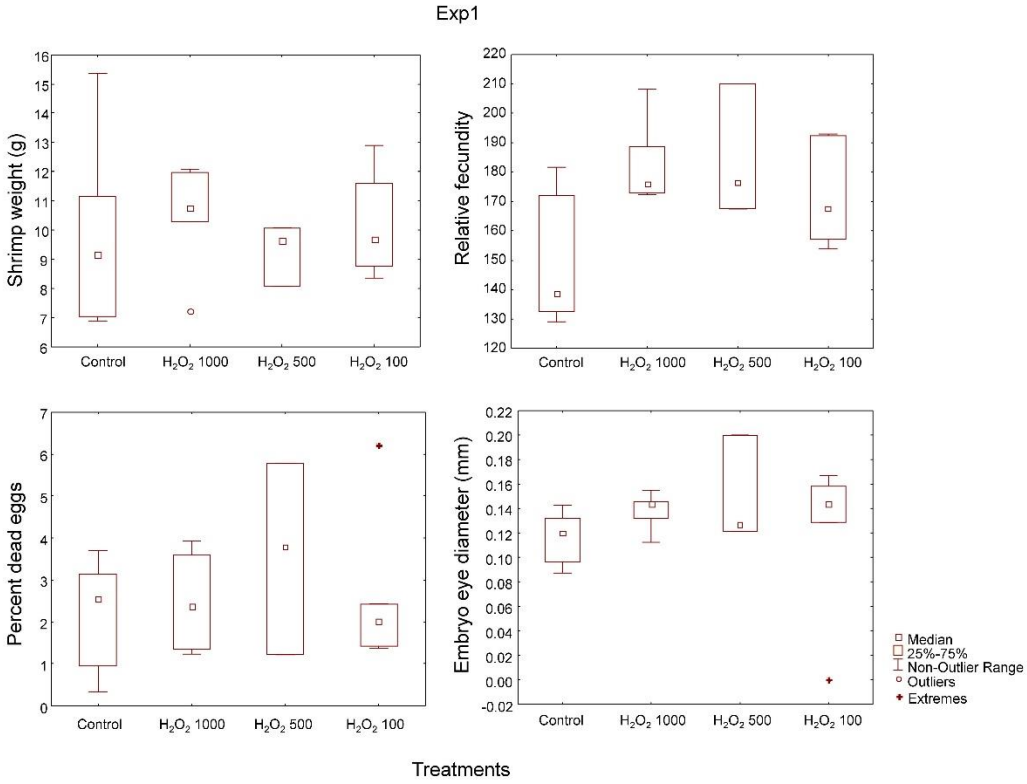

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806 Figure S3. Shrimp weight (g), relative fecundity (number of eggs per g shrimp), percent dead eggs and
807 embryo eye diameter measured at the end of Exp1 (T28d). N=3-9 shrimp per treatment. Embryo eye
808 diameters: average of 10 embryos per shrimp. No significant difference between treatments was revealed.

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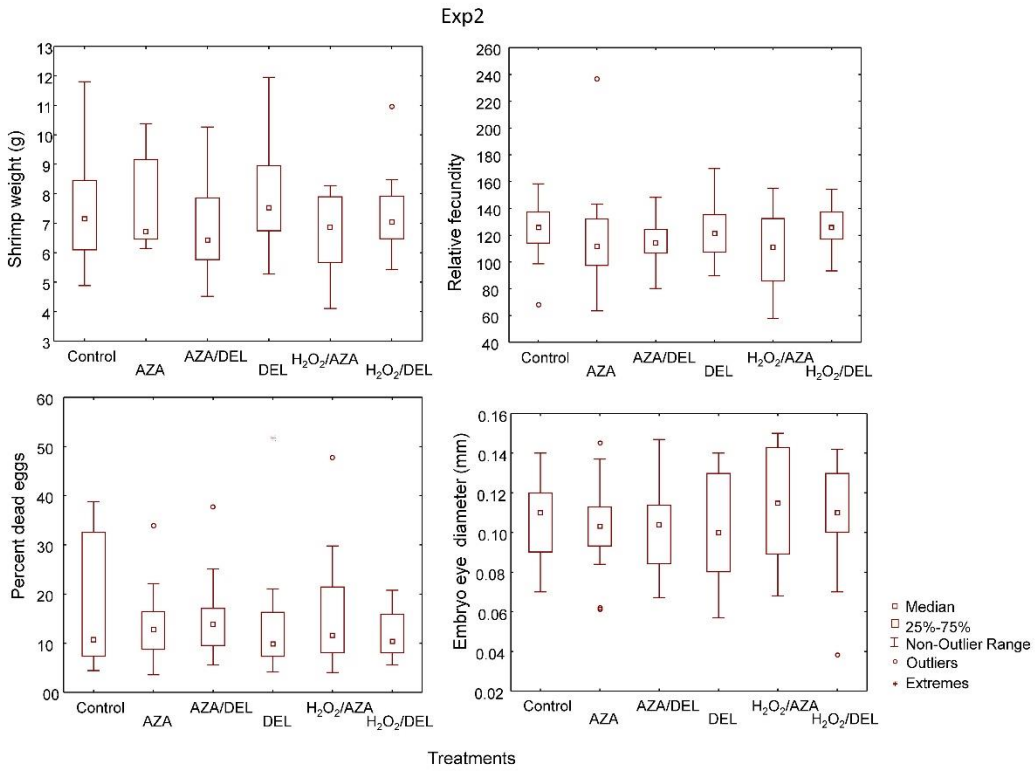

Figure S4. Shrimp weight (g), relative fecundity (number of eggs per g shrimp), % dead eggs and embryo eye diameter measured one day post exposure of Exp2 (T1d). N =15 shrimp per treatment. Embryo eye diameters: average of 10 embryos per shrimp. No significant difference between treatments was revealed.

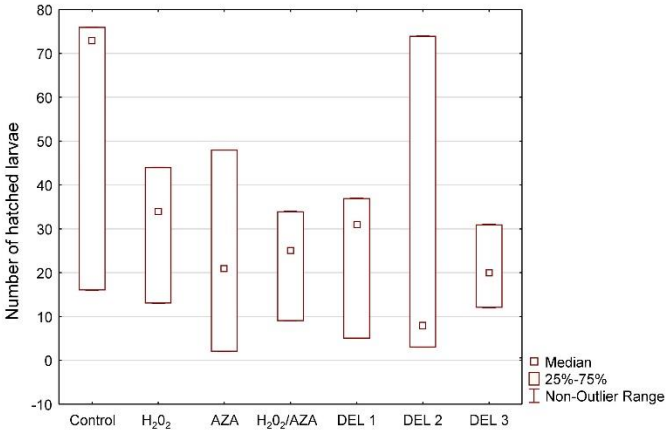

Figure S5. Number of hatched larvae in the different treatments of Exp3. For each treatment, 11-14 shrimp were still left during the period of hatching (3-5 shrimp per tank).