The effects of the chemotherapeutants hydrogen peroxide, deltamethrin and azamethiphos on non-target crustaceans

Rosa Helena Escobar Lux

Thesis for the degree of Philosophiae Doctor (PhD)
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Rosa Helena Escobar Lux

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SCIENTIFIC ENVIRONMENT

This doctoral work was carried out as a member of the Disease and Pathogen Transmission research group at the Institute of Marine Research (IMR), and the Faculty of Mathematics and Natural Sciences, University of Bergen.

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LIST OF ABBREVIATIONS

Abbreviation	Explanation
AChE	Acetylcholinesterase
AGD	Ameobic Gill Disease
EC_{50}	Effective median Concentration
H_2O	Water
H_2O_2	Hydrogen peroxide
HC_5	Hazardous concentration for 5% of species
LC_{50}	Lethal median Concentration
Log K_{ow}	Octanol-water partition coefficient
MRL	Maximum Residue Level
Na^+	Sodium
O_2	Oxygen
OCR	Oxygen Consumption Rate
PNEC	Predicted No Effect Concentration
SSD	Species Sensitivity Distribution

SUMMARY

I present new knowledge on the toxicity of three major bath treatment chemotherapeutants used in Norway. Previously, regarding the toxicity studies of hydrogen peroxide (H_2O_2) alone, a total of twelve non-target crustaceans have been examined across the globe, but only five species were relevant for the Norwegian marine ecosystem. The present study applied laboratory experiments to assess the toxicity of this chemotherapeutant to three non-target crustacean species that play a crucial role in the Norwegian marine ecosystem, bringing a better understanding of the risk posed by H_2O_2. Hydrogen peroxide has long been labeled as the most environmentally friendly bath treatment in use for the salmonid industry. It has also been considered that it poses little to no threat in terms of lethality to non-target crustaceans such as lobster, shrimps or crabs (Burridge *et al.,* 2014; Gebauer *et al.,* 2017). However, **papers I, II and III** show that the recommended H_2O_2 concentrations used by the salmonid industry across the globe are lethal to non-target crustaceans. Through the creation of species sensitivity distribution curves (SSD), this thesis identified the Northern krill (*Meganyctiphanes norvegica*) as the crustacean species that is most sensitive to H_2O_2 of those that have been tested so far. By including the sensitivity of six phyla other than the arthropods, this thesis takes a broader perspective on the impact of H_2O_2 on the marine environment. The hazardous concentration of H_2O_2 for 5% of the species (HC_5) derived from the available toxicity data for marine species is 5.11 (1.52 – 16.15) mg/L. As SSD curves are a central tool for ecological risk assessments, showing the different sensitivities and variations between species, it is crucial that this tool continues to be used for the risk assessment of the other chemotherapeutants.

Deltamethrin and azamethiphos have a detrimental effect on European lobster larvae (*Homarus gammarus*) in laboratory experiments (**Paper IV**). One-hour exposure to deltamethrin proved to be more toxic than H_2O_2 and azamethiphos to both stage I and stage II *H. gammarus* larvae. By examining the toxicity of all three chemotherapeutants to a single species this thesis, in combination with the results from previous studies, proposes a ranking of the toxicity of deltamethrin, H_2O_2 and azamethiphos based on the difference between the median lethal concentrations LC_{50} (**Papers II & IV**). With the

available data from other studies, the toxicity ranking for Norwegian relevant species is: deltamethrin > H_2O_2 > azamethiphos.

This thesis has also shown the importance of coupling sub-lethal studies with more conventional toxicity studies (**Papers I & II**). It was shown that behavior parameters linked with the predator avoidance and escape response of the European lobster juveniles and the copepod *Calanus* spp. were affected following short-term (1 h) exposures at concentrations ≤85 mg/L H_2O_2 (i.e. 5% of the recommended treatment). All three chemotherapeutants induced immobility at concentrations considerably lower than the reported lethal values. Furthermore, in **paper IV** the calculated effective median concentration EC_{50} values for both deltamethrin and azamethiphos were considerably lower than the reported LC_{50} values based on mortality.

The results from the hydrodynamic model presented in **paper IV** plus the lethality findings from **papers I, II and III** coupled with both field studies and models should be considered by regulatory authorities in Norway and can be an important tool for other salmonid producer nations when carrying out future environmental risk assessments of H_2O_2, deltamethrin and azamethiphos. These results should thus be used to evaluate the potential risks associated with the expansion of salmonid aquaculture into new locations. To have a better understanding of the risks of these chemotherapeutants in the Norwegian marine environment, further studies should evaluate their broader impact by assessing chronic or pulse-like exposures that are certainly closer to real life delousing scenarios where multiple pens are treated over a cumulative period of time. Likewise, data from the flushing of well-boats should also be included in new hydrodynamic models, as this bath treatment method dilutes the effluent of waste treatments and thus reduces its environmental impact (Ernst *et al.,* 2014).

Overall, this study has shown that the recommended H_2O_2, deltamethrin and azamethiphos concentrations used by the salmonid industry have a detrimental effect in the survival of the non-target crustaceans *Calanus* spp., *H. gammarus* and *M. norvegica*.

LIST OF PAPERS

This thesis is based upon the following papers, which are referred to in the text by their roman numerals:

Paper I:

Escobar-Lux, R.H., D.M. Fields, H.I. Browman, S.D. Shema, R.M. Bjelland, A.-L. Agnalt, A.B. Skiftesvik, O.B. Samuelsen & C.M.F. Durif. 2019. **The effects of hydrogen peroxide on mortality, escape response and oxygen consumption of *Calanus* spp**. *Facets* 4: 1–12.

Paper II:

Escobar-Lux, R.H., Parsons, A., Samuelsen, O.B., & Agnalt, A-L. 2020. **Short-term exposure to hydrogen peroxide induces mortality and alters exploratory behavior of European lobster (*Homarus gammarus*).** *Ecotoxicology & Environmental Safety*, 11111.

Paper III:

Escobar-Lux, R.H. and Samuelsen, O.B., 2020. **The Acute and Delayed Mortality of the Northern Krill (*Meganyctiphanes norvegica*) When Exposed to Hydrogen Peroxide**. *Bulletin of Environmental Contamination and Toxicology*, pp.1-6.

Paper IV:

Parsons, A., Escobar-Lux, R.H., Sævik, P., Samuelsen, O.B. & Agnalt, A-L. 2020. **The impact of anti-sea lice pesticides, azamethiphos and deltamethrin, on European lobster (*Homarus gammarus*) larvae in the Norwegian marine environment**. *Environmental Pollution*, 114725.

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INTRODUCTION

Global aquaculture has seen a rapid increase in the past 35 years with a rise in production from 5.2 million tons in 1981 to 110.02 million tons in 2016 (FAO, 2018). Salmonids are the most farmed marine fish with a global production of 2.6 million tons in 2016 (FAO, 2018). Furthermore, farmed Atlantic salmon (*Salmo salar*) represents more than 90 percent of the market, generating approximately 1.4 billion US dollars in revenue per year (Brauner *et al.,* 2012). Norway is the world's largest salmon producer followed by Chile and Scotland, with over 950 farms along its coastline and 1 million tons of salmon produced annually since 2011 (Norwegian Directorate of Fisheries, 2020; SSB, 2020), making farmed salmon a major component of the Norwegian economy.

The expansion in the production of Atlantic salmon in Norway has long raised concerns on its impact on the environment such as the negative impact on wild salmonid populations, the release of nutrients and chemical pollution, as well as the spread of diseases and parasites (Taranger *et al.,* 2015; Liu *et al.,* 2017). Salmonid aquaculture sites are distributed along the entire Norwegian west coast (Krkošek *et al*., 2013). The high number of fish or in this case hosts in the farms leads to high densities of parasites in areas of intensive aquaculture activity compared to farm-free areas (Krkošek *et al*., 2005; Serra-Llinares *et al.,* 2014).

The ectoparasitic copepod known as the sea lice is one of the most important challenges for the salmon industry, having the greatest economic impact on the industry (Costello 2009; Torrisen *et al.,* 2013). Abolofia *et al.* (2017) estimated the cost of sea lice to the industry to be US$301 million, equivalent to 8.81% of the total production for the same year. In 2011, the financial loss due to sea lice infestations was estimated to be US$334 million for the Norwegian industry alone by Liu and Bjelland (2014), but this was slightly low compared to calculations by Abolofia *et al.* (2017) pointing to a financial loss of US$436 million equivalent to 8.7% of the industries' total production for the year 2017.

Sea lice are crustacean copepods in the family *Caligidae* that are naturally occurring parasites of marine fish populations. In Norway, *Lepeophtheirus salmonis* is the major challenge for the salmonid industry, although unusually large numbers of

Caligus elongatus have been reported in infestations in the northern regions of the country (Hemmingsen *et al.,* 2020). *Caligus rogercresseyi* is a challenge for the Chilean industry. The most important difference between *L. salmonis* and *Caligus* spp. is that while *L. salmonis* is a parasite restricted to salmonids, *Caligus* spp. is less specific about its host. These species see their dispersal being limited by the natural low host density. However, this changed with the start of intensive salmonid farming providing the ideal conditions for the growth and dispersal of the parasites (Torrissen *et al*., 2013; Aaen *et al.,* 2015). As it happens with any other agricultural and aquaculture activity, the high-density conditions observed in the salmonid industry net pens have led to a high occurrence of parasitic infections. Roth *et al.* (1993) observed that in areas where the salmonid industry was not present, the hosts presented fewer lesions due to the low number of parasites. Nevertheless, changes in the coastal ecosystems where aquaculture has become predominant, place wild salmonid populations at risk of parasite transmission (Krkošek *et al*., 2006).

The sea lice *L. salmonis* life cycle comprises eight stages, each separated by a molt (Hamre *et al.,* 2019). Eggs are carried in a pair of strings (100-1000 eggs) which are extruded from the abdomen of the adult female (Costello, 1993). The first two stages are planktonic naupliar larvae. The planktonic and non-feeding larvae go through extensive morphological changes for about 5 to 15 days, depending on temperature, before molting into the third, infective stage that will later attach to the host using the second antennae that serve as small hooks. Studies suggest that copepodids could use water-borne chemical cues to recognize hosts (Bailey *et al.,* 2006). Before molting into the chalimus stage, the copepodids develop a special frontal filament, which is then used to stay attached to the host. The remaining five stages develop on the host and are strictly dependent on the host's skin for food (Hamre *et al.,* 2013). The chalimus stages I and II are followed by two pre-adult stages that can move freely over the host's skin. It is these pre-adult stages that cause the most harm to the salmon, which culminates at the adult phase of the louse.

Infected salmonids can suffer from substantial physiological and pathological consequences, which are highly dependent on the number and developmental stage of the *L. salmonis* (Torrisen *et al.,*2013). For the Atlantic salmon, the combination of

mobility and feeding behavior of the pre-adult and adult parasitic copepods, is the main cause for most of the severe consequences of the infection (Finstad *et al.,* 2000). The parasite feeds on the mucous, skin and blood of the host with the use of rasping mouth parts, resulting in skin erosion and sub-epidermal hemorrhaging (Costello, 2006). In an infection, the host fish can suffer from reduced appetite, changes in swimming behavior, reduced growth, reduced osmoregulatory and respiratory ability; all indications of a stressed and weakened fish (Costello, 2006). The transfer of sea lice from domesticated fish to the wild populations occurs through two major pathways, either from infected farmed escapees or from close proximity with an infected farm (Krkošek *et al*., 2009). Though effects from an infection can be characterized as sub-lethal, they may eventually be fatal for wild salmonid smolts migrating through the fjords where the farms are situated (Birkeland, 1996; Costello, 2009; Torrissen *et al.,* 2013; Serra-Llinares *et al.,* 2014; Aaen *et al.,* 2015).

In the last 25 years, severe annual sea lice infestations on migrating post-smolts, wild sea trout (*Salmon trutta*) and arctic char (*Salvelinus alpinus*) has been reported in Norway (Finstad & Bjørn, 2011). The negative impact of sea lice infestations on the survival of wild Atlantic salmon post-smolts is, therefore, a contributing factor in the decline of wild populations in Norway (Skilbrei *et al.,* 2013; Torrisen *et al.,* 2013). The severity of sea lice infestations on salmonid post-smolts depends on the size and condition of the fish (Wagner *et al.,* 2003, 2008; Heuch *et al.* 2005; Tveiten *et al.,* 2010; Thorstad & Finstad, 2018). Overall, 0.04-0.15 lice per g fish weight reduces the swimming ability and increases stress levels of the Atlantic salmon (Wagner *et al.* 2003; Tveiten *et al.,* 2010). Wagner *et al.* (2008) described that ~11 sea lice per fish can kill a wild smolt of 15 g. Another study indicated that post-smolts presenting > 10 lice would suffer higher mortalities (Heuch *et al.* 2005). The negative impacts of lice on wild populations also include delayed growth and delayed sexual maturation (Grefsrud *et al.,* 2019).

Anti-sea lice treatments

Due to its vast coastline and high number of suitable inland habitats for salmon, Norway has the highest number of spawning rivers for wild Atlantic salmon (Liu *et al.,*

2011), and therefore is home to most of the remaining wild populations. Through the Convention for the Conservation of Salmon in the North Atlantic Ocean (NASCO, 1982), Norway has the international responsibility to protect the remaining wild populations of *Salmo salar.* In Norway, the number of farmed salmon exceeds the number of wild ones, with an estimated ratio of 1 to 728 farmed harvested salmon in 2015 (Norwegian Directorate of Fisheries, 2018). This, increases the risk of diseases and parasite infestations for salmonid wild populations in areas with high density of aquaculture sites (Bjørn *et al*., 2001; Krkošek *et al*., 2005). Monitoring and controlling the parasite is therefore vital not only to minimize the losses in the industry and improve the welfare of the farmed fish, but also to protect the wild salmonid populations from negative effects associated with salmonid aquaculture.

To control the sea lice infestations in the farms and minimize the pressure on the wild stock, strict regulations have been put in place. The Salmon Lice Directive (FOR-2012-12-05-1140, 2020) requests that a plan for prevention and treatment of sea lice is prepared for each farm. The permitted number of sea lice per fish, according to the Norwegian authorities (2012), is 0.2 adult female or three mobile parasites per fish between 1 January and 31 August, and 0.5 adult female or five mobile individuals during the rest of the year. In order to comply with these regulations, the industry has relied on chemotherapeutants, but lately there have been development of alternative methods (Grefsrud *et al.,* 2019). Alternative methods include mechanical delousing systems such as: the Flatsetsund Engineering AS system that removes the lice through pressure washers, the SkaMk system using brushes, and the Hydrolicer ® system which uses the inverse turbulence principle to remove the lice from the salmon (Overton *et al.,* 2019). Other alternative methods to reduce lice infestation include the use of plankton shielding skirts, snorkel cages (Aaen *et al.,* 2015; Geitung *et al.*, 2019), and thermal treatments Thermolicer® and Optilicer® in which the salmonids are exposed to water temperatures of 20-34°C (Grøntvedt *et al.,* 2015; Roth, 2016). However, negative effects on the fish have been observed with the use of mechanical methods, including gill bleeding, skin wounds and increased mortality (Hjeltnes *et al.,* 2018). Finally, a biological alternative is the use of cleaner-fish, including wrasse species (*Labridae* sp.) and lumpfish (*Cyclopterus lumpus*) (Imsland *et al.,* 2014; Skiftesvik *et al.,* 2014).

Though several non-chemical methods are being used and new technologies are being developed, chemotherapeutants are still used in Norway as well as in the other salmon producing countries (Grefsrud *et al.,* 2019).

Chemotherapeutants tailored to combat sea lice are either applied as a bath treatment (hydrogen peroxide, organophosphates or pyrethroids) or in-feed treatment (emamectin-benzoate or flubenzurons). Two different approaches are in use for the bath treatments. One is by surrounding the cages with an impervious tarpaulin and mixing the solution directly into the enclosure and the other is by transferring the salmonids to well-boats. The recommended doses are then added, and the recommended treatment time is followed. Once the treatment is over, the chemotherapeutants are discharged into the surrounding environment (Burridge *et al.,* 2014). This will potentially affect non-targeted species that are present in the resulting plume of the discharged chemicals.

Indeed, hydrogen peroxide (H_2O_2), deltamethrin and azamethiphos as delousing agents, can negatively impact other species than the sea lice, i.e. non-target species. Crustaceans are at a higher risk as these chemotherapeutants, especially deltamethrin and azamethiphos, were tailored to remove sea lice, a crustacean. The extensive use of chemotherapeutants including the bath treatments over the years, has led to development of sea lice with reduced sensitivity towards one or several chemotherapeutants in heavily treated areas (Aaen *et al.,* 2015). This initiated a practice in which the frequency of treatments increased, the dose of the chemotherapeutant increased, or two drugs were used in combination, all of which increase the potential effect on non-target species.

The toxicity of these chemotherapeutants to non-target species has been reviewed (Urbina *et al.,* 2019). Acute toxicity studies often involve 24, 48 or 96h exposure periods, which do not necessarily reflect the exposures expected to occur in the marine environment following the discharge of chemotherapeutants from fish farms (Ernst *et al*., 2001; Ernst *et al.,* 2014; Urbina *et al*., 2019). Previous studies have observed that 1-3 h exposures occur during chemotherapeutant plume dispersion, therefore shorter exposure times, i.e. 1 h, can be more realistic as they can generally be expected from a single pen release (Ernst *et al.,* 2014). There is a need for toxicity studies to be performed under more environmentally relevant exposure periods; few

studies have followed shorter exposure times thus making a direct comparison between results of the chemotherapeutants toxicity difficult. Moreover, there is a lack of information concerning species relevant to Norwegian marine ecosystems even in relatively recent studies (Table 1). Therefore, it is crucial to improve our knowledge on the toxicity of chemotherapeutants to non-target crustacean species present in the Norwegian ecosystems. Especially important is the assessment of delayed sub-lethal effects of bath treatment plumes on these non-target species, resulting from short time exposures. Shorter exposure times i.e. 1h followed by a 24h post-exposure time can provide a more realistic assessment of the impacts of bath treatment plumes on non-target species (Medina *et al*., 2004; Van Geest *et al*., 2014; Bechmann *et al.,* 2019; Frantzen *et al.,* 2020).

Table 1. Summary of toxicity studies on H_2O_2, deltamethrin and azamethiphos performed on non-target marine crustacean species from the North-East Atlantic Ocean.

	Species	**Endpoints**	**Exposure Period**	**Post-Exposure Period**	**Reference**
H_2O_2	*Calanus finmarchicus*	Mortality, oxidative stress	96h	-	Hansen *et al*., 2017
	Corophium volutator	Mortality	96h	-	Smit *et al.,* 2008
	Paleamon elegans	Mortality	1h 24h	24h	Brokke, 2015
	Pandalus borealis	Mortality Behavior Embryo development	2h	28 days	Frantzen *et al*., 2020
	Pandalus borealis	Mortality Immobilization Feeding rate Gill histology	Pulse exposures	up to 12 days	Bechmann *et al*., 2019
	Praunus flexuosus	Mortality	1h 24h	24h	Brokke, 2015

Deltamethrin	*Monocorophium insidiosum*	Mortality Biochemical responses	10 days	-	Tucca *et al*., 2014
	Palaemon serratus	Mortality Swimming velocity Liver antioxidant status Energy metabolism Neurotransmission	96h	-	Oliveira *et al*., 2012
	Paleamon elegans	Mortality	1h 24h	24h	Brokke, 2015
	Pandalus borealis	Mortality Behavior Embryo development Reproductive output	2h	19-29 days	Frantzen *et al*., 2020
	Pandalus borealis	Mortality Swimming activity	2h (1x pulse) 2h (3x pulse)	13 days 48h	Bechmann *et al*., 2020
	Praunus flexuosus	Mortality	1h 24h	24h	Brokke, 2015
Azamethiphos	*Gammarus spp*	Mortality	96h	-	Ernst *et al*., 2001
	Paleamon elegans	Mortality	1h 24h	24h	Brokke, 2015
	Pandalus borealis	Mortality Behavior Embryo development Reproductive output	2h	19-29 days	Frantzen *et al*., 2020
	Pandalus borealis	Mortality Swimming activity	2h (1x pulse) 2h (3x pulse)	13 days 48h	Bechmann *et al*., 2020
	Praunus flexuosus	Mortality	1h 24h	24h	Brokke, 2015
	Tisbe battagliai	Mortality Developmental effects	7 days	-	Macken et al., 2015

Conventionally, assessments of the acute toxicity of pollutants to non-target crustaceans rely on the determination of lethal median concentrations (LC_{50}). However, these lethal values only provide a partial measurement of the real magnitude of the effects of these chemicals (Desneux *et al.,* 2007). Sub-lethal effects have been defined as an impact, either on a physiological or behavioral level, on individuals that survive the exposure. Ecologically relevant sub-lethal effects are defined as having an impact on the fitness of the individual: ability to grow, survive, and reproduce (Beiras, 2018). By influencing these endpoints, sub-lethal effects can have major consequences at a population level (Little and Finger, 1990). Sub-lethal endpoints are the first to be affected by pollutants and thus sub-lethal effects can occur at concentrations several orders of magnitude below the LC_{50} values (Beiras, 2018). Moreover, behavioral endpoints such as predator avoidance, burrowing activity, swimming activity, swimming speed, and oxygen consumption rates are possibly the most sensitive responses to pollutants (Beiras, 2018). Several behavioral responses in crustacean species alter the probability of successful predation. Ohman (1988), divided these behavioral responses into three major groups: avoidance behavior (through refuge, diel migration cycles, seasonal diapause and locomotor behavior), escape responses (through active motility, aggregation, bioluminescence and passive evasion), and defense responses (through chemical means and induced morphology). The avoidance behavior reduces the encounter probabilities with predators, the escape responses minimize the successful attacks, and the defense responses decreases the probability of ingestion by a predator. It is critical that the sub-lethal effects on the behavior of non-target organisms is considered in addition to traditional mortality measurements in order to have a better understanding of the real impact of pollutants. It is also important to assess whether these concentrations, calculated from laboratory based toxicity tests, are likely to threaten the wild non-target populations living in the vicinity of the aquaculture facilities. To better understand the environmental risk of these chemotherapeutants, a greater knowledge of the possible concentrations around the fish farms is required. Presently, there is limited information on the dilution and dispersal of H_2O_2, deltamethrin and azamethiphos in the Norwegian marine environment.

OBJECTIVES AND METHODOLOGICAL CONSIDERATIONS

Objectives

This doctoral thesis is part of the internally financed research project *Legemidler og Fremmedstoffer* (Institute of Marine Research), working to increase the knowledge of the risks associated with the discharges of chemotherapeutants from the aquaculture industry to the environment. The contribution of my studies to this project was to evaluate how bath treatment effluents may impact non-target crustaceans both in the water column and in the benthic habitats in proximity of the fish farming sites. To achieve this, keystone species of the North Atlantic, the copepods *Calanus* spp., the Northern krill (*Meganyctiphanes norvegica*) and the European lobster (*Homarus gammarus*), were exposed to the most frequently used anti lice bath treatments; H_2O_2, deltamethrin and azamethiphos. All three chemotherapeutants, H_2O_2, deltamethrin and azamethiphos, are non-specific when used as bath treatments, meaning that they can impact several non-target species other than the sea lice. The first objective was to elucidate whether these different species have distinct sensitivity when exposed to the same chemotherapeutant.

In Norway, H_2O_2 is the most used bath treatment but the knowledge around its toxicity to Norwegian species is limited. Thus, the first objective of this thesis was to answer the first question posed by this work. By calculating the median lethal concentration (LC_{50}) of different species exposed to H_2O_2 it was possible to estimate a species sensitivity distribution (SSD) curve, illustrating the different sensitivities to H_2O_2 for species present in marine ecosystems. The second objective was to compare how one species, European lobster, responded to the three chemotherapeutants H_2O_2, deltamethrin and azamethiphos. A toxicity ranking was established to elucidate the relative toxicity of each chemotherapeutant. The third objective was to quantify sub-lethal effects to assess the potential extent of the impact of these chemicals. Sub-lethal effects such as physiological or behavioral changes due to exposure to a chemotherapeutant, can occur at concentrations several orders of magnitude below the LC_{50} (Beiras, 2018). The chosen sub-lethal parameters were oxygen consumption and anti-predator behavior, after exposure to H_2O_2 of *Calanus* spp. and *H. gammarus*. The

fourth objective was to set the results of laboratory from this works' experiments into a more realistic context. To do this, data from field studies were combined with hydrodynamic models to understand the relevance of laboratory experiments in comparison to the expected environmental fate of the chemotherapeutants. The four aims of this studies can be summarized with the following questions:

i) Is one chemotherapeutant equally toxic to different marine species? (**Paper I, Paper II & Paper III**)

ii) Will a single species be equally sensitive to several chemotherapeutants? (**Paper II & IV**)

iii) Can non-lethal concentrations of hydrogen peroxide have impacts on the survival of non-target crustaceans? (**Paper I & Paper II**)

iv) Are laboratory toxicity results relevant in real-case dispersal scenarios? (**Paper IV**)

To answer these questions, the three major chemotherapeutants H_2O_2, deltamethrin and azamethiphos, were chosen for this work. Mortality that occurred within the 1 h exposures was defined as acute mortality whereas total mortality was defined as the cumulative mortality after 1 h exposure and the 24 and 48 h post-exposure periods. A description of the chemotherapeutants, a reasoning for the use of the selected species and the methods used in this work are presented below.

Chemotherapeutants

Hydrogen peroxide

Hydrogen peroxide (H_2O_2) (Fig. 1), was first introduced as an anti-sea lice agent in Norway in 1993 and was in use until 1997 when more efficient chemotherapeutants with a higher safety margin were introduced, like emamectin-benzoate, cypermethrin and deltamethrin (Thomassen, 1993; Kiemer and Black, 1997). However, an extensive use of these new drugs led to reduced sensitivity in sea lice (Costello, 2009; Aaen *et al.,* 2014) and brought back the use of H_2O_2 in 2010. Available formulations of H_2O_2 are Nemona® (49.5% w/w), Paramove 35® (35% w/w) and Paramove 50® (49.5% w/w) (Grant, 2002). In Norway, Nemona® and Paramove50® have marketing authorization. Though the mechanism of action of H_2O_2 on the sea lice is not entirely understood, it has been described as mechanical paralysis, inactivation of enzymes and DNA replication, and peroxidation of lipid and cellular organelle membranes by hydroxyl radicals (Cotran *et al.*, 1989). Studies have suggested that the mechanical paralysis is caused by the decomposition of H_2O_2 into water and O_2 bubbles in the gut and haemolymph, resulting in the immobilization of the sea lice, causing its detachment from the host and floating to the surface (Thomassen, 1993; Bruno Raynard, 1994; Aaen *et al*., 2014).

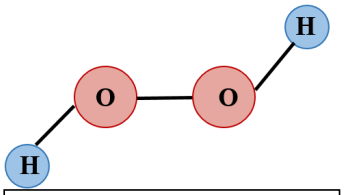

Fig. 1 Structural formula of hydrogen peroxide (H_2O_2).

Today, H_2O_2 is used for delousing purposes in almost all the salmon producing countries (Overton *et al.,* 2018), and in many countries, it is also used to treat amoebic gill disease (AGD) caused by the *Neoparamoeba perurans* (Young *et al.,* 2007). If untreated, AGD can be potentially fatal for salmonids as it causes multifocal lesions in the gills and its transmission has been associated with sea lice infestations (Nowak *et al.,* 2010). The recommended dose of H_2O_2 when used for delousing is between 1500 and 2100 mg/L with a treatment time of 20-30 minutes depending on the sea water temperature, and 1250 mg/L for 20 minutes to treat AGD (The veterinary catalogue, Norway 2020). An advantage of H_2O_2 over other compounds is that its MRL (Maximum Residue Level) value is not required and therefore there is no withdrawal period

between the treatment operation and time of slaughter of the fish (Haya *et al.*2005). Though the consumption of H_2O_2 has decreased in the past years as a result of reduced sensitivity in the sea lice, it is still the most prescribed chemotherapeutant in Norway with 4523 tons used in 2019 (Folkehelseinstituttet, 2019) (Table 2).

The effectiveness of H_2O_2 against the parasite differs between the different life stages of the sea lice: it removes pre-adult and adult stages but is not effective against the chalimus stages (Thomassen, 1993; Treasure *et al.,* 2000; Aaen *et al.*, 2014). Furthermore, H_2O_2 also has a detrimental effect on the maturation and reduced hatching viability of exposed egg strings of both *L. salmonis* and *C. rogercresseyi* (Aaen *et al.*, 2014; Bravo *et al.,*2015).

Table 2. Bath treatment chemotherapeutants used in Norway between the years 2011 and 2019. The values are given in kg of active substance, with the exception of H_2O_2 given in tons (from Folkehelseinstituttet, 2019, 2018).

	2011	**2012**	**2013**	**2014**	**2015**	**2016**	**2017**	**2018**	**2019**
Hydrogen peroxide (tons)	3144	2538	8262	31577	43246	26597	9277	6735	4523
Deltamethrin (kg)	54	121	136	158	115	43	14	10	10
Azamethiphos (kg)	2437	4059	3037	4630	3904	1269	204	160	154
Cypermethrin (kg)	48	232	211	162	85	48	8	0	0

The chemotherapeutants combating sea lice also affect non-target species following release into the sea. In the past years, several studies have tested the toxicity of H_2O_2 in 12 different non-target crustacean species (Smit *et al*., 2008; Burridge *et al*., 2014; Van Geest *et al*., 2014; Brokke, 2015; Gebauer *et al*., 2017; Hansen *et al*., 2017; Bechmann *et al*., 2019; Frantzen *et al.,* 2020) (Fig. 2). In addition, there is available literature for the toxicity of H_2O_2 on the polychaetes *Capitella* sp. and *Ophryotrocha* spp., and sugar kelp (*Saccharina latissima*) (Bruno and Raynard, 1994; Mitchell and Collins, 1997; Rach *et al.,* 1997; Fang *et al.,* 2018; Haugland *et al.,* 2019).

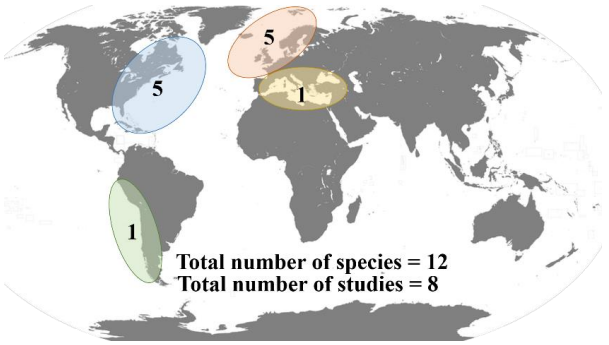

Fig. 2 Number of non-target crustaceans' species per geographical region used to study the toxicity of H_2O_2. Globally a total of 8 studies have looked at the toxicity of H_2O_2 at aquaculture relevant concentrations on non-target crustaceans.

Deltamethrin

The synthetic pyrethroids deltamethrin and cypermethrin were introduced in Norway in the mid-1990s, as the replacement of the organophosphates due to reduced sensitivity in the parasites and as a safer alternative to hydrogen peroxide (Jones *et al.,*1992; Hart *et al.,* 1997; Denholm *et al.,* 2002). Synthetic pyrethroids have a low toxicity on mammals (Davies, 1985) but are highly toxic for fish and crustaceans, including sea lice (Anderson, 1989; Coats *et al.,* 1989; Haya, 1989). Soon after its introduction, deltamethrin became the preferred chemotherapeutant in Norway, with more than 80% of the market share (Denholm *et al.,* 2002). However, due to this extensive use, both *L. salmonis* and *C. rogercresseyi* developed reduced sensitivity towards deltamethrin in the early-2000s decreasing the consumption (Sevatdal and Horsberg, 2003; Helgesen *et al.,* 2014). It was, however reintroduced in the market and became one of the most used treatments in Norwegian farms between 2010 and 2015, as the active ingredient of the commercial formulation AlphaMax®.

Deltamethrin is a wide spectrum insecticide which acts on the nerve transmission pathways (Miller and Adams, 1982; Kahn, 1983) (Fig. 3). More specifically, its mechanism of action involves interacting with the sodium (Na+) channels of nerve

membranes, resulting in the depolarization and overstimulation of nerve endings finally leading to paralysis (Haya *et al.,* 2005). The recommended deltamethrin treatment dose in salmon aquaculture is 2 µg/L for a time period of 30-40 minutes (The veterinary catalogue, Norway 2020).

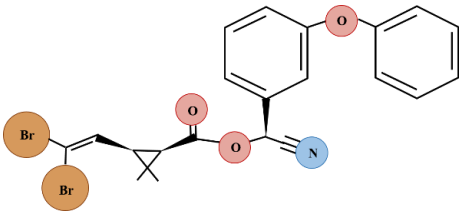

Fig. 3 Structural formula of deltamethrin ($C_{22}H_{19}Br_2NO_3$).

As deltamethrin is highly toxic to crustaceans, a number of different studies have examined its toxicity to non-target species (Fig. 4) (Dorts *et al.,* 2009; Fairchild *et al.* 2010; Oliveira *et al.*, 2012; Burridge *et al*., 2014; Tucca *et al.*, 2014; Brokke, 2015; Bechmann *et al.,* 2020; Frantzen *et al.,* 2020). However, only 45% of the studied species are relevant to Norwegian marine ecosystems. There is available literature on deltamethrin toxicity on other marine invertebrates such as the echinoderms *Paracentrotus lividus* and *Shaerechinus granularis*, the chorus mussel (*Choromytilus chorus*), and the polychaete *Nereis virens* (Van Geest *et al.,* 2014; Sanhueza-Guevara *et al*., 2018). Deltamethrin is rapidly metabolized and therefore unlikely to be accumulated in the aquatic food web (Kahn 1983).

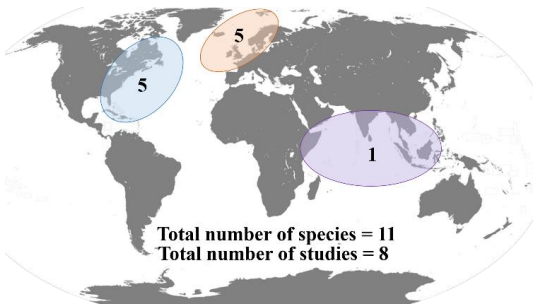

Fig. 4 Number of non-target crustaceans' species per geographical region used to study the toxicity of deltamethrin. Globally a total of 8 studies have looked at the toxicity of deltamethrin at aquaculture relevant concentrations on non-target crustaceans.

Azamethiphos

Until the mid-1990s, more than 80% of the delousing operations performed in Norway used the organophosphate dichlorvos, which is also the preferred chemotherapeutant in the rest of the salmon farming countries (Fallang *et al.,* 2004; Torrisen *et al.,* 2013). Azamethiphos was then introduced as a safer and more effective alternative to dichlorvos in 1994 and was in use until 1999, when reduced sensitivity of *L. salmonis* became a problem (Roth *et al.* 1993; Burka *et al.,*1997). However, reduced sensitivity in the salmon lice, this time against the pyrethroids and emamectin-benzoate, led to a reintroduction of azamethiphos in 2008 (Aaen *et al.,* 2015).

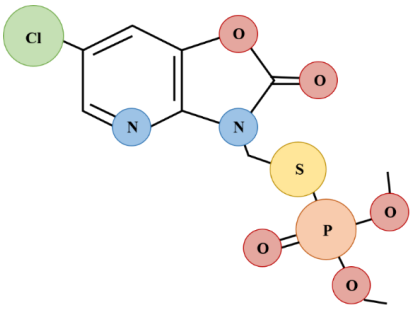

Fig. 5 Structural formula of azamethiphos ($C_9H_{10}ClN_2O_5PS$).

Azamethiphos is a neurotoxic insecticide (Fig. 5), which inhibits the acetylcholinesterase (AChE) activity leading to paralysis (Baillie, 1985). In Norway, azamethiphos is available as Azasure with a recommended treatment dose of 0.1mg/L and a treatment duration between 30 and 60 minutes. At temperatures of over 10℃ a treatment duration of 30 minutes is recommended. The effects of azamethiphos is visible within the first few hours after treatment (Roth *et al.,* 1996; Torrisen *et al.* 2013). Though azamethiphos is effective in removing pre-adult and adult lice, it is ineffective against chalimus stages (Roth *et al.,* 1993).

The toxicity of azamethiphos has been previously studied in a great number of crustaceans (Fig. 6) (Pahl and Opitz, 1999; Abgrall *et al.,* 2000; Ernst *et al*., 2001, 2014; Mayor *et al*., 2008; Burridge *et al*., 1999, 2000, 2008, 2014; Van Geest *et al*., 2014; Brokke, 2015; Macken *et al.,* 2015; Gebauer *et al*., 2017; Mill, 2019; Bechmann *et al.*, 2020; Frantzen *et al.,* 2020). However, relatively few studies (27%) involve species relevant to Norwegian marine ecosystems.

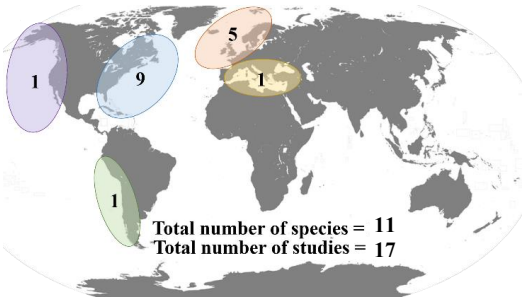

Fig. 6 Number of non-target crustaceans' species per geographical region used to study the toxicity of azamethiphos. Globally, 17 studies have looked at the toxicity of azamethiphos at aquaculture relevant concentrations on non-target crustaceans.

Study species

The abundance of zooplankton crustaceans varies seasonally, with a predominant occurrence following the phytoplankton spring bloom, usually taking place in the northern hemisphere between March and June, although some regional variations may occur (Grover, 1952; Plourde & Runge, 1993; Niehoff *et al.,*1999). In Norway, during the spring, chemotherapeutants are being used extensively to comply with sea lice regulations (Grefsrud *et al.,* 2018) (Fig. 7) and the chance of crustaceans being exposed to the substances is therefore higher. Delousing operations can involve concurrent and sequential applications within one or multiple net pens, leading to various discharges in a single fjord. These multiple discharges may expose non-target planktonic crustaceans to delousing plumes over extended periods of time (Grefsrud *et al.*, 2018). The copepods *Calanus* spp., the dominant zooplankton species of the North Atlantic, is of great ecological importance being a key trophic link in the marine food webs (Schminke, 2007). These dominant zooplankton species are intense grazers of primary production and are the main prey for other important species, such as the Northern krill, and several pelagic fish species such as herring and cod, both economically important species (Dalpadado *et al*., 2000; Sundby, 2000; Rullyanto *et al*., 2015). Eggs and nauplii of copepods are the main source of food for fish larvae of many species, which prey upon them almost exclusively, and the copepodite stages are preyed on by juvenile fish in the nursery areas (Runge and de Lafontaine, 1996; Heath and Lough, 2007). *Calanus* spp. are abundant in the Norwegian coastal zone where salmonid aquaculture sites are located (Broms *et al.,* 2009), but more importantly in

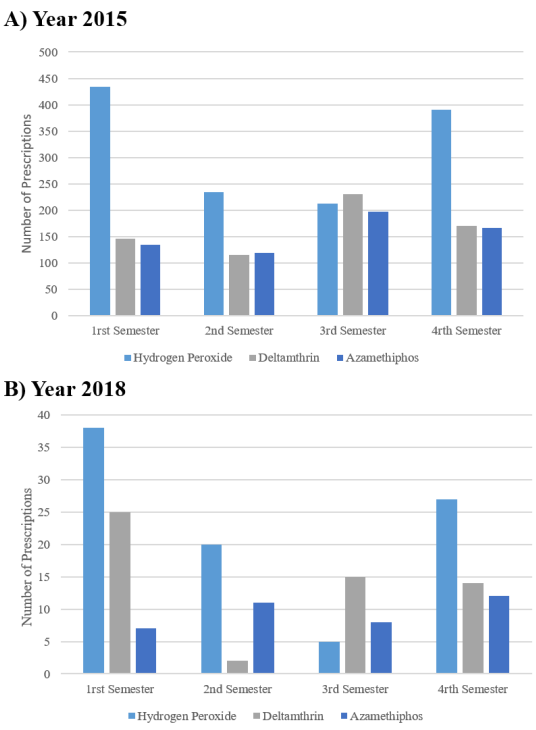

Fig. 7 Norway's yearly prescriptions for hydrogen peroxide, deltamethrin and azamethiphos per quarter, A) Prescriptions for the year 2015; B) Prescriptions for the year 2018. Overall, there has been a decrease in the number of prescriptions for the three chemotherapeutants between the years 2015 and 2018. Hydrogen peroxide is still the most used bath treatment in quarters 1, 2 and 4, followed by deltamethrin. Data from Grefsrud *et al.,* (2018).

spring when the zooplankton bloom is at its highest, adults reproduce almost exclusively in surface waters (< 50 m) and the egg production of *Calanus* spp. overlaps with the peak of chemotherapeutant operations. During the winter, large numbers of late juvenile stages (copepodite stage V) are found at depths of approximately 500 m (Hirche, 1996). The end of the diapause happens in the spring when the late juvenile stages migrate to surface and molt into adults. The effects of bath treatment chemotherapeutants on *Calanus* spp. are largely unknown. This is addressed in **Paper I.**

The Northern krill, *Meganyctiphanes norvegica*, another pelagic zooplankton species, inhabits offshore and coastal waters of the Norwegian sea (Kaartvedt *et al.,* 2002; Melle *et al.,* 2004; Tarling *et al.,* 2010). Its distribution is seasonal with a predominantly coastal distribution between the months of January and May (Grover, 1952), when the use of chemotherapeutants is at its highest. The Northern krill is a major component of the North Atlantic ecosystem, acting as a major contributor to the carbon pump cycle and a link between secondary and large predators (Kaartvedt *et al.,* 2005; Tarling *et al.,* 2010). It is preyed upon by several fish species, seabirds, and marine mammals (Brodie *et al*., 1978; Montevecchi *et al*., 1992; Sameoto *et al*., 1994; Onsrud *et al*., 2004; Stevick

et al., 2008). Mass death of krill washed up on a beach can occur and is considered a natural phenomenon. In recent years, there has been a higher frequency of reports in Norway describing this phenomenon in areas with salmon farms. Debates in the media of what might have caused the mass mortality often point to the use of chemotherapeutants for delousing of the salmon farms. However, the effects of bath treatment chemotherapeutants on the Northern krill is unknown. This is addressed in **Paper III.**

The European lobster, *Homarus gammarus*, is an important commercial species native to the Norwegian marine environment and found along the European continental shelf in the northeast Atlantic (Agnalt *et al.,* 2009). As a valuable marine resource, the European lobster has supported the northern Europe coastal fisheries for centuries, but overfishing and low recruitment has depleted its populations in Norway (Agnalt *et al.,* 2013). The distribution of *H. gammarus* in Norway overlaps with that of aquaculture sites (Agnalt, 2009), making it vulnerable to exposure following the release of bath treatment chemotherapeutants. The first four developmental stages of the European lobster are pelagic, thus potentially more vulnerable to bath treatments. The effects of chemotherapeutants on the European lobster are largely unknown. This is addressed in **Papers II & IV.**

Linking environmental fate with toxicity

Field surveys studying the dispersion and dilution of chemotherapeutants following discharge are difficult and expensive to implement. Another important objective of this thesis was to use models to link the environmental fate of the chemotherapeutants after discharge with the threshold concentrations found through the toxicology studies. This will allow us to establish the distances from the treatment site at which there are negative effects on wild non-target populations. The decrease in the concentration of chemicals after they have been released depends on several factors. Discharge from a farm will spread with the current (dispersal) and at the same time will be mixed with the surrounding water and be diluted. How far the chemotherapeutant spreads and how fast it is diluted depends on hydrographical variables such as current

velocity, wave exposure, temperature and stratification level of the water masses at the location. The rate of chemical breakdown can also be an important factor for some chemotherapeutants. Hydrogen peroxide has long been considered environmentally friendly as it is a highly polar compound which has an elevated oxidative capacity, therefore facilitating its decomposition into oxygen (O_2) and water (H_2O). After 7 days in sea water, 21% and 54% of H_2O_2 decomposes respectively at temperatures of 4°C and 15°C (Bruno & Raynard, 1994). If the water is aerated, decomposition occurs more rapidly, 45% and 67%, respectively. In real case scenarios, the decomposition of H_2O_2 may occur more rapidly due to the presence of organic matter in the water (Richard *et al*., 2007, Miller *et al*., 2009).

Deltamethrin ($C_{22}H_{19}Br_2NO_3$), has an octanol-water partition coefficient (log K_{ow}) of 6.2 (Urbina *et al.* 2019). The log K_{ow} is a physiochemical characteristic which indicates the capacity of a compound to accumulate in a sediment and to adhere to particulate material (Mayor *et al.,* 2008). Considering that deltamethrin has a high log K_{ow} and low water solubility (< 2 µg/L) (The veterinary catalogue, Norway 2020), it is expected that deltamethrin will have a greater tendency to accumulate in the sediment particularly in farms located in shallow areas and will be preserved for long periods of time (Haya *et al.*, 2005). Furthermore, deltamethrin may be absorbed not only by the sediment but also by organic materials and other materials like plastic. Deltamethrin can therefore be removed from the water phase following different pathways, decreasing the concentration in water and consequently the effect on non-target organisms.

Azamethiphos ($C_9H_{10}CIN_2O_5PS$) is highly soluble in water (1.1 g/L) and has a log K_{ow} of 1.05 (Worthing and Walker, 1987; Tomlin, 1997). This indicates that azamethiphos will remain in the aqueous phase and is not expected to accumulate in the sediment or adhere to organic matter. The estimated half-life of azamethiphos is between 8.9 and 10.8 days (Worthing and Walker, 1987; Burridge and Van Geest, 2014).

RESULTS AND DISCUSSION

In the work reported in **papers I, II and III**, a clear distinction was seen between acute and total mortality. Assessing both types of mortalities was essential to understand sensitivities of the different species to the chemotherapeutant used in this study. A 1 h exposure to 170mg/L H_2O_2, i.e 10% of the recommended treatment dose caused 0% mortality for *H. gammarus* pelagic stages (**Paper II**), on average 68% mortality for *Calanus* spp. (**Paper I**), and 100% mortality for *M. norvegica* (**Paper III**). Further, after a 24 h post-exposure period the mortalities for *Calanus* spp. and *H. gammarus* increased, leading to an average total mortality of 96% and 25%, respectively (**Paper I & II**). In **Paper III**, following the post-exposure period, the mortality of *M. norvegica* increased with time in all exposed groups resulting in successively lower median lethal concentrations (LC_{50}) values with 14.11 mg/L after 6 h (7.3-20.9), 4.92 mg/L (1.2-7.9) after 24 h and finally 0.86 mg/L after 48 h. These results show the importance of including a recovery period after the exposure.

Is one chemotherapeutant equally toxic to different marine species?

Hydrogen peroxide caused mortality in *Calanus* spp. (pre-adult and adult) (**Paper I**), to all pelagic stages of *H. gammarus* (**Paper II**), and to *M. norvegica* (**Paper III**) at concentrations below the treatment concentration of 1700 mg/L. These results suggest that H_2O_2 waste emissions from salmon farms could potentially be lethal to a wide range of non-target crustacean species in their vicinity.

In **Paper I and III** we observed that H_2O_2 was acutely toxic to wild-captured *M. norvegica* and to both stages copepodite V and adult *Calanus* spp.. The highest acute mortality recorded for European lobster larvae was 15.4% for stage I, and therefore no acute LC_{50} were calculated for any of the pelagic stages of *H. gammarus* (**Paper II**). While several studies have examined the toxicity of H_2O_2 to marine non-target crustacean species, few studies used a short-term exposure (1 h). Of the species studied with an East-North Atlantic geographical distribution, an acute LC_{50} value of $\geq$ 1700 mg/L H_2O_2 was found for the rock pool shrimp (*Palaemon elegans*) and chameleon

shrimp (*Praunus flexuosus*) (Brokke, 2015). A review of all toxicity studies reveals that most of the marine crustacean species tested as adults have a relatively high tolerance to H_2O_2 exposure at the recommended treatment concentrations, which is reflected in low acute mortality when they are exposed to concentrations similar to the recommended treatment dose (Burridge *et al.,* 2014; Van Geest *et al.,* 2014). The high acute mortality for *M. norvegica* is therefore an indication that krill is among the most sensitive species to H_2O_2 (**Paper III**).

The acute mortality was lower than the total mortality recorded after the post-exposure period for all the three-studied species (**Paper I, II & III**). From the species tested in this work, the Northern krill was the most sensitive with the highest acute and delayed mortality and lowest LC_{50} values, followed by the copepods *Calanus* spp., and leaving the European lobster as the least sensitive species to H_2O_2. For the Northern krill, the calculated LC_{50} value after a 24 h post-exposure period, represented a 3-fold dilution of the acute LC_{50} value (**Paper III**). In **Paper I**, the calculated LC_{50} values, after a 24 h post-exposure period, were subsequently lower, represented a 2.8 and 1.5-fold-dilution for the copepodite V and adult *Calanus* spp., respectively. Moreover, after a 24 h post-exposure period the lethality of H_2O_2 to all *H. gammarus* pelagic stages (I-IV) became evident (**Paper II**). The calculated total LC_{50} values for the European lobster represented approximately 10-, 4-, 3- and 2-fold dilutions for stages I, II, III and IV, respectively. Similarly, for rock pool shrimps and chameleon shrimps a significant mortality occurred during the 24 h post-exposure period (Brokke, 2015).

For these two species as well as for the Northern shrimp (*Pandalus borealis*), the acute mortalities were low but all three presented high mortalities post-exposure, classifying them as highly sensitive (Brokke, 2015; Frantzen *et al.,* 2020). In comparison, low mortalities were reported even after a 95 h post-exposure period for American lobster (*Homarus americanus*) larvae and adult, and sand shrimps (*Crangon septemspinosa*) (Burridge *et al*., 2014). Recommendations suggested in previous studies describe the importance of including a post-exposure period following the exposure to evaluate any delayed effects and obtain an accurate estimate of mortality (Van Geest *et al*., 2014; Brokke, 2015; Bechmann *et al*., 2020). Our findings support this.

In **papers I and II**, a difference in sensitivity to H_2O_2 exposure was observed in different developmental stages of the same species. Adult *Calanus* spp. showed higher acute and total mortality to H_2O_2 exposure than the copepodite stage V (**Paper I**). In the *Calanus* spp. life history, the late juvenile stage CV enters a diapause state during winter, which could explain the robustness of the CV to exposure to H_2O_2 relative to the adult copepod. In the case of the European lobster, stage I larvae were the most sensitive (**Paper II**) and stage IV the least sensitive. Similar stage specific differences in sensitivity to H_2O_2 exposure have been reported for other species including salmon lice *L. salmonis* and its eggs, and the copepod *Acartia* sp. (Mitchell and Collins, 1997; Van Geest *et al*., 2014; Aaen *et al*., 2015). Furthermore, stage-specific differences in sensitivity were also observed between stages I and II of the European lobster after exposure to azamethiphos (**Paper IV**). Stage-specific sensitivities observed in crustaceans towards chemotherapeutants have been explained as a result of differences in metabolism, moulting frequency, detoxification mechanisms and allometric (i.e. surface area to volume) differences, with older life stages often being less sensitive than earlier life stages (Medina *et al.,* 2002; Willis and Ling, 2004).

Species Sensitivity Distributions

To protect marine ecosystems, there must be standards in relation to water quality. Such standards require data from ecotoxicology studies and a method to convert those data into estimates of the concentration of pollutants that result in negligible impacts (Posthuma *et al.,* 2019). In order to understand the possible hazards of toxic compounds to an ecosystem, the effect on different species must be estimated. The concept of species sensitivity differences towards the same toxic compound has earlier been described as an important factor for ecotoxicology and environmental risk assessments (Van Straalen, 2002; Fourie *et al.,* 2007). Different species show different sensitivities to the same chemical substance and the variation between those species can be described by a statistical distribution. Laboratory results should not be used directly to perform a risk assessment, but must be extrapolated to calculate a predicted no-effect

concentration (PNEC). The variation that results from the observed sensitivities of different species can be modeled as a statistical distribution also known as a species sensitivity distribution (SSD).

An SSD is based on single species toxicity tests to a single compound fitted into a statistical model, most often log-logistic or log-normal. SSDs use toxicity values such as LC_{50} or EC_{50} when it comes to acute exposures and EC_{10} for chronic exposures. These values are then rank ordered and a statistical distribution is fitted to the values. Once the values have been fitted, the hazardous concentration for 5% of the species (HC_5) can be derived (with a 95% confidence interval). The threshold for observable impacts is defined as being above this value (Belanger and Carr, 2019). The HC_5 value can then be used to calculate the PNEC.

Species sensitivity distributions are recognized by a wide range of regulatory authorities across the globe (Belanger and Carr, 2019). However, the criteria for the data used in an SSD varies between countries and jurisdictions, often with differences in the minimum number of data points needed, leading to differences in the quality of the studies (Posthuma *et al.,* 2002).

The European guidelines for the creation of SSD curves do not specify what must be done when multiple points of toxicity data are provided for the same species but different life stages (ECHA, 2011). Therefore, this thesis first attempts to construct an SSD curve for the species relevant to Norway, including the stage-specific differences to assess the different sensitivities to H_2O_2 (Fig. 8). The SSD curve was based on mortality data (LC_{50} values) using the R-package *fitdistrplus* (Delignette-Muller & Dutang, 2015). The SSD curve shows that *M. norvegica* is the most sensitive species to H_2O_2 whereas the shrimp *P. elegans and P. flexuosus* are the most resilient non-target crustacean species of the Norwegian marine ecosystems. The derived HC_5 was 1.88 (0.5 - 6.7) mg/L H_2O_2. In order to have a better understanding of the toxicity of H_2O_2, three levels of sensitivity were included. The high sensitivity fraction corresponds to less than 25% of the affected species, which includes *C. finmarchicus* after a 1h exposure with a 24h, 48h and 96h post-exposure period and *M. norvegica* after a 1h exposure plus a 72h post-exposure period. The medium sensitivity fraction includes between 25% and 75% of the species affected. And in the low sensitivity fraction > 75% of the species are

affected. This low sensitivity fraction includes the *Calanus* spp. CV following 1h exposure and *H. gammarus* stages II to IV following a 1h exposure plus a 24h post-exposure period. The SSD curve also illustrates how the effect of the chemotherapeutants is influenced by both exposure time and the post-exposure period.

The revised method for deriving guideline values for toxicants in Australia and New Zealand (Warne *et al.*, 2018) provide a protocol when there are two or more studies presenting data for the same endpoint, species and different life-stages. These guidelines state that only a single toxicity value should be used to represent the sensitivity of each species in an SSD. However, there are often multiple toxicity values for each species. In that case, both selection and manipulation of the data is required. The geometric mean is calculated for toxicity values that come from studies with the same species, endpoints and duration of exposure. The lowest value for all combinations of species and endpoints is chosen and used for the SSD.

A second SSD curve was constructed including all available EC_{50}/LC_{50} for non-target crustacean species around the world following the Australian and New Zealand guidelines for multiple data points for the same species (Fig. 9). The additional species are *M. edwardsii* (Gebauer *et al.,* 2017), *H. americanus* larvae stage I (Burridge *et al.,* 2014), *Mysid* sp. (Burridge *et al.,* 2014), *Crangon septemspinosa* (Burridge *et al.,*2014) and *A. hudsonica* (Van Geest *et al.,* 2014). The derived HC_5 was 3.09 (0.6-14.9) mg/L H_2O_2. The same three sensitivity divisions are also included in this SSD. This new curve places *C. septemspinosa* as the most resilient species with an LC_{50} of 3182 mg/L H_2O_2. The species with lowest sensitivity relevant for Norway was *H. gammarus* stg. IV with an LC_{50} of 737 mg/L H_2O_2. Compared to the first SSD constructed (Fig. 8), the second SSD (Fig. 9) predicts a higher HC_5 value. By generating an SSD with more species the HC_5 value becomes more precise. Nevertheless, Fig.9 only represents species from the same taxonomic group. Though the determination of SSD curves for a single taxonomic group has previously been done in other studies to determine the differences in toxicity (de Souza *et al.,* 2020), the different environmental agencies require a minimum number of taxonomic groups to be represented in SSDs for their use in policy applications (Posthuma *et al.*, 2002).

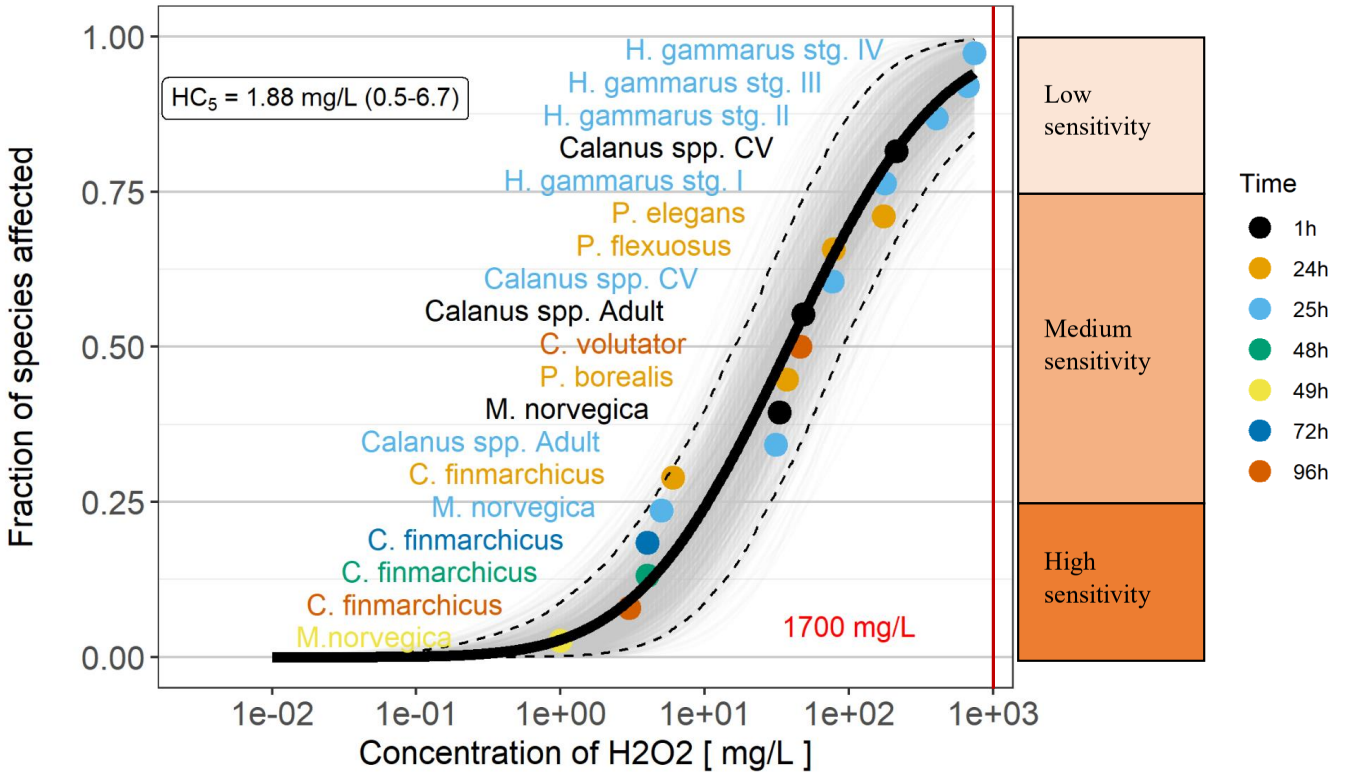

Fig. 8 Species sensitivity distribution (SSD) to H_2O_2 of non-target crustacean species relevant to the Norwegian marine ecosystem. The SSD was generated using the available LC_{50} data from this study and two previous studies that also calculated LC_{50} values (Smit *et al.,* 2008; Brokke, 2015; Refseth *et al.*, 2016; Hansen *et al.,* 2017). The graph depicts predictions (black), bootstrapped values (grey), CI (dashed) and the LC_{50} values (dots).

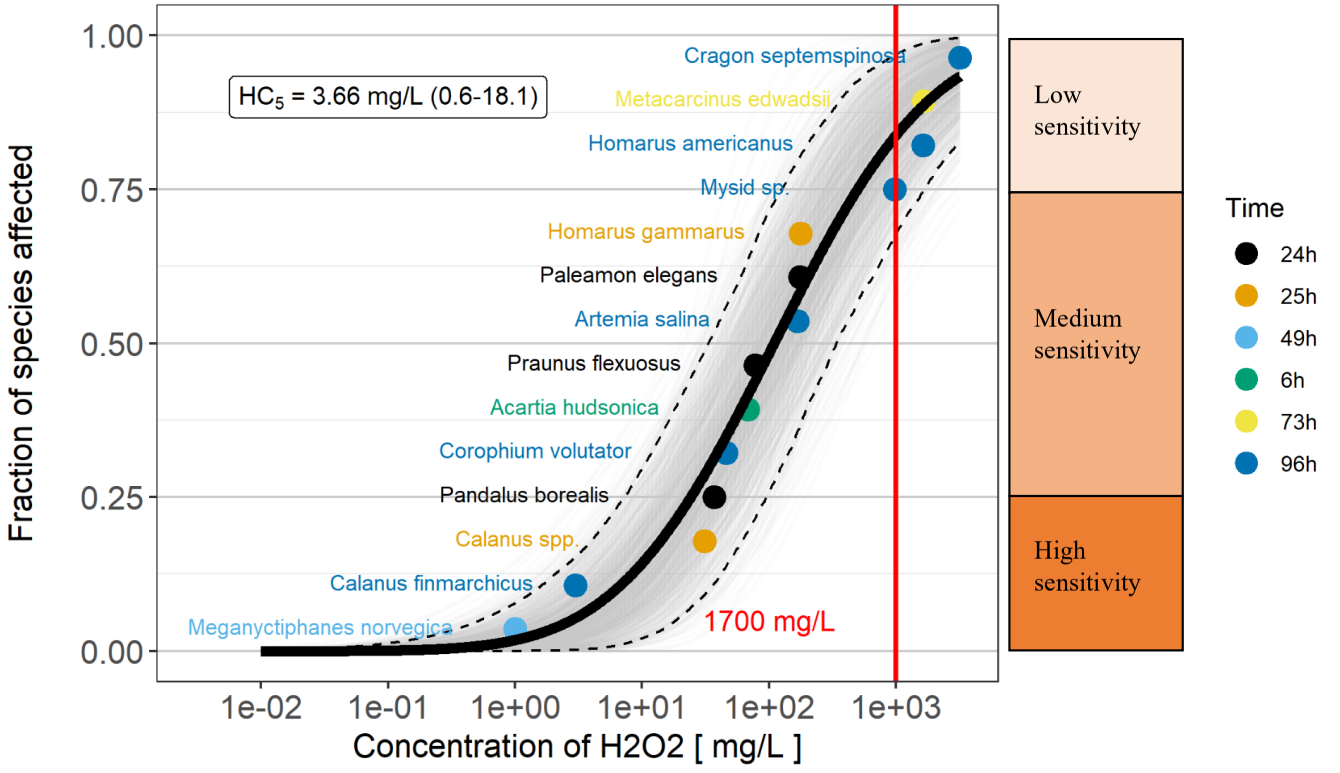

Fig. 9 Species sensitivity distribution (SSD) to H_2O_2 of non-target crustacean species. The SSD was generated using the available LC_{50} data from this study and five previous studies that also calculated LC_{50} values (Smith *et al.*, 2008; Burridge *et al,* 2014; Van Geest *et al.,* 2014; Brokke, 2015; Refseth *et al.*, 2016; Gebauer *et al.,* 2017; Hansen *et al.,* 2017). The graph depicts predictions (black), bootstrapped values (grey), CI (dashed) and the LC_{50} values (dots).

The available toxicity data for marine species exposed to H_2O_2 was evaluated. Preference was given to toxicity data published in peer-reviewed journals. All of the data used complied with a range of criteria following the Guidelines for Fresh & Marine Water Quality (2018): 1) The study should be publicly available. 2) The study should be available in English. 3) Toxicity data should be published after 1980. 4) Duration of exposure should be stated. 5) Test concentrations should not differ by a large amount (e.g. more than 10-fold difference). 6) Toxicological endpoints should be stated and/or endpoints should be ecologically relevant. 7) Toxicity values should not be greater than twice the aqueous solubility. 8) Mortality in the controls must be stated either in the text or in a table. 9) Mean mortality in the controls should not exceed 20%.

Toxicity data that fulfilled all of these criteria were then quality assessed, according to the methods of Zhang *et al*, 2015. Toxicity data with a quality score ≥80% were classified as 'high' quality, data with a quality score of ≥50 to <80% were classified as 'acceptable' quality, while data with a quality score of <50% were classified as 'unacceptable' quality. Only 'high' and 'acceptable' quality data were used for the creation of the SSD in Fig. 10 (Appendix A).

By including the sensitivity of six other phyla, the SSD presented in Fig. 10 shows a broader perspective of impact of H_2O_2 on the marine environment. The HC_5 value derived from the available toxicity data for marine species is 5.11 (1.52 – 16.15) mg/L. This value is a HC_5 with a higher degree of precision than the first derived value, reinforcing the need for data to achieve better SSDs. The single-celled green algae, *Dunaliella tertiolecta* (phylum Ochrophyta) was the most sensitive. Overall, the different species of arthropoda exhibit different sensitivity. Figure 10 also shows that the toxicity of H_2O_2 is not specific to crustaceans, which is consistent with earlier observations on this chemotherapeutant. By being non-specific H_2O_2 can negatively impact a broader number of phyla.

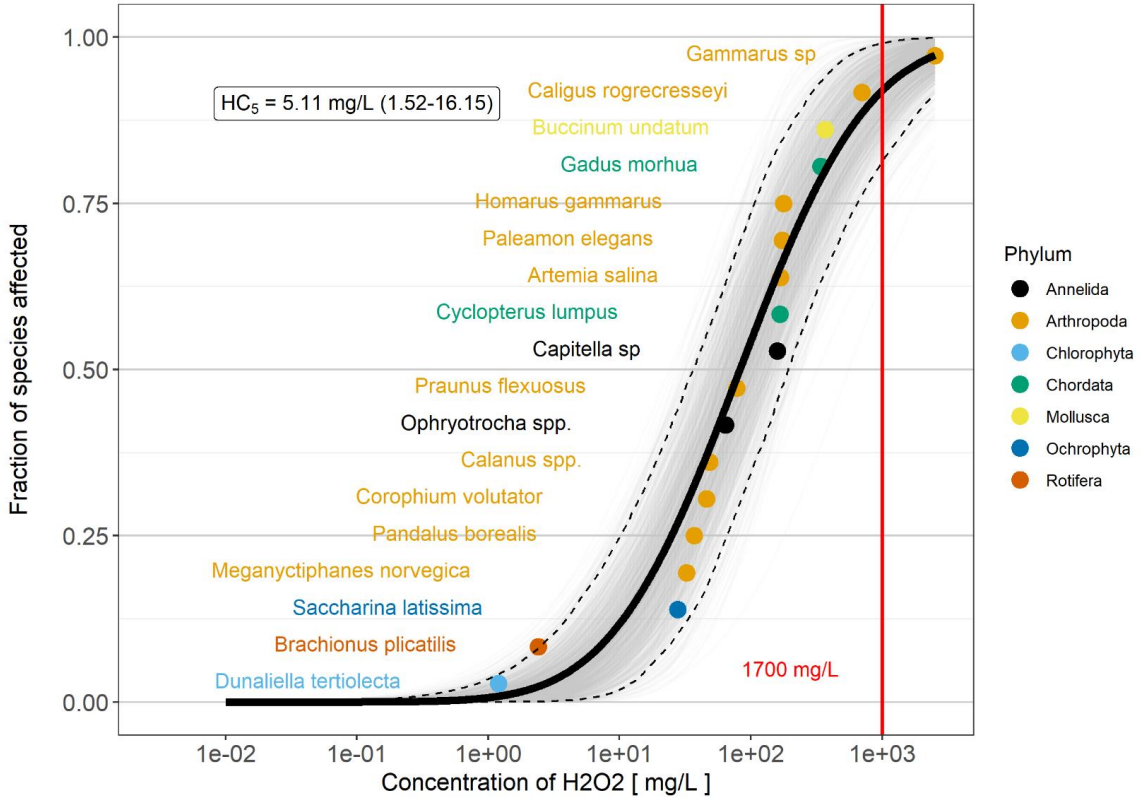

Fig. 10 Species sensitivity distribution (SSD) to H_2O_2 of marine species. The SSD was generated using the available toxicity data for marine species. The graph depicts predictions (black), bootstrapped values (grey), CI (dashed) and the LC_{50} values (dots

The review of studies presenting toxicity data of marine species towards H_2O_2 shows that arthropods were over-represented with 10 species in the SSD, while the other six phyla only had data for one or two species. The strict criteria for the SSD ecotoxicology data selection constrained the SSDs that could be developed to a low number of chemical compounds. The current methods for aquatic toxicity data evaluation are mainly based on freshwater species, and some of the data selection criteria are not applicable to marine species. As a result, the risks of most marine pollutants cannot be evaluated and, therefore, managed (Posthuma *et al.*, 2019). The European Environment Agency (2018) reports that environmental quality standards have been assessed for approximately only 300 compounds out of over 146000 compounds registered in the REACH website. ECHA requires a minimum of 10 tests with species from different taxonomic groups (minimum 8) for the derivation of an SSD (ECHA, 2008). As is most often the case, the modeled SSD curve in this thesis for the toxicity of H_2O_2 in marine species does not comply with ECHA's minimum of 8 taxonomic groups for the extrapolation of a PNEC value from the HC_5 value. Further toxicity studies on non-target species other than arthropods are needed for the completion of the H_2O_2 SSD curve and the extrapolation of a PNEC value for this pollutant.

Will a single species be equally sensitive to several chemotherapeutants?

A risk ranking, illustrating the relative risk of each chemical against another for key species, is an important tool to direct where regulatory efforts should be focused on to best protect Norway's and other salmonid producing country's marine ecosystems. Therefore, available data from previous studies was reviewed, in order to propose a preliminary ranking of the toxicity of these chemotherapeutants to non-target crustaceans. All the available information on species that have been exposed to the three chemotherapeutants and for which LC_{50} values have been calculated was collated (Table 3). Eight different crustacean species have been tested with H_2O_2, deltamethrin and azamethiphos: *Acartia hudsonica*, *Crangon septemspinosa, Homarus americanus*

(multiple stages), *Homarus gammarus* (multiple stages), *Metacarcinus edwardsii*, *Mysid* sp., *Praunus flexuosus* and *Palameon elegans.* Overall, there are different lethal threshold rankings for the different non-target crustaceans exposed to H_2O_2, deltamethrin and azamethiphos.

Table 3. Summary of all the available toxicity studies for non-target crustacean species exposed to H_2O_2, deltamethrin and azamethiphos.

Species	Chemicals	Exposure time	Post-Exposure time	LC_{50}	References
Acartia hudsonica	H_2O_2	1h	6h	2.6-10 mg/L*	Van Geest *et al.,* 2014
	Deltamethrin	1h	6h	0.017-0.067 µg/L*	
	Azamethiphos	1h	6h	-*	
	Cypermethrin	1h	6h	0.098-0.36 µg/L*	
Crangon septemspinosa	H_2O_2	1h	96h	3182 mg/L	Burridge *et al.* 2014
	Deltamethrin	1h, 24h	96h	142 ng/L	
	Azamethiphos	1h, 24h	96h	> 85.5 µg/L	
Homarus americanus Adult	H_2O_2	1h	96h	> 3750 mg/L	Burridge *et al.* 2014
	Deltamethrin	1h, 24h	96h	18.8 ng/L	
	Azamethiphos	1h, 24h	96h	24.8 µg/L	
Homarus americanus Stage I	H_2O_2	1h	96h	1673 mg/L	Burridge *et al.* 2014
	Deltamethrin	1h, 24h	96h	3.4ng/L	
	Azamethiphos	1h, 24h	96h	> 86.5 µg/L	
Homarus gammarus Stage I	H_2O_2	1h	24h	177 mg/L	**Paper II & IV**
	Deltamethrin	1h	24h	2.6 ng/L	
	Azamethiphos	1h	24h	43.1 µg/L	
Homarus gammarus Stage II	H_2O_2	1h	24h	404 mg/L	**Paper II & IV**
	Deltamethrin	1h	24h	2.9 ng/L	
	Azamethiphos	1h	24h	20.5 µg/L	
Metacarcinus edwardsii zoea I	H_2O_2	40min	48h	1642 mg/L	Gebauer *et al.,* 2017
	Deltamethrin	40min	48h	1.25 µg/L	
	Azamethiphos	40min	48h	2.84 µg/L	

	Cypermethrin	30min	48h	-	
Mysid sp.	H_2O_2	1h	96h	> 973 mg/L	Burridge *et al.* 2014
	Deltamethrin	1h	96h	13.9ng/L	
	Azamethiphos	1h	96h	> 86.5 µg/L	
Palameon elegans	H_2O_2	1h, 24h	24h	179 mg/L	Brokke 2015
	Deltamethrin	1h, 24h	24h	8.3 ng/L	
	Azamethiphos	1h, 24h	24h	47 µg/L	
Praunus flexuosus	H_2O_2	1h, 24h	24h	24.9 mg/L	Brokke 2015
	Deltamethrin	1h, 24h	24h	6.3 ng/L	
	Azamethiphos	1h, 24h	24h	4.6 µg/L	

* In the study conducted on *A. hudsonica*, LC_{50} values were not determined. The calculated effective concentrations (EC_{50}) based on the immobilization are given in the table.

In the case of the early life stages of the European lobster, although the recommended treatment concentrations of H_2O_2, deltamethrin and azamethiphos were all lethal (**Papers II & IV**), a difference in lethal threshold ranking was observed. After 1 h exposure to H_2O_2 followed by a 24 h post-exposure period the calculated LC_{50} values for stages I and II of *H. gammarus* represent approximately 9- and 4-fold dilutions of a treatment concentration of 1700 mg/L (**Paper II**). Exposures to deltamethrin were considerably more toxic to both larval stages I and II, with LC_{50} values representing approximately an 800-fold dilution of the recommended treatment dose of 2000 ng/L (**Paper IV**). Lastly, the LC_{50} for azamethiphos were calculated to approximately 2- and 5-fold dilutions respectively of the treatment solution of 100 µg/L for stage I and stage II *H. gammarus* (**Paper IV**). The ranking of toxicity is therefore: deltamethrin > H_2O_2 > azamethiphos, from more toxic to less toxic (Fig. 11). The results reported on the shrimp species *P. elegans* and *P. flexuosus* (Brokke, 2015) and *Mysid* sp. (Burridge *et al.,* 2014)*,* support the conclusion in **Papers II & IV** for the proposed ranking of lethal thresholds. In contrast, following from the *H. americanus* larvae LC_{50} (Burridge *et al.,* 2014), H2O2 was less toxic than azamethiphos. The data for *H. americanus* larvae by Burridge *et al*. (2014) result in a ranking of deltamethrin > azamethiphos > H_2O_2. Overall, deltamethrin is the more toxic of the three.

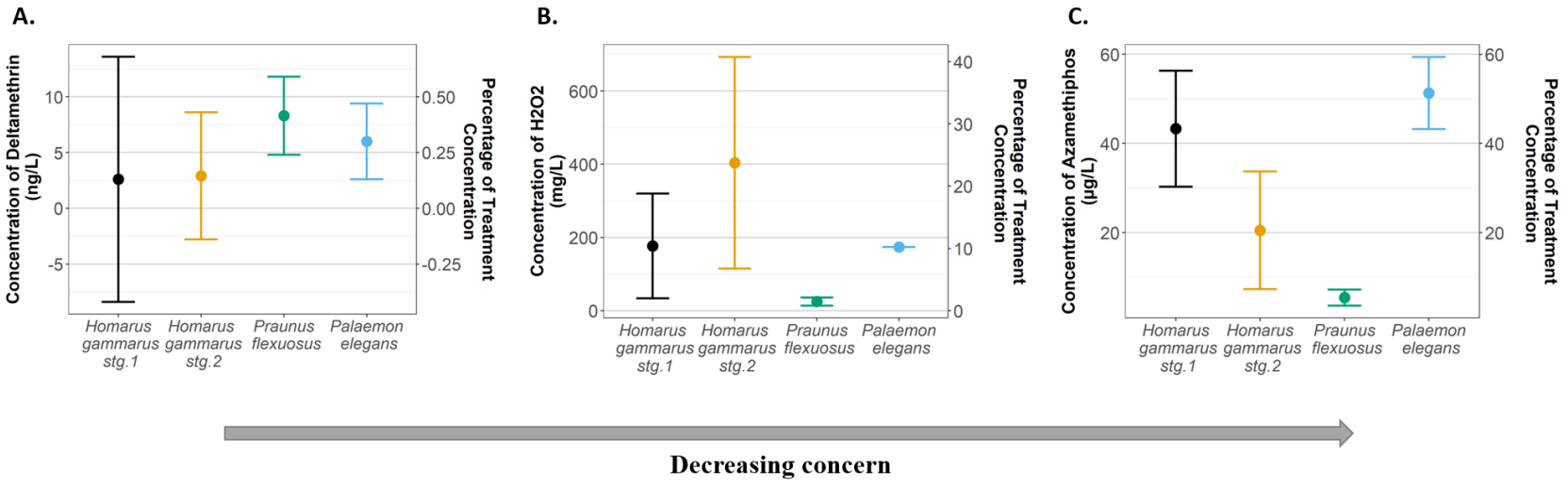

Fig. 11 Ranking of the toxicity of bath treatment chemotherapeutants, deltamethrin, H_2O_2 and azamethiphos based on the difference between the median lethal concentrations LC_{50} (95% C.I). The LC_{50} values for Norwegian native species, *Homarus gammarus* larvae stages I and II (**Papers II & IV**), *Praunus flexuous* and *Palaemon elegans* (Brokke, 2015), are plotted as circles, with the 95% confidence intervals in brackets. The recommended treatment concentrations in Norway are 2µg/L deltamethrin, 100 µg/L azamethiphos, and 1700 mg/L H_2O_2.

The sub-lethal effects of chemotherapeutants

Though the importance of examining sub-lethal effects is clear, the available literature regarding these on behavioral endpoints by the delousing chemotherapeutants is limited. The effects of deltamethrin, azamethiphos and H_2O_2 have been studied on the swimming performance of *M. edwardsii* larvae (Gebauer *et al.,* 2017). The sub-lethal effects of the same three chemotherapeutants were examined on the behavior, embryonic development and reproductive output of *P. borealis* (Frantzen *et al.,* 2020). Another study focused on the effects of H_2O_2 on the deep-water shrimp *P. borealis*, and investigated immobilization, swimming activity and feeding rates after exposure (Bechmann *et al.,* 2019). Even at low concentrations, reported effects were, inability to swim or increased swimming activity (depending of the chemical), inability to capture food and delayed molting (Gebauer *et al.,*2017; Bechmann *et al.,* 2019; Frantzen *et al.*, 2020).

In this work, two categories of non-lethal effects were measured. In **papers I & II**, short-exposure (1h) to sub-lethal concentrations of H_2O_2 caused both metabolic stress and led to the impairment of anti-predator behaviors (escape response and shelter seeking behavior) of non-target crustacean species. In addition to these effects two observations were made; bubble formation inside the carapace and immobilization when exposed to sub-lethal concentrations of H_2O_2. The exposure to H_2O_2 even at low doses, resulted in the formation of bubbles inside the carapace of all pelagic stages of *H. gammarus* (**Paper II**). The formation of bubbles led the animals to float to the surface of the water. In field conditions, this would be detrimental as animals would be unable to feed or escape predators. The formation of O_2 bubbles has also been described in sea lice (Bruno and Raynard, 1994), and is considered the primary cause of detachment from the salmonid host following H_2O_2 treatment (Cotran *et al.,* 1989; Treasurer *et al*., 2000; Aaen *et al.,* 2014). During a 24 h exposure to H_2O_2, stage I larvae of the American lobster were also floating, however there was no mention of bubbles inside the carapace (Burridge *et al.,* 2014). Bubble formation was not observed for the Northern krill (**Paper III**), nor reported for the Northern shrimp, the mola rock crab larvae, or copepods (*A. hudsonica* and *C. finmarchicus*) (Van Geest *et al.,* 2014;

Gebauer *et al.,* 2017; Bechmann *et al.,* 2019; Frantzen *et al.,* 2020), nor for pre-adult or adult stages of *Calanus* spp. (**Paper I**). The reason for these inconsistencies requires further investigation.

Immobilization was observed throughout experiments with H_2O_2. It was described in the copepod *A. hudsonica,* the crab *M. edwardsii*, and the shrimp *P. borealis* after exposures equal to or below the recommended treatment doses (Van Geest *et al*., 2014; Gebauer *et al.,* 2017; Bechmann *et al.,* 2019; Frantzen *et al.,* 2020). Though none these studies reported bubble formation, they all described a mechanical immobilization induced by the exposure to H_2O_2. Hydrogen peroxide, even at the sub-lethal doses, also caused some degree of immobilization in *Calanus* spp. and *H. gammarus* (**Paper I & II**). Immobilization was also observed in *H. gammarus* stages I and II after exposure to deltamethrin and azamethiphos (Table 4) (**Paper IV**). In the wild, paralyzed zooplankton would be unable to feed or to maintain their position in the water column and their predator avoidance behavior would also be severely affected.

Table 4. Summary of the EC_{50} values based on the combination of mortality and immobilization by deltamethrin and azamethiphos for *H. gammarus* larvae stages I and II.

Chemical	Life stage	Endpoint measured	Exposure Period	EC_{50}	Fold-Dilution	Reference
Deltamethrin	*H. gammarus* Stage I	Immobility + Mortality	1h	0.6 ng/L	4	**Paper IV**
	H. gammarus Stage II	Immobility + Mortality	1h	0.4 ng/L	7	**Paper IV**
Azamethiphos	*H. gammarus* Stage I	Immobility + Mortality	1h	15.5 ng/L	2	**Paper IV**
	H. gammarus Stage II	Immobility + Mortality	1h	9.2 ng/L	3	**Paper IV**

After a 1h exposure to sub-lethal concentrations of H_2O_2, measurable effects were observed on the escape response of *Calanus* spp. (**Paper I**). Both the distance at which the copepods initiated their escape response and the distance traveled decreased with increasing H_2O_2 concentration. These two parameters in the response of copepods are decisive factors in escaping from predators (Fields and Yen, 1997). The success of

copepods in planktonic communities is highly dependent on their ability to initiate a rapid response (Fields and Yen, 2002). Additionally, the oxygen consumption rates (OCR) significantly decreased with increased concentrations of H_2O_2 (**Paper I**). Considering that the energetic cost of an escape reaction in copepods is high and that the strength of each response decreases with increasing frequency of predator attacks (Strickler, 1975; Fields, 2000), a lower OCR (metabolic activity) may impact the escape response of copepods. Our behavioral results suggest that *Calanus* spp., the dominant component of the North Atlantic zooplankton communities, will be more susceptible to predation due to impairment of their escape response as a result of the paralysis induced by H_2O_2 even at a concentration equivalent to 1% of the recommended treatment dose.

In **paper II**, all sub-lethal concentrations 85-510 mg/L H_2O_2, negatively affected various behavioral parameters associated with the shelter seeking of the stage V *H. gammarus*. After a short-term (1h) exposure, lobsters traveled a shorter distance and inspected the potential shelter significantly fewer times compared to non-exposed individuals. Furthermore, at concentrations equivalent to 10 and 30% of the recommended H_2O_2 dose, it took the lobsters a longer time to recognize the shelter (**Paper II**). Other studies also report negative effects of anti-sea lice drugs on lobster juveniles. Sub-lethal concentrations of azamethiphos had a negative effect on the use of shelters by juvenile *H. americanus*, with an increase in the lobsters' latency to re-enter the shelter observed with increasing azamethiphos concentrations (Abgrall *et al.,* 2000). Similarly, juvenile *H. gammarus* exposed to the in-feed drug teflubenzuron used significantly more time to find and recognize shelter than those given unmedicated feed (Cresci *et al*., 2018). Together these studies demonstrate that shelter-seeking behavior of juvenile lobsters is impacted by chemotherapeutants given both in-feed and as bath treatment. The survival of newly settled lobsters depends on the ability to avoid predators and rapidly find a shelter (Hudon, 1987; Lawton & Lavalli, 1995; van der Meeren, 2001; Mehrtens *et al*., 2005). Young lobsters that reside in the vicinity of salmon farms treating with H_2O_2, may therefore be at a higher risk of predation. Our results suggest that H_2O_2, as low as 5% of the recommended treatment concentration, poses a risk to the pelagic life stages as well as bottom-dwelling lobster life stages. Twenty-four hours after exposure, there were no significant differences between control

and H_2O_2 -exposed lobsters for any of the behavioral parameters assessed. This suggests that the sub-lethal effects of H_2O_2 on the shelter seeking behavior of *H. gammarus* may only be short lived, with the risk of predation in the wild likely to be highest in the immediate aftermath of an exposure scenario.

In the previous section, the toxicity of H_2O_2, deltamethrin and azamethiphos was ranked to define their relative risks based on their LC_{50} values. A similar ranking of the compounds has also been used for non-lethal effects. In the case of the copepod *A. hudsonica,* the feeding inhibition threshold ranking was: H_2O_2 > deltamethrin > cypermethrin > azamethiphos (Table 5) (Van Geest *et al.,* 2014). Ernst *et al.* (2001) recorded immobilization, based on the loss of mobility of *E. estuarius* even though the movement of the appendages was still present. They calculated the EC_{50} value for azamethiphos to be 3.0 µg/L (33-fold dilution) and for cypermethrin <0.05 µg/L (2000-fold dilution). Given these values the immobility threshold ranking for *E. estuarius* was: azamethiphos > cypermethrin.

Table 5. Toxicity ranking for *Acartia hudsonica* based on the EC_{50} values from feeding inhibition (data taken from Van Geest *et al.* 2014).

Chemical	H_2O_2	Deltamethrin	Cypermethrin	Azamthiphos
Treatment Concentration	1200 mg/L	2 µg/L	5 µg/L	100 µg/L
EC_{50} value	2.6-10 mg/L	0.017-0.067 µg/L	0.017-0.067 µg/L	ND
Fold-dilution	460 to 120	30 to 117	13 to 51	-

Preliminary results indicate that 1h exposure to sub-lethal concentrations of H_2O_2 caused structural alterations in the gill of *H. gammarus* larvae (Fig. 12, Escobar-Lux unpublished data). Gill alterations on non-target crustaceans, *P. montague* and *P. borealis*, following H_2O_2 exposure have also been reported in previous studies (Fagereng, 2016; Bechmann *et al.,* 2019). The present study hypothesizes that gill damage can be an indicator for delayed effects and increased mortality with increasing post-exposure periods. Further research is necessary to corroborate this.

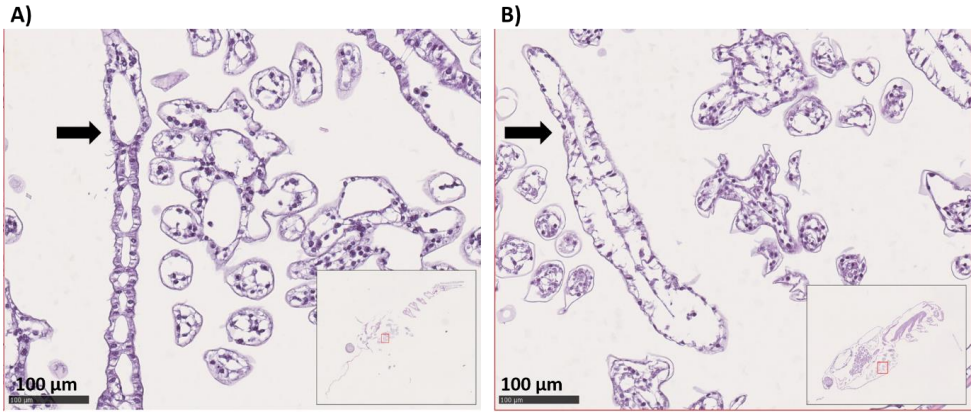

Fig. 12. Gills from control *H. gammarus* larvae stage I (A), and larvae exposed for 1h to 170mg/L H_2O_2 (B) (Escobar-Lux, unpublished data). Significant structural alterations are observed in the gill of exposed larvae. The epithelium is observed to be lifted from the basal membrane in the H_2O_2 exposed larvae.

Low doses of H_2O_2 may have detrimental consequences on the survival of non-target species, but relatively few studies have focused on the sub-lethal effects of other chemicals such as deltamethrin and azamethiphos. Our results have also shown immobilization of stages I and II of *H. gammarus* after exposure to deltamethrin and azamethiphos (**Paper IV**). Acute toxicity studies are not sufficient to evaluate effects of chemotherapeutants. Sub-lethal endpoints that are ecologically relevant should be implemented in future toxicity studies to avoid underestimation of risk.

Contextualizing laboratory studies

This work and previous studies have reported the negative effects of chemotherapeutants on non-target marine organisms at concentrations in line with current usage in salmonid aquaculture. So far, these effects have been assessed only in controlled laboratory settings. Ideally, field studies should be prioritized, however, limitations such as higher overall costs and sampling occurring at a single point in time and location, may restrict the outcome of such studies.

As of today, no study has exposed non-target species in-situ following a H_2O_2 plume discharge, but two separate field studies have made direct measurements of the chemotherapeutant concentrations in the surrounding waters of a Norwegian salmonid farm following the discharge effluents (Andersen & Hagen, 2016; Fagereng, 2016). The concentrations decreased with time and distance from the farm (Andersen and Hagen, 2016). Low concentrations of H_2O_2, 25 to 60 minutes after the discharge were measured in water sampled at 20-60 m from the edge of net pens after discharge (Fagereng, 2016). Still, these concentrations were higher than the calculated LC_{50} for the Northern krill and European lobster (**Paper II and III)**.

In the case of deltamethrin and azamethiphos two field studies have measured their concentration in the surrounding waters after discharge (Ernst *et al.,* 2014; Langford *et al.,* 2015). Using rhodamine dye, one of the studies measured the fate of the dispersing chemotherapeutants following six treatments to facilitate the tracking of the plume (Ernst *et al.* 2014). The study showed that azamethiphos was mostly present in the aqueous phase, indicating that non-target organisms are primarily exposed in the water column, while deltamethrin was observed primarily associated with particles. High concentrations of azamethiphos (25 µg/L) in water were sampled within 1 m from the edge of the pen (Ernst *et al.,* 2014). These concentrations are similar to the lethal concentrations described in this thesis for *H. gammarus* (**Paper IV**). Another study indicated that azamethiphos might dilute more slowly, with concentrations of 26 ng/L in the water samples one week after the treatment (Langford *et al.,*2015). Overall, the concentrations reported by the available field studies support that the lethal and effective concentrations found in this thesis for H_2O_2, deltamethrin and azamethiphos can be found in the surrounding areas of farms following their discharge.

Laboratory studies coupled with hydrodynamic models are useful for predicting the environmental impact of chemotherapeutants. By incorporating the topography of farm locations and the lethal/effective data from toxicity studies, these models can predict the dilution, dispersion and impact areas of the chemicals. A previous mathematical model predicted the dispersal of H_2O_2 from Norwegian aquaculture sites (Refseth *et al.*, 2016). This model describes a more extensive spread of H_2O_2 than the field studies implied, predicting that concentration under 100 mg/L H_2O_2 can be present in surface

waters (0-3 m depth) up to several hours after discharge at distances up to 2 km away. These simulations suggest that both the pre-adult stage CV and adult stage of *Calanus* spp. and *M. norvegica* located within 2 km of H_2O_2 treated salmonid farms may be exposed to lethal concentrations (Fig 13) (**Papers I & III**). Furthermore, the model also predicted that areas within 1 km of the farm may experience concentration > 300 mg/L H_2O_2 within the first hour after discharge indicating that pelagic life stages of *H. gammarus*, particularly stages I and II living within 1–2 km of a salmon farm may be exposed to lethal concentrations of H_2O_2 (**Paper II**).

This thesis, with the use of a hydrodynamic model, simulated the dispersal of deltamethrin and azamethiphos into the Norwegian marine environment and mapped the potential risk areas for wild European lobster (Fig. 13) (**Paper IV**). Our results show that large areas around salmonid farms, ranging from 21.1 to 39.0 km^2, were within high impact zones of deltamethrin. The azamethiphos impact zones around the farms were relatively small and thus its effect would be less severe (**Paper IV**). The difference in the impact zones is large and is caused by the difference in toxicity between the two drugs. For azamethiphos the LC_{50} values for stage I and II *H. gammarus* larvae represented approximately 2- and 5-fold dilutions of the treatment concentration used in Norway. For deltamethrin, on the other hand, these values were approximately 800-fold dilution of the treatment concentration. Compared to previous models, the present study indicates that these low levels of deltamethrin could disperse to approximately 10 times greater distances.

The model in **paper IV** demonstrates that large areas around aquaculture sites are exposed to lethal and effective concentrations of deltamethrin following treatments, and therefore this compound may have widespread adverse effects on sensitive non-target crustacean species living in the vicinity. However, we must also highlight some major limitations of the model. First, several of the model's underlying assumptions could result in its predictions being worst case scenarios of the impact zones. For example, the model may over-estimate the dispersal of deltamethrin as it assumes that the chemical is not absorbed by organic matter. Secondly, though the impact zones were delimited by the effective concentrations, time was not considered and therefore, how long the harmful concentrations were present in an area was not determined. The model

simply maps areas surrounding the net pens that experience lethal and effective concentrations at any point in time during the 24 h simulation.

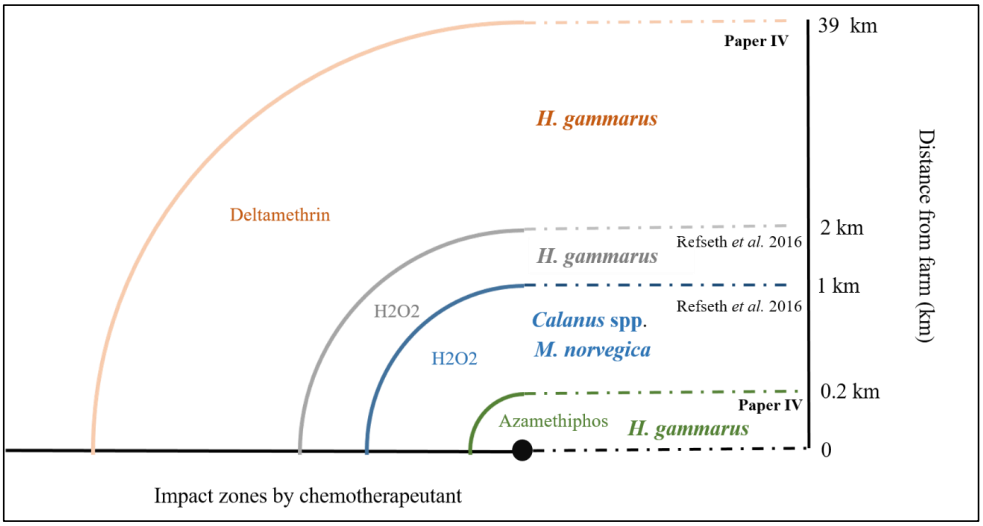

Fig. 13. Simplified illustration of the impact zones of H_2O_2, deltamethrin and azamethiphos after discharge from a salmonid farm. Based on the hydrodynamic model data from Refseth *et al.* 2016 and **Paper IV.** The figure does not illustrate the depth of the impact zones.

Hydrodynamic models have shown that a single 1 h exposure to H_2O_2, deltamethrin and azamethiphos had lethal and sub-lethal effects on non-target organisms. Delousing operations can involve parallel and consecutive chemotherapeutant treatments in many sea cages within a single fjord. Thus, non-target crustaceans are likely exposed to multiple chemotherapeutant plumes over a few days (Grefsrud *et al*., 2018). In previous studies, lower LC_{50} and EC_{50} values have been described as a result of pulse-like exposures for crustaceans (Burridge *et al*., 2000, 2008; Bechmann *et al*., 2019). Therefore, the real impact of H_2O_2, deltamethrin and azamethiphos on wild populations of non-target crustaceans may be higher than the effects observed for a single exposure. Laboratory studies can provide further results to be implemented in future models and reduce this knowledge gap.

ADDITIONAL CONSIDERATIONS

In this thesis, the tests that were carried out were static; that is, the exposure water was not changed over time. The limitations of such static studies are: 1) The toxicant concentrations drop due to degradation, uptake or adsorption by the container, and 2) the water quality may diminish due to the accumulation of waste and decrease of O_2. To mitigate these limitations, I conducted short one-hour exposures in glass beakers, in which neither H_2O_2, deltamethrin and azamethiphos degrade after 1h exposure (Bruno & Raynard, 1994; Lyons et al., 2014; Fagereng, 2016; Burridge *et al.,* 2014; Bechmann *et al.,* 2020).

The LC_{50} values in this work are based in nominal concentration. These three compounds have been approved as drugs, suggesting that the nominal concentrations are an official procedure as they comply with a minimum quality standard. Nevertheless, the measurement of pollutant concentrations before and after their use should be carried out in ecotoxicology studies to ensure consistency of exposure concentrations and nominal concentrations.

This study identifies some general lessons and considerations for ecotoxicology studies in the marine environment. Ecotoxicology studies are often based on guidelines design for freshwater environments in which conventional laboratory species such as *Daphnia magna* are used. However, we cannot rely on the results of freshwater species for the approval of pesticides to be used in the marine environment. It is of utmost importance to take into consideration the ecology of the species tested and their role in the ecosystem. By considering the relative toxicities across species and life-stages, this thesis has highlighted the need to perform tests with species relevant to the ecosystems in question. This work, together with previous studies, has shown the importance of including different life stages when performing ecotoxicology tests. Data for the most sensitive life stage should be included in the creation of SSD curves, as we do not know which life stage is the most sensitive, larval, juvenile or adult stages and which should be tested. Additionally, the 3Rs (Replacement, Reduction and Refinement) should be taken into account when conducting ecotoxicology studies. Although, enough data should be collected to produce reliable SSD curves, PNECs, NOEC values in order to

provide better risk assessments. If the studies are conducted with high standards, completing as much of the quality criteria from a standardized guideline, and on relevant species, enough data for management advice will be available.

PERSPECTIVES FOR THE FUTURE

Standardized toxicity tests for chemotherapeutants should be conducted to allow a better comparison between studies. Shorter exposure times followed by a prolonged observation period, will continue to provide insight on the delayed effects on non-target crustaceans and other marine species. Likewise, this thesis supports the necessity to assess the negative impacts of these chemicals at a local level. Variation in species sensitivity has been observed, therefore the outcome of this thesis highlights that there is a need for agrochemical companies to carry out toxicity tests with local keystone species from different taxonomic groups when applying for authorization from the appropriate regulatory body. Sub-lethal endpoints should also become required in the implementation of risk assessments to avoid underestimating risk. Lastly, risk assessments can be further improved with the inclusion of time-to-event data. By incorporating both exposure concentration and duration at which the first effects appear, a better overview of the impact of these compounds can be attained.

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APPENDIX A

Studies from which the toxicity data was used for the derivation of the SSD, Figure 10.

Reference	Phylum	Species Scientific Name	Endpoint	Duration (h)	LC/EC50	Mean Value	Lowest Value
Fang et al., 2018	Annelida	*Capitella* sp	Mortality	1	159,3	159,3	159,3
Fang et al., 2018	Annelida	*Ophryotrocha* spp.	Mortality	1	64,3	64,3	64,3
Smit et al., 2008	Chlorophyta	*Dunaliella tertiolecta*	Growth Inhibition	72	1,2	1,2	1,2
Smit et al., 2008	Arthropoda	*Artemia salina*	Mortality	72	188	188	
Smit et al., 2008	Arthropoda	*Artemia salina*	Mortality	96	168	168	168
Paper I	Arthropoda	*Calanus spp.*	Mortality	1	30,6		
Paper I	Arthropoda	*Calanus spp.*	Mortality	1	77,1	48,57	48,57
Marin et al., 2017	Arthropoda	*Caligus rogrecresseyi*	Immobilisation	0,3	700	700	700
Smit et al., 2008	Arthropoda	*Corophium volutator*	Immobilisation	24	611	611	
Smit et al., 2008	Arthropoda	*Corophium volutator*	Immobilisation	96	46	46	46
Refseth et al., 2016	Arthropoda	*Gammarus sp*	Mortality	24	2520	2520	2520
Paper II	Arthropoda	*Homarus gammarus*	Mortality	1	177		
Paper II	Arthropoda	*Homarus gammarus*	Mortality	1	404		
Paper II	Arthropoda	*Homarus gammarus*	Mortality	1	665		
Paper II	Arthropoda	*Homarus gammarus*	Mortality	1	737	432,67	432,67
Paper III	Arthropoda	*Meganyctiphanes norvegica*	Mortality	1	32,5	32,5	32,5
Brokke, 2015	Arthropoda	*Paleamon elegans*	Mortality	24	173	173	173

Refseth et al., 2016	Arthropoda	*Pandalus borealis*	Mortality	24	37	37	37
Brokke, 2015	Arthropoda	*Praunus flexuosus*	Mortality	24	43	43	43
Refseth et al., 2016	Chordata	*Cyclopterus lumpus*	Mortality	24	167	167	167
Refseth et al., 2016	Chordata	*Gadus morhua*	Mortality	24	342	342	342
Refseth et al., 2016	Mollusca	*Buccinum undatum*	Mortality	24	367	367	367
Haugland et al., 2019	Ochrophyta	*Saccharina latissima*	Mortality	1	80,7	80,7	
Haugland et al., 2019	Ochrophyta	*Saccharina latissima*	Photosynthetic Capacity	1	35,4	35,4	
Haugland et al., 2019	Ochrophyta	*Saccharina latissima*	Photosynthetic Efficiency	1	27,8	27,8	27,8
Smit et al., 2008	Rotifera	*Brachionus plicatilis*	Mortality	24	2,4	2,4	2,4

Paper I

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RESEARCH ARTICLE

The effects of hydrogen peroxide on mortality, escape response, and oxygen consumption of *Calanus* spp.

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Abstract

Hydrogen peroxide (H_2O_2), a pesticide used in salmonid aquaculture, is released directly into the environment where nontarget organisms are at risk of exposure. We determined threshold concentrations for mortality of *Calanus* spp., the dominant zooplankton species in the North Atlantic, and assessed sublethal effects, focusing on the escape response and oxygen consumption rates (OCRs) as behavioral and physiological assays. One-hour exposure to 170 mg·L^{-1} (i.e., 10% of the recommended H_2O_2 treatment) was lethal to copepodite stage V (92% mortality) and adult females (100% mortality). The acute median lethal concentration (1h-LC_{50}) was 214.1 (150.67–277.4) and 48.6 (44.9–52.2) mg·L^{-1} for copepodite V and adults, respectively. The 25-h LC_{50} was 77.1 (57.9–96.2) and 30.63 (25.4–35.8) mg·L^{-1} for copepodite V and adults, respectively. At concentrations of 0.5% and 1% of the recommended treatment level, *Calanus* spp. showed a decrease in escape performance and lower OCRs with increased concentration. At H_2O_2 concentrations of 5% of the recommended treatment levels (85 mg·L^{-1}), exposed copepods showed no escape reaction response. These results suggest that sublethal concentrations of H_2O_2 will increase the risk of predation for *Calanus* spp. Furthermore, this study provides supporting evidence that theoretical "safe" values, traditionally used for predicting toxicity thresholds, underestimate the impact of H_2O_2 on the physiological condition of nontarget crustaceans.

Key words: aquaculture, behavior, ecotoxicology, hydrogen peroxide, sublethal effects, zooplankton

Introduction

Lepeophtheirus salmonis, salmon louse, is a parasitic copepod affecting farmed and wild salmonids (Costello 2006; Torrissen et al. 2013). Addressing the economic and ecological impact of salmon lice is considered one of the most important challenges for the salmon industry. The parasite feeds on skin, causing damage associated with osmotic stress and secondary infections (Finstad et al. 2000; Johnson et al. 2004). Salmon lice infestations increase the overall cost of salmon aquaculture due to high expenses associated with delousing and the concomitant reduction in fish growth and reduced marketability due to skin lesions (Costello 2009; Liu and Bjelland 2014). In natural populations, smolts from wild salmon and trout can suffer high mortality if infested with a high density of salmon lice (Liu et al. 2011).

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To control salmon lice infestations strict regulations on the number of lice per fish—0.2 adult female lice in the spring, and 0.5 adult female lice per fish for the rest of the year—have been established in Norway, and a plan for management of salmon lice is requested from each farm by the Salmon Lice Directive (www.lovdata.no/dokument/SF/forskrift/2012-12-05-1140). To meet these regulations, commercial farms rely partly on chemical therapeutants to control salmon lice populations. One of the therapeutants used in Norway to treat salmon lice and amoebic gill disease (Young et al. 2007) is hydrogen peroxide (H_2O_2). Hydrogen peroxide is administered by bath treatment, either directly in the net pens or using well boats (Ernst et al. 2001). After treatment, the chemical is discharged into the surrounding water. The use of H_2O_2 peaked in 2015 at 43 246 tons, but declined to 9277 tons in 2017 (www.fhi.no/hn/legemiddelbruk). The mechanism of action of H_2O_2 on salmon lice includes mechanical paralysis, inactivation of enzymes and DNA replication, and peroxidation of lipid and cellular organelle membranes by hydroxyl radicals (Cotran et al. 1989). Mechanical paralysis is caused by decomposition of H_2O_2 to water and O_2 bubbles in the haemolymph, which causes detachment of the pre-adult and adult lice from the fish (Thomassen 1993; Aaen et al. 2014). Hydrogen peroxide is not effective on the chalimus stages.

The pelagic zooplankton, *Calanus* spp., is a key component in the north Atlantic food web (Melle et al. 2014) and is abundant in the coastal zone where aquaculture sites are located (Broms et al. 2009). *Calanus* spp. is an important grazer of primary production (Runge and de Lafontaine 1996; Heath and Lough 2007). The younger life stages are important food for juvenile fish in the nursery areas, and the adults are main prey for several pelagic fish stocks such as herring and cod (Dalpadado et al. 2000; Sundby 2000; Rullyanto et al. 2015). In the spring, egg production for *Calanus* spp. overlaps with the peak application of pharmaceuticals to keep the level of salmon lice below 0.2 lice females per fish. Its effect on *Calanus* spp. is largely unknown.

Only a few studies have examined the effect of exposing nontarget organisms to H_2O_2 (Burridge et al. 2014; Van Geest et al. 2014; Brokke 2015). Brokke (2015) reported that exposure to 1700 $mg \cdot L^{-1}$ H_2O_2 for 1 h resulted in 10% mortality in chameleon shrimp (*Praunus flexuosus*) and 20% in rockpool shrimp (*Palaemon elegans*) after a 24-h recovery period. Consequently, the median lethal concentration (LC_{50}) values were higher than the treatment concentration for both species. In contrast the opossum shrimp (*Mysid* sp.) were considerably more sensitive with a LC_{50} of 973 $mg \cdot L^{-1}$ (i.e., lower than the recommended treatment concentration (Burridge et al. 2014)) and late copepodide stage *Calanus* spp. had a LC_{50} of 6 $mg \cdot L^{-1}$ H_2O_2 following a 24-h exposure, indicating a time-dependent effect (Hansen et al. 2017).

The objective of this study was to determine the threshold concentrations at which H_2O_2 causes mortality in *Calanus* spp. and to assess possible sublethal effects, focusing on the escape response as a behavioral assay and oxygen consumption rates as a physiological indicator.

Materials and methods

Copepods were collected from the dock at Austevoll Research Station, Institute of Marine Research, Norway (60°05′20″N, 5°15′57″E) at a depth of 20–30 m using light traps and plankton nets. The light traps (mesh size 500 µm; 0.45 m in diameter; BellaMare, San Diego, California, USA) were equipped with a white LED light and deployed overnight. A standard plankton net (mesh size, 200 µm; diameter, 30 cm) was used to collect copepods from 20 m to the surface. Copepods were collected at least 3 km away from any commercial fish farm and were transported to the laboratory at Austevoll Research Station where *Calanus* spp. adult females and copepodite V stages were sorted. Copepods were maintained overnight in 10 L containers at 8 °C. Seawater used in the experiments was pumped from a depth of 160 m in Bjørnafjorden and filtered through a sand filter. Copepods were tested within 24 h of capture and each copepod was tested only once.

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Commercial H_2O_2 (Nemona, Akzo Nobel Pulp and Performance Chemicals AB, Bohus, Sweden) is 49.50% H_2O_2. For treating salmon, the recommended concentration for a H_2O_2 bath treatment is 1500–2100 $mg \cdot L^{-1}$ depending on temperature, for 20 min (https://www.felleskatalogen.no/medisin-vet/atc-register/QD08A). Typically, toxicity studies use exposure times of 24, 48, 72, and 96 h; however, this is not representative of the scenario following a bath treatment on a salmon farm. Therefore, we followed the recommendations of Burridge et al. (2014) and Van Geest et al. (2014) and limited the exposure time to 1 h.

A preliminary study was conducted to select the concentrations to be used in the main experiment. Testing was undertaken with concentrations of 1700 and 340 $mg \cdot L^{-1}$ H_2O_2 (~100% and 20% of the recommended treatment concentration). These concentrations caused 100% mortality after 1 h exposure for both copepodite Vs and adult females. Thus, the concentrations chosen in this study were 170, 85, 17, and 8.5 $mg \cdot L^{-1}$, corresponding to 10%, 5%, 1%, and 0.5% of a recommended treatment dose of 1700 $mg \cdot L^{-1}$.

The copepods were randomly divided into five groups, each group consisting of 30 individuals. H_2O_2 was added to each of four 4 L tanks and mixed to the target concentrations. One tank contained clean seawater and served as a control. Three PVC pipes (25 cm diameter, 25 cm tall, 500 µm Nitex screen bottom) were added to each tank; each pipe contained approximately 10 individuals. The experiment was conducted in triplicate using a total of approximately 450 copepods. The temperature in the tanks was 13 °C.

Mortality

Stage V and adult female copepods were used in all experiments. The exposure time was 1 h and, after exposure, the copepods were transferred to 10 L tanks in which they were held for 24 h (recovery period). The copepods were observed under a dissecting microscope immediately after the 1-h exposure and after the 24-h recovery period. Dead individuals were counted at each time point. Individuals were considered dead if they were discolored, deformed (urosome and pleopods folded back), or if there was no movement of the antenna and pleopods after a gentle stimulus. Copepods laying on the bottom of the tank, with retracted antennae but showing uncontrolled limb motion, (e.g., twitching of the antennae) were considered immobilized. Mortality that occurred during the 1-h exposure is defined as acute mortality. Total mortality was defined as the sum of mortality during the 1-h exposure plus that after the 24-h recovery period.

Sublethal effects

The escape response of the copepods was measured as a behavioral assay and oxygen consumption rates as a physiological indicator of sublethal effects of exposure to H_2O_2. Exposure concentrations used in these experiments were 8.5, 17.0, and 85.0 $mg \cdot L^{-1}$ H_2O_2, in addition to a control. The set-up was identical to the mortality experiment. After the 1-h exposure the copepods were transferred to 10 L tanks containing filtered seawater and the behavioral responses and oxygen consumption rates (OCRs) were measured (described below). All copepods were tested within 5 h of exposure in a randomized order. The entire experiment was repeated on three consecutive days with freshly collected copepods.

Escape response

Silhouette video photography (Browman et al. 2003), was used to observe the swimming behavior of copepods. This system allows high-quality observations of small transparent organisms at high resolution and is unaffected by ambient light intensity. Two video cameras were mounted orthogonally, each camera illuminated with a 20 cm collimated beam generated by a small red (720 nm)

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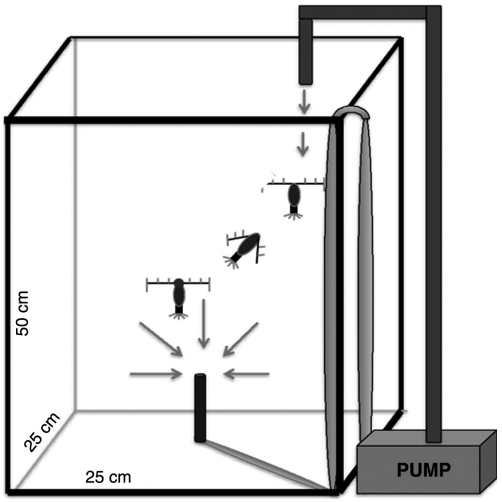

Fig. 1. Schematic diagram of the siphon tank used to observe the escape reaction of *Calanus* spp. in response to a suction flow. Illustration of the escape response of *Calanus* spp. The distance from the copepod to the siphon at the initiation of the escape response represents the detection threshold distance. Once the animal initiated an escape reaction (retracts its antennae and jumps), the total distance traveled, and the speed of the escape were measured.

LED light source. A glass aquarium holding the organisms was placed at their intersection of the two light paths. The two simultaneous orthogonal views allow particles in the field of view to be tracked in three dimensions. The software packages TRAKFISH, MANTRACK, and ANAPATHS (Racca Scientific Consulting and JASCO Research Ltd., Victoria, British Columbia, Canada) were used to analyze the video records (Browman et al. 2003).

Escape response was tested in a 31 L tank (25 cm × 25 cm × 50 cm) (**Fig. 1**). To stimulate the escape response, a siphon (a 16-gauge, stainless steel, flat-tip hypodermic needle that acted as a mimic of a suction predator) was mounted in the center of the tank, 70 mm above the bottom (Fields et al. 2012). The flow rate into the siphon was maintained at 1 mL·s^{-1}. The velocity (V) of the water entrained by the siphon decreases exponentially with distance (r) from the siphon as: $V = Q(4\pi \times r^2)^{-1}$ where Q is the volume exiting the siphon (Kiørboe et al. 1999). At 5 cm from the siphon, the flow was calculated to be 30 µm·s^{-1}, which is below the threshold for the escape response of this species of copepod (Fields et al. 2012). As the water drained through the siphon, filtered seawater was re-introduced at the top of the tank to maintain a constant water level. This type of set-up has been used previously to initiate an escape response in other copepod species (Fields and Yen 1997). All trials (treatment and controls) were filmed for 60 min in a climate-controlled room at 13 °C (±0.5 °C). The tank contained approximately 200 copepods. Each animal was used only once.

The distance from the predator at which the copepod initiates their escape (threshold distance), and how fast and how far, are the decisive factors in the ability of a copepod to avoid predation (Fields et al. 2012). An escape response involves a single or a series of jumps during which the copepod draws its antennae in to the sides of its body followed by rapid motion of the swimming legs (Strickler 1975; Fields 2000). In case of multiple sequential escape jumps, only the first escape was used. The end of

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the escape response was defined as the moment when the antennae returned to their original position. Copepods that initiated an escape response but were captured by the siphon were designated as unsuccessful escapes and were not included in the behavioral analysis. The threshold and magnitude of the escape response was evaluated by measuring three parameters: immediate escape response (distance of the copepod from the siphon when the first escape response was initiated), the average speed of the escape and the total distance traveled during the escape. Exposed copepods that were completely paralyzed were not included in the assessment of escape performance or oxygen consumption rate.

Oxygen consumption rates (OCRs)

Only adult female copepods were used in this experiment. Three replicate measurements were made on the same day using three individuals from each concentration and the control. Test animals were held in experimental chambers (4.3 mL) filled with filtered seawater and sealed with a ground glass top that has a small access hole (0.4 mm) to accommodate the oxygen microelectrode. The experimental chambers were stirred (10 rpm) using a glass-encased magnetic stir bar (2 mm). Dissolved oxygen concentrations were measured using a Clark-type oxygen microelectrode (Unisense; Aarhus, Denmark). The linear response of each electrode was calibrated with 0.2 µm filtered seawater bubbled for a minimum of 1 h to set the 100% dissolved oxygen calibration point (Runge et al. 2016). For the anoxic calibration, seawater was placed into a silicone tube immersed in a solution of 0.1 mol·L^{-1} sodium ascorbate and 0.1 mol·L^{-1} sodium hydroxide overnight (for over 4 h). All oxygen measurements were made at 12 °C (±0.01 °C) in a ThermoScientific water bath (Model A10B with a thermostat SC100).

Oxygen concentration in the chambers was measured every 2 s for 1.5 h. The oxygen concentration never decreased below 20% saturation. Control chambers, without copepods, were measured to determine background levels of microbial and algal respiration. The oxygen consumption was computed as the difference between the beginning and end of the incubation and then corrected with the values obtained from the control chambers. Activity level of the copepods was assessed under a dissecting microscope before and after the OCR analysis to ensure that all of the copepods were alive.

Statistical analysis

Statistical analysis was conducted using the software R (R Studio, version 3.4.3). The concentration of H_2O_2 that caused 50% mortality (LC_{50}), and their 95% confidence intervals (CI), were calculated for each stage using a generalized linear model with binomial error structures and probit links according to Finney (1971). Pesticide concentrations were $\log_{10}$ transformed to linearize the data. At sublethal concentrations, changes in the escape performance variables and OCR as a function of H_2O_2 concentration were tested using a linear regression with a significance level (p) of 0.05 after significance between replicates within treatments was tested using ANOVA.

Results

Mortality

No mortality was recorded in any of the control groups. After 1-h exposure to 170 mg·L^{-1} H_2O_2, the acute mortality was 38.0% ± 0.08% for copepodite stage V and 97.0% ± 0.03% for adult *Calanus* spp. females (**Table 1**); all of the surviving animals in this treatment were immobilized. During the 24-h recovery period, the mortality increased to 92.0% ± 0.01% for copepodite stage V and 100% for adult females. At 85 mg·L^{-1}, total mortality was 34% ± 0.09% (copepodite stage V) and 89% ± 0.17% for adult females. At 17 mg·L^{-1} total mortality was 30% ± 0.12% and 14% ± 0.03% for stage V and adults, respectively. No delayed mortality or immobilization was observed when exposed to 8.5 mg·L^{-1} in

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Table 1. Acute (1-h exposure) and total mortality (1-h exposure + 24-h recovery) shown in percentages (%) with ±SD, of copepodite stage V ($N = 324$) and adult females ($N = 327$) of *Calanus* spp. exposed to different concentrations of H_2O_2.

	Acute mortality (%)		Total mortality (%)	
H_2O_2 (mg·L^{-1})	Copepodite V	Adult	Copepodite V	Adult
0	0	0	0	0
8.5	0	0	0	0
17	14 ± 0.03	14 ± 0.09	30 ± 0.12	14 ± 0.03
85	14 ± 0.12	74 ± 0.08	34 ± 0.09	89 ± 0.17
170	38 ± 0.08	97 ± 0.03	92 ± 0.01	100
340[a]	100	100	—	—
1700[a]	100	100	—	—

[a]From the preliminary study.

Table 2. LC_x with corresponding 95% confidence intervals for copepodite stage V and adult *Calanus* spp. following 1-h exposures to hydrogen peroxide (1h-LC_x) and following a 24-h recovery period (25h-LC_x).

Hydrogen peroxide (mg·L^{-1})				
Copepodite V	1h-LC_{10}	29.3 (18.8–39.7)	25h-LC_{10}	10.9 (6.5–15.3)
	1h-LC_{50}	214.1 (150.7–277.4)	25h-LC_{50}	77.1 (57.9–96.2)
	1h-LC_{90}	1566.1 (673.75–2458.6)	25h-LC_{90}	545.6 (284.8–806.5)
Adults	1h-LC_{10}	17.2 (14.6–19.8)	25h-LC_{10}	11.6 (9.1–14.2)
	1h-LC_{50}	48.3 (44.9–52.2)	25h-LC_{50}	30.6 (25.4–35.8)
	1h-LC_{90}	135.2 (121.5–148.9)	25h-LC_{90}	80.7 (60.6–100.8)

either stage V or adults. Based on the total mortality rates, the acute 1h-LC_{50} and total 25h-LC_{50} values with 95% CIs were calculated (Table 2).

Escape response

Biological replicates within each treatment were not significantly different (ANOVA $p < 0.5$) so they were pooled for further statistical analysis. Copepods from the control, 8.5 and 17.0 mg·L^{-1} H_2O_2 treatments successfully escaped the siphon 94% of the time. There was a significant difference in the escape performance between the control copepods and those exposed to 8.5 or 17.0 mg·L^{-1}. The escape threshold decreased significantly with increased concentration ($y = 8.24 - 0.14x$; $R^2 = 0.13$; $p = 0.015$) (Fig. 2A). Every 10 mg·L^{-1} increase in concentration caused a 1.4 mm decrease in distance from the siphon at which the copepod initiated the escape reaction. Similarly, once the copepod initiated the escape response the distance that *Calanus* spp. traveled decreased with increasing H_2O_2 ($y = 31.84 - 0.8x$; $R^2 = 0.103$; $p = 0.031$) (Fig. 2B). Every 10 mg·L^{-1} increase in concentration caused an 8-mm decrease in distance traveled away from the siphon. However, there was no significant difference in the escape speed between the control and treatment levels up to 17 mg·L^{-1} ($p = 0.32$) (Fig. 2C). At higher concentrations (85 mg·L^{-1}), none of the copepods made a successful escape reaction from the siphon or even initiated an escape response. Although alive, the copepods exposed to

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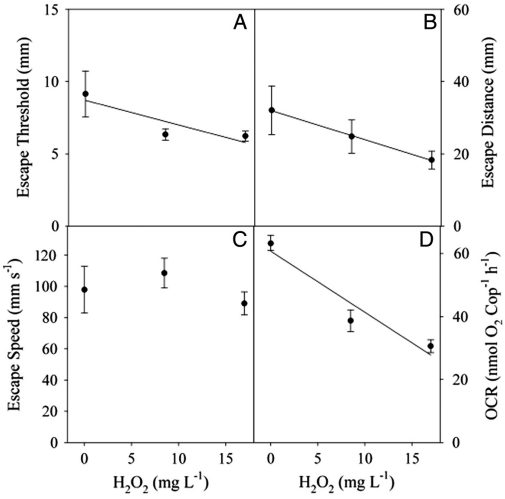

Fig. 2. Changes in the escape performance of *Calanus* spp. at sublethal concentrations of H_2O_2. (A) Escape threshold distance from the siphon at which the *Calanus* spp. initiates the escape reaction ($p = 0.015$), (B) the distance traveled during the escape response ($p = 0.031$), (C) the escape speed, and (D) oxygen consumed ($p = 0.005$). Values are means (±SE). Lines show significant linear regressions of the data. OCR, oxygen consumption rate.

concentrations of 85 mg·L^{-1} showed limited swimming ability and were most often lying on the bottom of the tank.

Oxygen consumption rate (OCR)

OCR was measured at H_2O_2 concentrations at which copepods showed a behavioral response to the siphon (0, 8.5, and 17 mg·L^{-1} H_2O_2 treatments). OCR decreased significantly in the H_2O_2 treatments relative to controls ($y = 60.75 - 1.93x$; $R^2 = 0.886$; $p = 0.005$). At 12 °C, OCR for *Calanus* spp. decreased from 64 nmol O_2 ind^{-1}·h^{-1} in the control group to 24 nmol O_2 ind^{-1}·h^{-1}and 39 nmol O_2 ind^{-1}·h^{-1} in 8.5 and 17.0 mg·L^{-1} H_2O_2 respectively (Fig. 2D). At 85 mg·L^{-1} H_2O_2, the copepods were still alive but were unresponsive; their OCR was not significantly different from that of controls (ANOVA, $p > 0.5$).

Discussion

The recommended concentration of H_2O_2 used to treat salmon lice (1700 mg·L^{-1}) causes acute mortality in wild-captured *Calanus* spp. A 1-h exposure to the recommended treatment concentration, and to 20% of the recommended concentration (340 mg·L^{-1}), caused 100% mortality in both copepodite stage V and adult females. As the concentration of H_2O_2 is decreased, the mortality in *Calanus* spp. also decreased. At the lowest concentration tested (8.5 mg·L^{-1}) no acute or delayed mortality was observed. The acute mortality (recorded immediately after exposure), was lower than the total mortality recorded after a 24-h recovery period. These results suggest that it is important to include a 24-h recovery period in these types of experiments to obtain an accurate estimate of mortality.

Adult *Calanus* spp. showed higher mortality to H_2O_2 exposure than copepodite stage V. Similar stage-specific differences in sensitivity to H_2O_2 exposure have been reported for other copepod species

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(*Acartia* sp.; Van Geest et al. 2014), including salmon lice (*L. salmonis*; Mitchell and Collins 1997) and their eggs (Aaen et al. 2014; Bravo et al. 2015). We found no available data on the sensitivity of *Calanus* spp. eggs or nauplii to H_2O_2. However, given the abundance of ovigerous copepod females present during the spring period when H_2O_2 application is the highest (Grefsrud et al. 2018), this should be tested in future.

The immobilization of the copepods observed in this study was expected as it is one of the effects of H_2O_2 on salmon lice. Bubbles of O_2 gas in the haemolymph are the primary cause of sea lice detachment from the host following treatment with H_2O_2 (Cotran et al. 1989; Treasurer et al. 2000; Aaen et al. 2014). However, the formation of gas bubbles observed in salmon lice (Bruno and Raynard 1994) was not reported in *Acartia hudsonica*, *Metacarcinus edwardsii*, or in *Calanus* spp. (Van Geest et al. 2014; Gebauer et al. 2017; Hansen et al. 2017), so the mechanism of immobilization observed in *Calanus* spp. is unclear.

The distance from the predator at which a copepod initiates an escape reaction, and the strength of the escape reaction, are decisive factors in the copepod's ability to avoid predation (Fields and Yen 1997). *Calanus* spp. that were not exposed to H_2O_2 (control) showed escape performance metrics consistent with earlier studies on copepods (Fields et al. 2012). At sublethal levels, exposure to H_2O_2 had measurable effects on the escape reaction of *Calanus* spp. At a concentration of 85 $mg{\cdot}L^{-1}$, approximately 25% of the exposed adult copepods survived a 1-h exposure, yet none of these survivors made a successful escape from the siphon. Many of the copepods exposed to 17 $mg{\cdot}L^{-1}$ were partially immobilized and sank to the bottom of the aquarium during exposure, unlike copepods exposed at 8.5 $mg{\cdot}L^{-1}$ at which no immobilization was observed. *Calanus* spp. showed a decrease in the threshold distance at which they initiated their escape reaction with increased H_2O_2 concentration and after the copepods initiated the escape response, they traveled a significantly shorter distance from the siphon. These behavioral results suggest that *Calanus* spp. exposed to sublethal concentrations of H_2O_2 (at 1% of the recommended treatment levels) will be more susceptible to predation because of an impaired escape response. These results are consistent with the findings of Van Geest et al. (2014) who observed immobilization of *A. hudsonica* after 15 min of exposure to concentrations of $\geq$10 $mg{\cdot}L^{-1}$.

Exposed copepods also experienced reduced OCR in response to increased concentrations of H_2O_2. The lower metabolic activity is a likely cause of the decreased distance traveled during the escape. In addition, the lower metabolic activity may impact the repetitive escape reaction of copepods. The escape reaction of copepods is energetically costly (Strickler 1975) and the strength of the response decreases with increased escape frequency (Fields 2000). A lower OCR will decrease the ability of the copepod to perform multiple escape reactions and thereby further increase their predation risk (Fields 2000).

At the highest sublethal concentrations tested (85 $mg{\cdot}L^{-1}$; 5% of treatment levels), *Calanus* spp. were unable to swim and sank to the bottom of the tank. These individuals exhibited no response to the predator mimic. Paradoxically, the respiration rates of the copepod at these higher levels of exposure were similar to levels measured in the controls. These results suggest a change in the mode of action of the H_2O_2 (Rand 1995). At higher concentration (above 17 $mg{\cdot}L^{-1}$), H_2O_2 may produce narcosis, causing partial paralysis. The risk of predation for these copepods with no escape reactions is extremely high. The data show that some of these animals may recover normal escape behavior; however, since predation can have a significant effect on the population dynamics of invertebrates (Pangle et al. 2007), sublethal effects on escape behavior potentially have important ecological implications for the affected population. The results of this study provide supporting evidence that theoretical "safe" values, traditionally used for predicting toxicity thresholds, underestimate the impact of H_2O_2 on the physiological condition of nontarget crustaceans. This warrants additional research.

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Our results indicate that the No Observable Effect Concentration for *Calanus* spp. is between 8.5 and 17 $mg{\cdot}L^{-1}$. This is considerably higher than the concentration reported for another calanoid copepod species, *A. hudsonica*, for which the sublethal concentration level of 2.6–10.0 $mg{\cdot}L^{-1}$ (EC_{50}) was determined based on feeding rate measurements (Van Geest et al. 2014). This suggests that the impact of H_2O_2 on copepods is species specific. Copepods as a group may be more sensitive to H_2O_2 than other planktonic crustaceans. For example, Gebauer et al. (2017) reported a LC_{50} for the mola rock crab larvae (*M. edwardsii*) of 1642 $mg{\cdot}L^{-1}$, two orders of magnitude higher than thresholds for *Calanus* spp.

While it is clear that even 0.5% of the standard treatment concentration of H_2O_2 has a detrimental effect on *Calanus* spp., the dispersal and dilution processes that affect the effluent plumes after treatments at aquaculture sites are still unclear (Ernst et al. 2001). Development and testing of dispersion models, including field studies to verify the models, will be important to evaluate the broader impact of H_2O_2 on the organisms living around salmon farms.

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Author contributions

RHE-L, DMF, HIB, RMB, ABS, and CMFD conceived and designed the study. RHE-L, DMF, SDS, and RMB performed the experiments/collected the data. RHE-L, DMF, and CMFD analyzed and interpreted the data. HIB, A-LA, ABS, and OBS contributed resources. RHE-L, DMF, HIB, SDS, RMB, A-LA, ABS, OBS, and CMFD drafted or revised the manuscript.

Competing interests

The authors have declared that no competing interests exist.

Data availability statement

All relevant data are within the paper.

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Paper II

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Short-term exposure to hydrogen peroxide induces mortality and alters exploratory behaviour of European lobster (*Homarus gammarus*)

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ABSTRACT

Bath treatment chemotherapeutants, used to control sea lice infestations in the salmonid aquaculture industry, are released directly into the marine environment around fish farms and pose a serious risk to non-target species, particularly crustaceans. Hydrogen peroxide (H_2O_2) is the most frequently used bath treatment chemotherapeutant on Norwegian fish farms, however, limited information is available on its toxicity to European lobsters (*Homarus gammarus*), a commercially important species at risk of exposure due to its distribution overlapping with salmon farm locations. The aim of this study was to investigate the lethal effects of H_2O_2 on pelagic (stage I-IV) larvae/post-larvae and its sub-lethal effects on the benthic stage V *H. gammarus*. To assess the lethal effects of H_2O_2, we carried out a series of 1 h toxicity tests and assessed mortality after a 24 h post-exposure period. Exposure to H_2O_2 was toxic to all pelagic larval stages tested, with estimated median lethal concentrations (LC_{50}) of 177, 404, 665 and 737 mg/L for stage I, II, III and IV, respectively. These concentrations represent approximately 10, 23, 40 and 43%, of the recommended H_2O_2 concentrations used for delousing salmon on Norwegian fish farms, respectively. To assess the sub-lethal effects of H_2O_2 on *H. gammarus*, stage V juveniles were exposed to H_2O_2 at concentrations of 85, 170 and 510 mg/L for 1 h and shelter-seeking behaviour and mobility endpoints were assessed. Numerous behavioural parameters including distance travelled to shelter, time to locate shelter and the number of shelter inspections, were negatively affected in lobsters exposed to H_2O_2 when assessed immediately after the exposure period. However, no differences between control and exposed lobsters were detected after a 24 h post-exposure period. Our results demonstrate that short term exposures to H_2O_2 are lethal to pelagic *H. gammarus* life stages and can negatively affect the shelter seeking behaviour of benthic life stages, though these behavioural changes may be short-lived.

1. Introduction

Sea lice (*Lepeophtheirus salmonis*) infestations are a major challenge to the salmonid farming industry around the world (Costello, 2006; Torrisen et al., 2013; Vollset et al., 2016). The lice are naturally occurring parasitic copepods that affect both farmed and wild salmonid populations, causing skin damage and sub-epidermal hemorrhages that can lead to osmotic stress and secondary infections (Johnson et al., 2004; González et al., 2015). The high density of sea lice in the surrounding water of the salmon farms may lead to high mortality of the migrating post smolts of wild Atlantic salmon (*Salmo salar*) and the sea trout (*Salmo trutta*) (Costello, 2009; Vollset et al., 2016). In order to manage sea lice infestations on Norwegian fish farms, the Norwegian Salmon Lice Directive has limited the number of adult female lice per fish to 0.2 in spring and 0.5 for the rest of the year (FOR-2012-12-05-1140, 2012). To comply with these regulations, the industry relies on the use of chemotherapeutants, either dissolved in the water and applied as a bath treatment (hydrogen peroxide [H_2O_2], deltamethrin, azamethiphos) or applied as an in-feed drug (emamectin-benzoate, diflubenzuron, teflubenzuron) or on other non-medicated treatments e.g. mechanical removal or the use of warm or fresh water (Grefsrud et al., 2019).

Recently, Norway has seen a major decrease in the consumption of all chemotherapeutants (Folkehelseinstituttet, 2019), as a consequence of the development of resistance amongst the sea lice and the introduction of new delousing methods. Hydrogen peroxide is still, however, the predominate chemotherapeutant used in Norway, with 4523 tons used in 2019 (Folkehelseinstituttet, 2019). It acts on sea lice by inducing

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mechanical paralysis, inactivation of enzymes and DNA replication, and peroxidation of lipid and cellular organelle membranes by hydroxyl radicals (Cotran et al., 1989; Valenzuela-Muñoz et al., 2020). Studies have shown that the mechanical paralysis is caused by decomposition of H_2O_2 to water and O_2 gas/bubbles in the gut and hemolymph, resulting in the release of the pre-adult and adult lice from the fish which subsequently float to the surface (Thomassen, 1993; Bruno and Raynard, 1994; Aaen et al., 2014). On salmon farms, the target concentration of H_2O_2 for bath treatments is between 1500 and 2100 mg/L and treatment period can last for 20–40 min, depending on temperature (Treasure et al., 2000). Once the treatment is complete, the waste treatment water is released into the surrounding environment as the tarpaulin enclosing the net pens is removed or from wells boat release while in transport. As the plume of H_2O_2 disperses into the marine environment, pelagic non-target organisms may be exposed to the effluent (Burridge et al., 2014).

Several studies have found lethal effects of exposure to H_2O_2 on different marine crustacean species, including American lobster (*Homarus americanus*) larvae and adults, sand shrimp (*Crangon septemspinosa*), *Mysid* sp., amphipods (*Corophium volutator*), *Metacarcinus edwardsii*, brine shrimp (*Artenia salina*), northern shrimp (*Pandalus borealis*), and the copepods *Acartia hudsonica* and *Calanus* spp. (Smit et al., 2008; Burridge et al., 2014; Van Geest et al., 2014; Gebauer et al., 2017; Hansen et al., 2017; Bechmann et al., 2019; Escobar-Lux et al., 2019). A limited number of studies have also shown that exposure to H_2O_2 can have sub-lethal effects on crustaceans. For example, exposures to relatively low concentrations of H_2O_2 for short periods of time caused mechanical paralysis in copepod *Acartia hudsonica* ($\geq$10 mg/L), *Calanus* spp. ($\geq$17 mg/L) and *Pandalus borealis* (15 mg/L) (Van Geest et al., 2014; Bechmann et al., 2019; Escobar-Lux et al., 2019).

Acute toxicity tests often involve 24, 48 and 96 h exposure periods, which do not necessarily reflect acute exposures expected to occur in the marine environment (Ernst et al., 2001; Urbina et al., 2019). In recent years, there has been an increasing demand for toxicity tests to be performed under more environmentally relevant exposure conditions (Urbina et al., 2019). Shorter exposure times i.e. 1 h followed by a 24 h r post-exposure time (to assess delayed effects), would therefore provide a more accurate assessment of the impacts of bath treatment plumes on non-target species (Medina et al., 2004; Van Geest et al., 2014; Escobar-Lux et al., 2019).

The aim of this study was to investigate the toxicity of H_2O_2 to European lobster (*Homarus gammarus*), a non-target crustacean species native to the Norwegian marine environment. *H. gammarus* is an important commercial species and is at risk of exposure to bath treatment chemotherapeutants as its distribution overlaps with the location of salmon farms along the coast of Norway (Agnalt, 2008). The life history of *H. gammarus* includes a number of distinct developmental stages including a planktonic larval phase (stages I-III), a post-larva phase (stage IV) which marks the transition from planktonic to benthic living, followed by a fully benthic phase from stage V and onwards (Sars, 1874; Lawton and Lavalli, 1995). During the pelagic life stages, lobsters are most at risk of exposure to H_2O_2 when the pesticide disperses from the salmon cages into the surrounding marine environment following the operational release of bath treatment effluents. Our first objective, therefore, was to perform a series of 1 h toxicity tests to environmentally relevant concentrations of H_2O_2 with each of the *H. gammarus* pelagic larval stages (I-IV) in order to establish lethal concentrations. The benthic lobster life stages are also at risk of exposure to H_2O_2 under certain environmental concentrations. For example, when the water column is well mixed, H_2O_2 can potentially sink under salmon cages and undergo horizontal dispersion along the seafloor instead of in the surface layers (Refseth et al., 2017). Stage V lobsters naturally exhibit an exploratory and shelter-seeking behaviours when placed in new environments (Agnalt et al., 2017; van der Meeren, 2001) which can potentially be negatively affected by exposure to chemical pollutants. Therefore, our second objective was to examine the sub-lethal effects of H_2O_2 on *H. gammarus* stage V post-larvae following short (1 h) exposures, and specifically assess changes in their shelter seeking behaviour.

2. Material and methods

2.1. Chemicals

Commercial H_2O_2 (Nemona, 49,50% H_2O_2 or 600 g L^{-1}) was purchased from Akzo Nobel, Pulp and Performance Chemicals, AB Sweden.

2.2. Animal collection and handling

This experiment was approved by the Norwegian Food Safety Authority (ID 15510) and was carried out according to The Code of Ethics of the World Medical Association for animal experiments (The Norwegian Ministry of Agriculture and Food, 2010, 2015). Six ovigerous *H. gammarus* females were purchased from a lobster dealer on May 22, 2018 and transferred to Austevoll Research Station, Institute of Marine Research (HI) (N60°05′15.36″, E5°15′54″).

The lobsters were subsequently kept in holding tanks (1.5 m × 1.5 m) containing sand filtrated seawater from 160 m depth (salinity of 34.7 ppt), with a flow rate of 30 L min^{-1} and a photoperiod of 16-h/8-h day/night. The water temperature was maintained at 8 °C to control hatching. In August 2018, the seawater temperature was gradually increased to 16 °C to stimulate hatching. Newly hatched larvae, staged according to Sars (1874), were collected and transferred to aerated 40 L incubators (Hughes et al., 1974) supplied with running seawater at 14 °C. In order to limit cannibalism, all larvae in an incubator had an age difference no greater than three days. Correspondingly, each incubator was stocked with a maximum of 1500–2000 larvae. The larvae were fed daily with frozen artemia and Otohime C2 (Marubeni Nisshin Feed Company, Japan). When the larvae reached stage IV, they were transferred to separate 170 ml^3 (7.0 cm × 3.5 cm x 7.0 cm) housing compartments made of white plastic PVC with 2.5 mm diameter holes in the bottom to allow water exchange. Coarse-grained sand was added to each compartment to induce normal claw development (Govind and Pearce, 1989; Agnalt et al., 2017). The compartments were held in holding tanks at 14 °C and the lobster juveniles were fed frozen shrimp once a day. The incubators were treated twice a week with chloramine T (0.02 g L^{-1}) for 1 h to control Leucatrix minor infections in the larvae (Dr. D. Boothroyd, pers. comm.).

2.3. Toxicity studies

Lethality studies were performed with the pelagic larvae (stages I-IV). Exposures were conducted for 1 h and were followed by a 24 h post-exposure period. The temperature in the water-system was set to 14 °C, and in order to keep the temperature in the exposure units and in the post-exposure period, the room temperature was regulated to keep the temperature accordingly. The water temperature ranged between 13 and 14 °C. As no previous studies have assessed the toxicity of H_2O_2 on *H. gammarus* larvae, the chosen concentrations were based on the recommended dose for treating salmon (1700 mg/L). All four larval stages were exposed to H_2O_2 at concentrations of 170, 510, 850, 1190, 1530 mg/L corresponding to 10%, 30%, 50%, 70% and 90% of the recommended treatment dose. The mean carapace length for stage I, II, III and IV was 2.3 mm ± 0.1, 3.3 mm ± 0.1, 3.8 mm ± 0.2 and 5.0 mm ± 0.5, respectively.

For larval stages I & II, exposures were carried out in glass tank containing five larvae with five replicates per concentration; for stages III & IV (due to increased cannibalism and the number of available animals) each tank had approximately four larvae with four replicates per concentration (Burridge et al., 2014; Parsons et al., 2020). The glass tanks used for exposure had a volume of 700 ml. Prior to the start of exposure (within 5 min), the tanks were filled with fresh sand filtered

seawater at 14 °C and mixed with the appropriate H_2O_2 volumes. Following the exposures, larvae were transferred to 1 L individual post-exposure tanks supplied with continuously aerated seawater at 14 °C. Mortality and general condition of the larvae were assessed at 0, 6 and 24 h (for larval stages I & II) post exposure. The lobsters were considered to be immobilised when normal swimming was absent, but there was movement of the pleopods and mouth parts after gentle prodding. Larvae were considered dead if they were discoloured, deformed (detached carapace), or if there was no movement of the pleopods after gentle stimuli. Mortality that occurred during the 1 h exposure was defined as acute mortality whereas total mortality was defined as the combined mortality of the 1 h exposure and the 24 h post-exposure period.

2.4. Behavioural studies

Sixty-four stage V lobsters were randomly divided into four groups, control and three exposure groups, which were exposed for 1 h to sub-lethal concentrations of H_2O_2 (85, 170 and 510 mg/L) in individual containers. The selected concentrations were based on the estimated LC_{50} values established for stage IV and represented 0, 5, 10 and 30% of the recommended treatment dose, respectively. Exposures were carried out in glass tanks containing 500 ml of the appropriate test solution at 13.5–14.0 °C, where solutions were made as described above for the toxicity tests. Thirty-two lobsters were randomly selected and photographed for length measurements. Carapace length (CL) was recorded as the distance from the posterior rim of the eye socket to the posterior edge of the carapace, using the open source software ImageJ (Image Processing and Analysis in Java, mean CL = 6.04 ± 0.06 mm).

Immediately after exposure, the lobsters were transferred to individual containers filled with fresh seawater and aeration, until the commencement of the behavioural studies (within approximately 2 min). To ensure that the lobsters had enough space to walk freely and explore the environment, four wide light acrylic diffusers (65 cm × 12 cm x 6 cm) were used as lanes for the behavioural studies (Fig. S1). The lanes were filled with 3.12 L of seawater and maintained at 13.5–14.0 °C. To observe and record the behaviour of the lobsters, two GoPro Hero5® cameras where position at a height of 53.5 cm above the lanes. White sand was used as a substrate to ensure a better contrast between the lobster and the bottom of the tank. Shelters (5.5 cm × 2 cm), made from white PVC pipes cut in half, were placed at one end of each lane. The four lanes were simultaneously recorded, with one lobster from each exposure group placed in each lane at the opposite end of the shelter. This set-up has been used previously to study the shelter-seeking behaviour and activity levels of *H. gammarus* juveniles (Taormina et al., 2020). The lobsters were recorded for 30 min, after which the following parameters were recorded: 1) total distance travelled (cm); 2) time to locate shelter (s); 3) total number of inspections of the shelter; 4) time to accept shelter (s)-defined as time of entering and remaining inside the shelter for the rest of the observation; 5) proportion of lobsters that accepted shelter by the end of the observation.

Once the recording period was over, the lobsters were returned to their individual holding tanks. This marked the beginning of the 24 h post-exposure period. During this period the lobsters were fed frozen deep-water shrimp (*Pandalus borealis*). After the 24 h post-exposure period, the behavioural assays were repeated, in order to assess if there was any improvement in their behaviour. Between each trial, the lanes were cleaned, and the water was changed.

2.5. Statistics

All statistical analyses were conducted in R (Version 3.4.3 (2018-07-02) Copyright © 2018 The R Foundation for Statistical Computing).

2.5.1. Toxicity studies

Median lethal concentrations (LC_{50} values), and their 95% confidence intervals (CI), were calculated for each stage using generalised linear models (GLM) with binomial error structures and probit links, according to Finney (1971). Concentrations were log10 transformed to linearize the data. The dose-response curves were plotted using the ggplot2 R package.

2.5.2. Behavioural studies

Behavioural data were firstly tested for normality using the Shapiro-Wilk Test. If the data met the requirement for normality, an unpaired two-sample *t*-test was performed to compare the measured endpoint between treatment groups. If the data did not meet the requirement for normality, a non-parametric Mann-Whitney *U* Test was performed. Multivariate repeated measures ANOVA was carried out to test if there was any difference between the data acquired after 1 h exposure and the data acquired after a 1 h plus a 24 h post-exposure period.

3. Results

3.1. Toxicity studies

Acute mortality was low for all the treatment groups (Table 1), and the highest mortality of 15.4 ± 0.1% was obtained for stage I larvae exposed to the highest concentration of 1530 mg/L. No acute mortality was recorded for stage IV larvae, in any of the treatment groups. Immobilization and bubble formation on the inside of the carapace occurred in all larval stages but time-to-event was only recorded for stages I & II. In all of the H_2O_2 treatment groups, all stage I & II larvae developed air bubbles inside the carapace (Fig. 1), floated to the surface and subsequently became immobilised. This occurred within the first 5 min of the exposure period. Since many of the immobile and floating larvae did not recover, mortality increased during the 24 h post-exposure period. No acute mortality was recorded for any of the control groups immediately after the 1 h exposure.

Total mortality reached 100% for stage I larvae exposed to 1530 mg/L H_2O_2, and correspondingly, 92 ± 0.1% for stage II, 81 ± 0.2% for stage III and 75 ± 0.2% for stage IV (Table 1). In the groups exposed to 170 mg/L the total mortality observed after the 24 h post-exposure period was 44 ± 0.3%, 24 ± 0.1%, 25 ± 0.3% and 6.3 ± 0.1% for stages I, II, III and IV, respectively. Mortality was also observed in the control group for stage IV after the 24 h post-exposure period (12.5 ± 0.1). Estimated LC_{50} values and their CI for stage I, II, III and IV were 177 mg/L (142–212 mg/L), 404 mg/L (289–519 mg/L), 665 mg/L (423–906 mg/L) and 737 mg/L (507–967 mg/L), respectively (Fig. 2).

3.2. Behavioural studies

Independent of their treatment groups, the naïve stage V lobsters i.e. no previous encounter with shelter, started exploring their new environment as soon as they were released. The exploratory behaviour principally consisted of the lobsters freezing just as they were released in the lane, and then moving towards one of the lane borders. With the use of their antennae and claws, the lobsters maintained physical contact with the border, and then explored the long side of the lane in either direction. Once the lobsters made physical contact with the shelter, it was inspected multiple times occasionally followed by a second exploration of the lane before entering and accepting the shelter.

When examined immediately after the 1 h exposure period, the exposed lobsters (85, 170 and 510 mg/L H_2O_2) travelled significantly shorter distances compared to control (Mann-Whitney *U* Test, $p < 0.01$) (Fig. 3). The mean distances travelled were 569 ± 119 cm, 179 ± 40 cm, 242 ± 120 cm, and 130 ± 34 cm for lobsters in control, 85, 170 and 510 mg/L treatment groups, respectively.

Furthermore, the time spent by the lobsters to locate shelter for the first time was also greatly influenced by H_2O_2 exposure (Fig. 4). In particular, lobsters exposed to the two highest doses of H_2O_2 spent significantly longer times to locate the shelter compared to lobsters in

Table 1
Summary of the mean (±SD) acute mortality (1 h exposure) and total mortality (1 h exposure + 24 h post-exposure) of *H. gammarus* stage I (n = 150), stage II (n = 150), stage III (n = 109), and stage IV (n = 96) larvae after exposures to a range of H_2O_2 concentrations.

	Acute Mortality (%)				Total Mortality (%)			
	1-h exposure				1-h exposure + 24-h post-exposure			
H_2O_2 (mg/L)	Stage I	Stage II	Stage III	Stage IV	Stage I	Stage II	Stage III	Stage IV
1530	15.4 ± 0.1	0	4.8 ± 0.1	0	100	92 ± 0.1	81 ± 0.2	75 ± 0.2
1190	8.3 ± 0.1	4 ± 0.1	0	0	100	88 ± 0.1	45 ± 0.1	68.8 ± 0.3
850	8 ± 0.1	4 ± 0.1	0	0	100	80 ± 0.2	56.3 ± 0.2	62.5 ± 0.3
510	4 ± 0.1	0	0	0	100	44 ± 0.3	16.7 ± 0.1	37.5 ± 0.3
170	0	0	0	0	44 ± 0.3	24 ± 0.1	25 ± 0.3	6.3 ± 0.1
Control	0	0	0	0	0	5 ± 0.1	0	12.5 ± 0.1

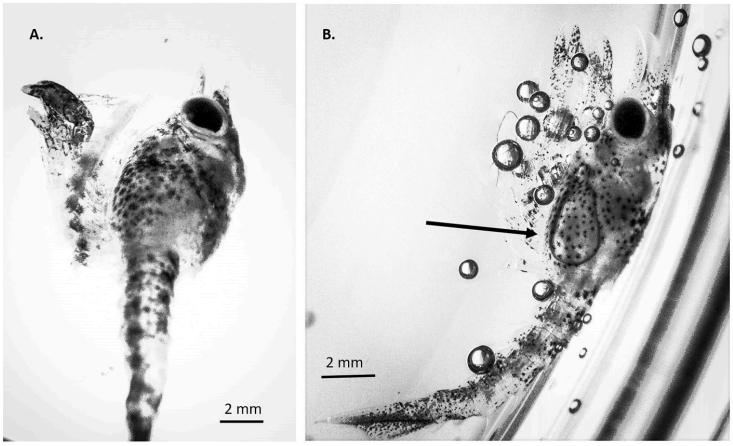

Fig. 1. Representative images of *H. gammarus* stage I larvae in the (A) control and (B) 850 mg/L H_2O_2 group. The black arrow indicates the presence of an air bubble inside the carapace.

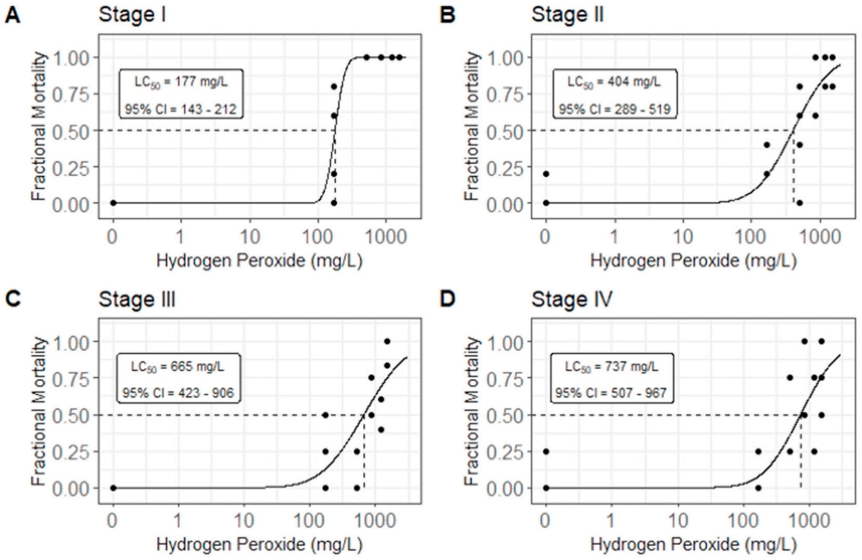

Fig. 2. The toxicity of H_2O_2 to *H. gammarus* larvae following a 1 h exposure and 24 h post-exposure period. Dose-response curves show mortality amongst pelagic *H. gammarus* (A) stage I, (B) stage II, (C) stage III and (D) stage IV larvae.

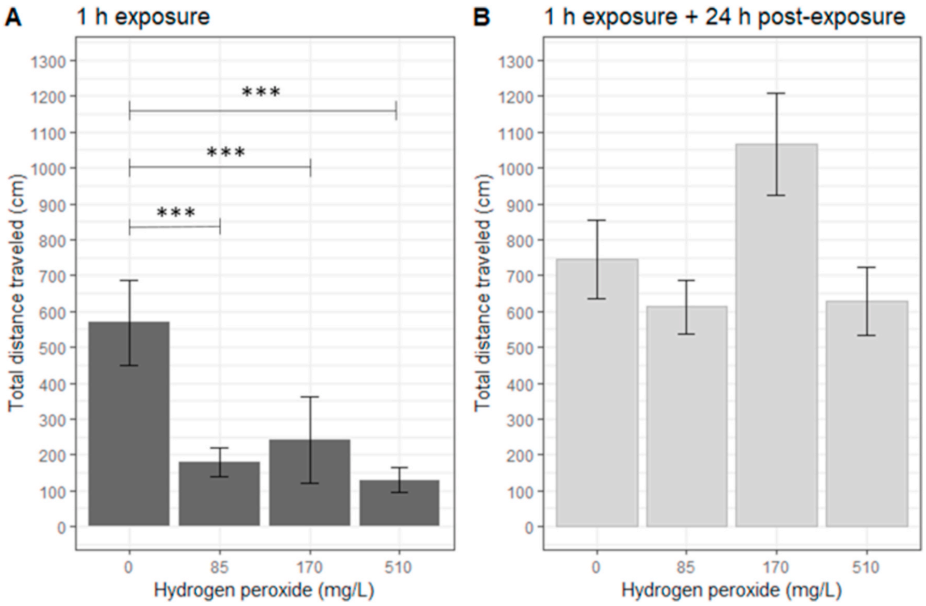

Fig. 3. The total distance travelled (cm) by *H. gammarus* stage V post-larvae in the 30 min behavioural assays performed following 1 h exposures to sub-lethal concentrations of H_2O_2. ***$p < 0.001$ treatment vs. control. Data is presented for behavioural assays performed A) immediately after the exposure period and B) after a 24 h post-exposure period. n = 16 per concentration.

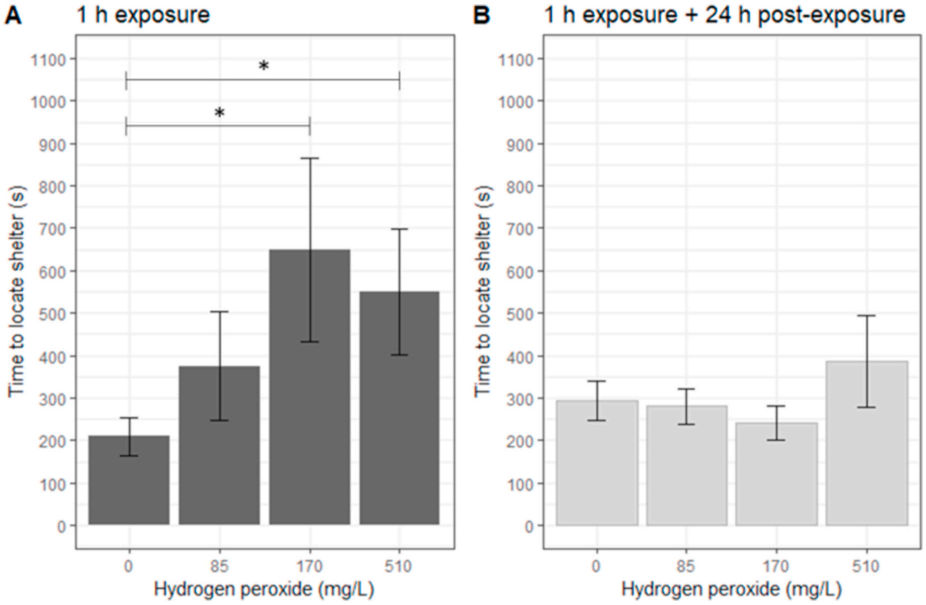

Fig. 4. Time (seconds) taken by *H. gammarus* stage V post-larvae to find and inspect the provided shelter for the first time during 30 min behavioural assays performed following 1 h exposures to sub-lethal concentrations of H_2O_2. *$p < 0.05$ treatment vs. control. Data is presented for behavioural assays performed A) immediately after the exposure period and B) after a 24 h post-exposure period. n = 16 per concentration.

the control group (Mann-Whitney *U* Test, $p < 0.05$). The mean time taken to locate the shelter for the first time were 210 ± 45 s, 375 ± 129 s, 649 ± 215 s, and 551 ± 148 s for lobsters in control, 85, 170 and 510 mg/L treatment groups, respectively.

Similarly, the total number of shelter inspections were affected by H_2O_2 exposure, where individuals in the control group inspected the shelter at a significant higher rate than the lobsters in all the treatment doses lesser (Mann-Whitney *U* Test, $p < 0.01$) (Fig. 5). There was, however, no significant effect of H_2O_2 exposure on the time (s) taken by the lobsters to accept the shelter (data not shown), though based on a limited data set since only one lobster in the 170 mg/L treatment group accepted the shelter. The proportion of lobsters that had accepted their shelters at the end of the experimental period (30 min) were 44, 12, 6 and 12% in the control, 85, 170 and 510 mg/L treatment groups, respectively, showing a significant influence by H_2O_2 exposure (Mann-Whitney *U* Test,$p < 0.01$) (Fig. 6).

Twenty-four hours after the exposure, there were no significant differences between control and H_2O_2-exposed larvae for any of the behavioural parameters assessed (Figs. 3–5) (Mann-Witney *U* Test, $p > 0.05$).

4. Discussion

4.1. Mortality

Exposure to H_2O_2 was lethal to *H. gammarus* larval stages (I-IV). In this study, we have shown that a 1 h exposure to H_2O_2, at environmentally relevant concentrations, was lethal to each of the pelagic *H. gammarus* larval stages (I-IV). The stage I larvae were the most sensitive life stage tested with an LC_{50} value for H_2O_2 of 177 mg/L, followed by stage II (LC_{50} = 404 mg/L), stage III (LC_{50} = 676 mg/L) and stage IV (LC_{50} = 738 mg/L). Consistent with our results, stage-specific differences in sensitivity to H_2O_2 were also observed in toxicity studies with sea lice (*L. salmonis*), *Calanus* spp. and *Acartia* sp. (Aaen et al., 2014; Van Geest et al., 2014; Bravo et al., 2015; Escobar-Lux et al., 2019).

In line with our findings, previous studies have also reported that short term (1 h) exposures to H_2O_2 were toxic to non-target marine crustaceans, and where species-specific differences in sensitivity are apparent. For example, while Burridge et al. (2014) observed that a short-term exposure (1 h + 96 h post-exposure period) to H_2O_2 was lethal to *Mysid* spp., C. septemspinosa and *H. americanus* larvae, the estimated LC_{50} values (973, 3182 and 1637 mg/L, respectively) were much higher than those reported here, especially when compared to *H. gammarus* stage I. Furthermore, a number of other studies have reported that H_2O_2 was not acutely toxic to crustacean species like *P. flexuosus*, *P. elegans* and adult *H. americanus* (Brokke, 2015; Burridge et al., 2014) following a 1 h exposure.

In comparison, a recently published paper reported that 1 h exposures (followed by a 24 h post-exposure period) to H_2O_2 were acutely toxic to copepodite V and adult *Calanus* spp., and both life stages were more sensitive than *H. gammarus* larvae (as examined here), with LC_{50} values of 77.1 mg/L and 30.6 mg/L calculated, respectively (Escobar Lux et al., 2019). Taken together these studies demonstrate that there are species- and life-stage specific differences in sensitivity to H_2O_2 exposure amongst crustaceans, and especially *H. gammarus* stage I larvae appears to be one of the most sensitive species tested to date.

While H_2O_2 exposures were lethal to all of the *H. gammarus* larval stages tested, the deleterious effect of H_2O_2 appeared to be delayed, with larval mortalities mostly occurring during the 24 h post-exposure period. For example, the acute mortality amongst stage I larvae ranged between 0 and 15%, but the total mortality reached 44–100% at 24 h post-exposure. Delayed effects following a post-exposure period was also observed in H_2O_2 toxicity studies with *P. borealis* (Bechmann et al., 2019), *Calanus* spp. (Escobar-Lux et al., 2019) and zoea *M. edwarsii* (Gebauer et al., 2017). These studies combined demonstrates the importance of including a post-exposure period in the experimental design to prevent an underestimation of the toxic effects of H_2O_2 on non-target crustaceans.

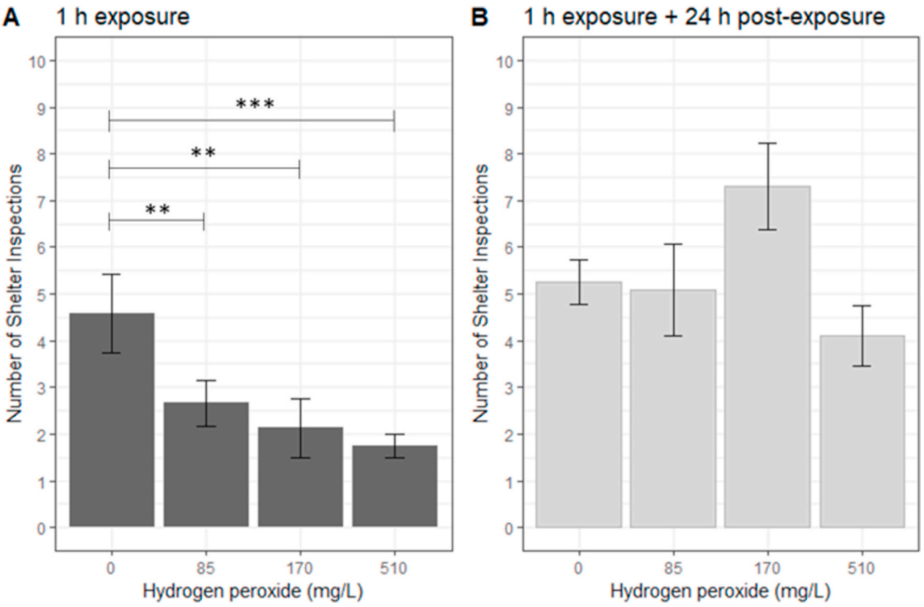

Fig. 5. Total number of shelter inspections by *H. gammarus* stage V post-larvae during 30 min behavioural assays performed following 1 h exposures to sub-lethal concentrations of H_2O_2. **$p < 0.01$; ***$p < 0.001$ treatment vs. control. Data is presented for behavioural assays performed A) immediately after the exposure period and B) after a 24 h post-exposure period. n = 16 per concentration.

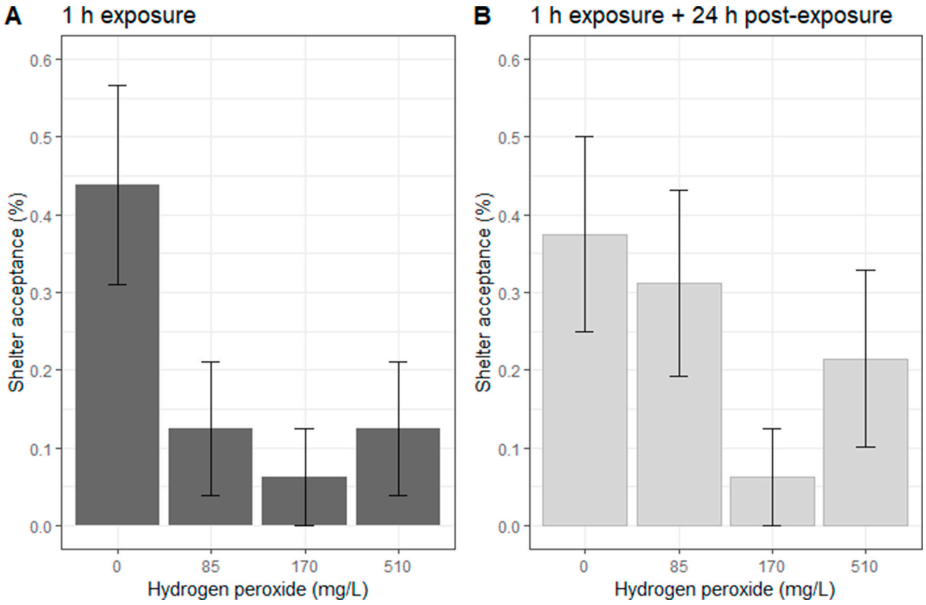

Fig. 6. Proportion (%) (±SD) of *H. gammarus* stage V post-larvae that accepted the shelter during 30 min behavioural assays performed following 1 h exposures to sub-lethal concentrations of H_2O_2. Data is presented for behavioural assays performed A) immediately after the exposure period and B) after a 24 h post-exposure period. n = 16 per concentration.

4.2. Immobilization and bubble formation

We observed that exposure to H_2O_2, even at the lowest dose tested, resulted in the formation of bubbles almost immediately (within 5 min of the exposure commencing) inside the carapace of all *H. gammarus* stage I and II larvae. Bubbles were also observed inside the carapace of stages III and IV though time to event was not monitored. As these larvae were subsequently paralysed at the surface of the water and only a limited number of individuals recovered after 24 h post-exposure period, our results suggest that the negative effects of H_2O_2 exposure on these larval stages may be substantial and rapid. In the wild, paralysed larvae would be unable to feed and unable to maintain their position in the water column and negatively impact their predator avoidance behaviour. These larvae may, therefore, be considered as ecologically dead and the effect in the wild may be larger than what is indicated by the LC_{50} value alone. While mechanical paralysis and the formation of O_2 bubbles in the hemolymph has previously been observed amongst H_2O_2-exposed adult sea lice (Thomassen, 1993; Bruno and Raynard, 1994; Aaen et al., 2014), this was not reported for *H. americanus* larvae in H_2O_2 acute toxicity tests (Burridge et al., 2014). Interestingly, while *M. edwardsii* larvae and copepods (*A. hudsonica* and *Calanus* spp.) were paralysed following short-term exposures to H_2O_2, the formation of bubbles was not reported/observed (Van Geest et al., 2014; Gerbauer et al., 2017; Escobar-Lux et al., 2019), suggesting differences in mechanistic pathways of toxicity amongst crustacean species.

It is interesting to note, that although we observed that a single 1 h H_2O_2 exposure had lethal and sub-lethal effects on *H. gammarus* larva, delousing operations can involve the concurrent and sequential pesticide applications in many cages within a single fjord. Consequently, multiple discharges and cumulative loading of the pesticides can occur and non-target crustaceans are likely to be exposed to multiple H_2O_2 plumes over a longer period (Grefsrud et al., 2018). Lower LC_{50} and EC_{50} values have been reported as a result of longer exposure times or pulse-like exposures for both *H. americanus* and *P. borealis* (Burridge et al., 2000, 2008; Bechmann et al., 2019). Therefore, the impact of H_2O_2 on wild lobster larvae may be more pronounced under these conditions than the effects observed here for single exposures.

4.3. Effects of H_2O_2 on the shelter-seeking behaviour

Here we have shown that short (1 h) exposures to sub-lethal concentrations of H_2O_2 negatively affected several behavioural parameters associated with shelter-seeking in stage V *H. gammarus* lobsters when examined immediately after the exposure period. In all H_2O_2 treatment groups (85–510 mg/L), the lobster juveniles moved significantly less (total distance travelled) and inspected the shelter fewer times compared with control juveniles. Such negative impacts on locomotion observed in short-term sub-lethal exposures to pesticides have previously been linked to a failure in predator avoidance for other crustacean species (Farr, 1977; Rasmussen et al., 2013). Furthermore, juveniles exposed to the two higher H_2O_2 concentrations (170 and 510 mg/L) spent a longer period of time exploring their surroundings and to locate and recognise the shelter. As far as we are aware, no published studies to date have examined the effect of H_2O_2 on the shelter seeking behaviour of *H. gammarus* or any other lobster species, though exposure to H_2O_2 did have measurable effects on the escape behaviour of *Calanus* spp. (Escobar-Lux et al., 2019). Interestingly, a recent study reported reduced exploratory behaviour amongst *H. gammarus* juveniles exposed to sub-lethal concentrations of the in-feed anti-sea lice drug teflubenzuron (Cresci et al., 2018). Specifically, and in line with our findings, the study found that teflubenzuron exposed juveniles took significantly more time to find and recognise shelter (Cresci et al., 2018). Furthermore, sub-lethal concentrations of the organophosphate pesticide azamethiphos negatively affected the use of shelters by juvenile *H. americanus*, with an increase in the lobsters' latency to re-enter the shelter observed with increasing azamethiphos concentrations (Abgrall et al., 2000). Taken together, these studies demonstrate that shelter seeking

behaviour of juvenile lobsters is negatively affected following exposure to a range of anti-sea lice pesticides, including H_2O_2, and this may have negative consequences on the lobster's ability to avoid predators. Post-larvae or early benthic juvenile lobsters are more dependent on the rapid attainability of their shelters than adults (Mehrtens et al., 2005), and multiple studies have shown that the vulnerability of newly settled juveniles due to lack of protective shelters is high, and therefore important for survival (Hudon, 1987; Lawton and Lavalli, 1995; van der Meeren, 2001). Juveniles that reside in the vicinity of salmon farms treating with H_2O_2, may therefore be at a higher risk of predation if they cannot rapidly attain a shelter. Interestingly, however, all of the behavioural endpoints affected immediately after the exposure period returned to baseline levels at 24 h post-exposure, with no significant differences between exposed and control lobsters. This suggests that the effects of H_2O_2 on the shelter seeking behaviour of *H. gammarus* larvae may only be short lived, with the risk of predation in the wild likely to be highest in the immediate aftermath of an exposure scenario.

4.4. Potential effects of H_2O_2 to wild populations

Hydrogen peroxide has previously been described as the most environmental friendly bath treatment chemotherapeutant on the market and it is estimated that it poses little threat in terms of lethality to non-target crustaceans, such as lobster and shrimp, after short term exposures (Burridge et al., 2014). Here, however, we have shown that the 1 h-LC_{50} values calculated for stage I, II, III and IV *H. gammarus* larvae represent approximately 10, 23, 40 and 43%, respectively, of the recommended H_2O_2 concentrations used for treating sea lice infestations on Norwegian fish farms. Furthermore, we have also shown that lobster juvenile behavioural parameters associated with shelter seeking were also affected following short-term exposure to H_2O_2 at concentrations as low as 85 mg/L (or 5% of the recommended treatment dose). It is important, however, to assess whether these concentrations, calculated from laboratory based toxicity tests, are likely to pose a risk to lobster larvae living in the wild near aquaculture facilities. While it has previously been reported that H_2O_2 breaks down into water and oxygen, the speed of this process is influenced by several parameters including temperature and the amount of organic matter in the water. Degradation studies have estimated that the half-life of H_2O_2 ranged between 1 and 56 days (Bruno and Raynard, 1994; Lyons et al., 2014; Fagereng, 2016; Parsons and Samuelsen unpubl. data), and even the shortest of these estimated degradation times is considerably longer than the 1 h needed to induce mortalities, paralysis and altered exploratory behaviours amongst the pelagic and benthic larval stages of *H. gammarus*. Since H_2O_2 is expected to rapidly dilute in receiving waters, it is, however, reasonable to assume that the degradation rate will have limited impact on the environmental concentrations and dispersal dynamics instead will greatly influence the impact of H_2O_2 on non-target species. Considering that H_2O_2 is extensively used as an anti-sea lice pesticide around the world, relatively few field studies have, however, measured the concentration of H_2O_2 in the waters surrounding fish farms after the discharge of bath treatment effluents. One such study from the west coast of Norway, found that concentrations of H_2O_2 were either below the limit of detection or relatively low in water sampled 20–60 m from the edge of a salmon cage after the bath treatment water was discharged (Fagereng, 2016). In contrast, a later Norwegian study measured relatively high concentration of H_2O_2 (up to 778 mg/L), similar to or greater than the LC_{50} values observed here for *H. gammarus* larvae (177–738 mg/L) in the water directly under (at depths up to 60 m) and surrounding (within 15 m) a salmon cage post treatment. These higher H_2O_2 concentrations did, however, decrease with time (Andersen and Hagen, 2016).

Recently, studies have started to use mathematical models to predict the dispersal of bath treatment pesticides from Norwegian farms and indicate that the spread of H_2O_2 in the marine environment may be more substantial than field studies imply (Refseth et al., 2017; Parsons et al., 2020). Model simulations, performed by Refseth et al. (2017), found that low concentrations of H_2O_2 (<100 mg/L) should be detected in surface waters (0–3 m depth) at large distances from Norwegian farms, up to several hours after the discharge. This study also reports that areas closer to the farm (within 1 km) may experience higher H_2O_2 concentrations (>300 mg/L) for the first hour after discharge, while areas within a 2 km radius may be exposed to concentrations of 100 mg/L (Refseth et al., 2017). These simulations suggest that pelagic life stages of *H. gammarus*, in particular stage I and II larvae, that are living within 1–2 km of a salmon farm may be exposed to lethal concentrations of H_2O_2.

It is interesting to note that both field measurement and model simulation studies report that when environmental conditions result in a well-mixed water column, H_2O_2 plumes can sink to the seafloor within minutes of discharge. These findings have serious implications for benthic non-target species and life stages, such as juvenile and adult lobsters, living in the vicinity of fish farms. For example, Andersen and Hagen (2016), measured H_2O_2 concentrations that were 43% of the treatment concentration on the sea floor (at 70 m depth) 8 min after a discharge. Similarly, Refseth et al. (2017), predicted that 50% of the initial treatment doses (800 mg/L) could sink to the seafloor under fish cages and horizontal transport along the bottom would be reduced compared to the surface layers, meaning that these higher concentrations would persist for longer periods of time (up to 5–10 h). Considering that we observed behavioural changes in newly settled stage V *H. gammarus* juveniles, at 5% of the recommended treatment concentration, these studies suggest that H_2O_2 poses a risk to bottom-dwelling lobster life stages as well as the pelagic life stages.

In summary, the results presented here clearly demonstrate that short-term exposures to H_2O_2, at and below recommended industry concentrations, have lethal and sub-lethal effects on multiple life stages of the commercially important European lobster. In order to better understand the potential effects of H_2O_2 in the Norwegian marine environment, further studies which assess the impact of acute and chronic exposures to H_2O_2 on a wide variety of native non-target species are required.

Credit author statement

Rosa H. Escobar-Lux: Conceptualization, Methodology, Formal analysis, Writing - original draft, Writing - review & editing, Aoife E. Parsons: Conceptualization, Methodology, Writing - review & editing, Ole B. Samuelsen: Conceptualization, Methodology, Writing - review & editing, Ann-Lisbeth Agnalt: Conceptualization, Methodology, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2020.111111.

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Paper III

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The Acute and Delayed Mortality of the Northern Krill (*Meganyctiphanes norvegica*) When Exposed to Hydrogen Peroxide

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Abstract
Bath treatment pharmaceuticals used to control sea lice infestations in the salmonid industry, such as hydrogen peroxide (H_2O_2), are released directly into the environment where non-target organisms are at risk of exposure. The aim of this study was to determine the threshold concentrations for mortality of the Northern krill, *Meganyctiphanes norvegica,* a major component of the north Atlantic marine ecosystem. To assess the lethal effects of H_2O_2, we carried out a series of 1 h acute toxicity tests and assessed mortality through a 48 h post-exposure period. One-hour exposure to 170 mg/L, corresponding to 10% of the recommended H_2O_2 treatment, caused 100% mortality and a subsequent acute median-lethal concentration LC50 value of 32.5 mg/L. Increased mortality was observed with time in all exposed groups, resulting in successively lower LC_{50} values during the post-exposure period. The suggested H_2O_2 concentrations have the potential of causing negative effects to the Northern krill.

Keywords Crustacean · Toxicity · LC_{50} · Aquaculture

Sea lice (*Lepeophtheirus salmonis* and *Caligus rogercresseyi*), naturally occurring parasitic copepods affecting both farmed and wild salmonid populations, are a major challenge for the salmonid industry worldwide (Costello 2006; Torrissen et al. 2013; Vollset et al. 2016). The parasites feed on the mucous, skin, and blood of its host, and if present in significant numbers they can cause damage associated with osmotic stress and secondary infections (Finstad et al. 2000; Johnson et al. 2004; González et al. 2015). Norwegian wild salmonid populations, migrating post smolts from Atlantic salmon and local populations of sea trout (*Salmon trutta*), can suffer high mortality if there is high density of salmon lice larvae in the surrounding water (Costello 2009; Vollset et al. 2016). In farmed fish, salmon lice infestations reduce the general welfare of the fish and lead to an increase of the overall cost of the industry due to reduced growth and marketability due to skin lesions, and high costs associated with delousing treatments (Costello 2009). Therefore, both the economic and ecological impact of salmon lice infestations are significant challenges for the salmonid industry.

In order to control salmon lice infestations, the industry has relied on the use of different chemotherapeutants, through the application of bath treatments and the use of in-feed drugs. Bath treatments can be applied either by enclosing the fish cages with an impervious tarpaulin or transferring the fish into well-boats, and after treatment the waste water is directly released into the surrounding water (Ernst et al. 2001; Burridge et al. 2010). At a global level, hydrogen peroxide (H_2O_2) was introduced as an antiparasitic agent after the loss of sensitivity in both *L. salmonis* and *C. rogercresseyi* to other delousing agents (Bravo et al. 2015; Urbina et al. 2019). In Norway alone, H_2O_2 is still the most used bath treatment therapeutant with a consumption of 4523 tons in 2019 (www.fhi.no/hn/legemiddelbruk).

Hydrogen peroxide acts on salmon lice by hydroxyl radicals attacking lipid and cellular organelles resulting in inactivation of enzymes and DNA replication (Cotran et al. 1989; Urbina et al. 2019). Previous studies have also shown that decomposition of hydrogen peroxide to water and O_2 bubbles in the gut and the haemolymph may cause mechanical paralysis leading to detachment of the pre-adult and adult salmon lice from the fish and causing them to float towards the surface (Bruno and Raynard 1994;

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Aaen et al. 2014). A bath treatment involves the release of a large volume of H_2O_2 containing waste water and the chemical can potentially be dispersed over a wide area (Burridge et al. 2010, 2014, Parsons et al. 2020, Refseth et al. 2017). Therefore, there is a growing concern about the possible toxic effects of H_2O_2 on non-target aquatic invertebrate species living in the vicinity of fish farms, and specifically crustaceans which has been proven as particularly vulnerable (Smit et al. 2008; Burridge et al. 2014; Van Geest et al. 2014; Gebauer et al. 2017; Hansen et al. 2017; Bechmann et al. 2019; Escobar-Lux et al. 2019).

The pelagic zooplankton, *Meganyctiphanes norvegica,* Northern krill, is a species at risk as its distribution overlaps with the location to many salmon farms in Norway, as it inhabits both coastal and offshore waters (Kaartvedt et al. 2002; Melle et al. 2004; Tarling et al. 2010). Furthermore, the distribution of this boreal krill species has been described to be seasonal, with a predominant coastal distribution between the months of January and May (Grover 1952). In Norway, during this period of the year, pharmaceuticals are being used to keep the level of salmon lice below 0.2 female lice per fish as specified in the Norwegian Ministry of Trade, Industry and Fisheries (FOR-2012-12-05-1140, 2012) (Grefsrud et al. 2019). The total biomass of euphasiid stocks in the Norwegian Sea has been previously estimated to 42 million tons (Mt), with around 40–75% of this stock being Northern krill (Lindley 1982; Melle et al. 2004). Thus, the northern krill is a major component of the north Atlantic marine ecosystem, acting as a keystone organism between lower trophic levels and larger predators and plays an important role in the sequestration of carbon (Kaartvedt et al. 2005; Tarling et al. 2010). It is preyed upon by several commercially important fish species (Sameoto et al. 1994; Onsrud et al. 2004), seabirds (Montevecchi et al. 1992; Stevick et al. 2008), and marine mammals (Brodie et al. 1978). Moreover, the commercial exploitation of Northern krill is gaining interest in the salmonid industry as a potential protein alternative to the fishmeal (Tarling et al. 2010). Mass death of krill washed up on a beach can occur and is considered a natural phenomenon. Previously the mass stranding of *M. norvegica* has been explained as predation events in which predators' chase krill ashore (MacDonald 1927), transported to land by oceanic currents or by special events like upwellings (Aitken 1960; Cox 1975), or because special lightning conditions that might interfere with the krill's behavior (Wiborg 1966). However, in recent years there has been a higher frequency of reports in Norway describing this phenomenon near areas with salmon farms. This started a debate in public media of what might have caused the mass mortality and one of the most frequently cited suggestions has been the use of pesticides for delousing of the salmon farms, and especially H_2O_2. However, the effects of H_2O_2 exposure on the Northern krill have until now been unknown.

For treating salmon, the recommended concentration for a H_2O_2 bath treatment is 1500–2100 mg/L for 20 min depending on temperature (https://www.felleskatalogen.no/medisin-vet). Typically, toxicity studies use exposure times that vary from 24 to 96 h. However, these may not be representative of the real-life scenarios following a release of waste water after a bath treatment on a salmon farm (Ernst et al. 2001; Urbina et al. 2019). The use of 1 h exposures, is considered a more realistic exposure scenario, but to date only a limited number of species have been tested under those conditions (Medina et al. 2004; Fairchild et al. 2010; Burridge et al. 2014; Van Geest et al. 2014; Escobar-Lux et al. 2019; Parsons et al. 2020). What these previous studies also have shown is that the mortality observed immediately after exposure tends to be lower than the mortalities registered if a post-exposure period is included in the experimental set-up. A longer post-exposure observation period is therefore recommended.

The main objective of this study was to examine the toxicity of H_2O_2 to *M. norvegica*, a non-target crustacean and keystone species of the Norwegian marine environment. Our objective was to expose the Northern krill to a short 1 h pulse of H_2O_2 and assess the acute and delayed mortality during a post-exposure period of 48 h in clean seawater.

Materials and Methods

In the present study, krill (*M. norvegica*) were collected from the dock at Austevoll Research Station, Institute of Marine Research Norway (60° 05′ 20″ N 5° 15′ 57″ E) using light traps. The light traps (mesh size 500 µm; 0.45 m in diameter; BellaMare USA) were equipped with a white LED light and deployed at a depth of 20 m overnight. The research station is at least 3 km away from the nearest commercial salmon farm. Krill from the traps were transported to the laboratory at Austevoll Research Station and kept overnight in 10 L buckets supplied with sand filtered seawater from a depth of 160 m (Bjørnafjorden) holding a temperature of 8 °C (salinity of 34.2 ppt; pH 7.94). The experiment was performed within 48 h of capture and prior to exposure the krill were sorted and only krill in excellent physical condition were used in the experiments.

Commercial H_2O_2 (Nemona, Akzo Nobel Pulp and Performance Chemicals AB Sweden) at a concentration of 49.50% (600 g/L) was purchased from Akzo Nobel, Pulp and Performance Chemicals, AB Sweden. Since no previous studies had assessed the toxicity of H_2O_2 on *M. norvegica*, the chosen concentrations were based on the recommended dose for treating salmon (1700 mg/L). The krill were exposed to concentrations of 1.7, 8.5, 17, 170, 850 and

1700 mg/L H_2O_2, corresponding to 0.1, 0.5, 1, 10, 50 and 100% of the recommended treatment dose. All exposures were conducted in glassware units with a volume of 500 mL. A total of 140 krill were randomly divided into seven treatment groups, including a control group, with four replicates for each treatment and each replicate counting five individuals. After the 1 h exposure, acute mortality was recorded and the krill were transferred to 10 L recovery tanks where mortality was checked successively at 6, 24 and 48 h post-exposure using a dissecting microscope. Krill were considered dead if there was no movement of the pereopods, pleopods or antenna after a gentle stimulus. Mortality that occurred during the 1 h exposure was defined as acute mortality. Total mortality was defined as the cumulative mortality after the 48 h post-exposure period.

The statistical analyses for mortality were done in the software R (Version 3.5.3 (2019-03-11) Copyright © 2019 The R Foundation for Statistical Computing). The LC_{50} values, and their 95% confidence intervals (CI), were calculated using generalized linear models (GLM) with binomial error structures and probit links, according to Finney (1971). Hydrogen peroxide concentrations were log10 transformed to linearize the data.

Results and Discussion

This study clearly show that H_2O_2 was acutely toxic to wild-captured Northern krill *M. norvegica*. While no mortality was recorded in the group exposed to the lowest dose of 1.7 mg/L or in the control group, a 1 h exposure to 170 mg/L, i.e. 10% of recommended dose, caused 100% mortality and a subsequent acute LC_{50} value of 32.5 mg/L (16.8–48.2) was calculated (Fig. 1a). During the post-exposure period, increased mortality with time was observed in all exposed groups resulting in successively lower LC_{50} values with 14.11 mg/L after 6 h (7.3–20.9), 4.92 mg/L (1.2–7.9) after 24 h and finally 0.86 mg/L after 48 h (Fig. 1b–d). No mortality was registered in the control groups during the post-exposure period. The calculated LC_{50} value at 24 h represents a threefold dilution of the acute 1 h LC_{50} value. These findings clearly support the recommendations suggested in previous studies to include a post-exposure period following the exposure to H_2O_2 to assess any delayed effects (Van Geest et al. 2014; Brokke 2015; Escobar-Lux et al. 2019).

While several studies have examined the toxicity of H_2O_2 on marine crustacean species, the number of studies using an exposure time of 1 h is more limited. A review of those studies reveals that some crustaceans

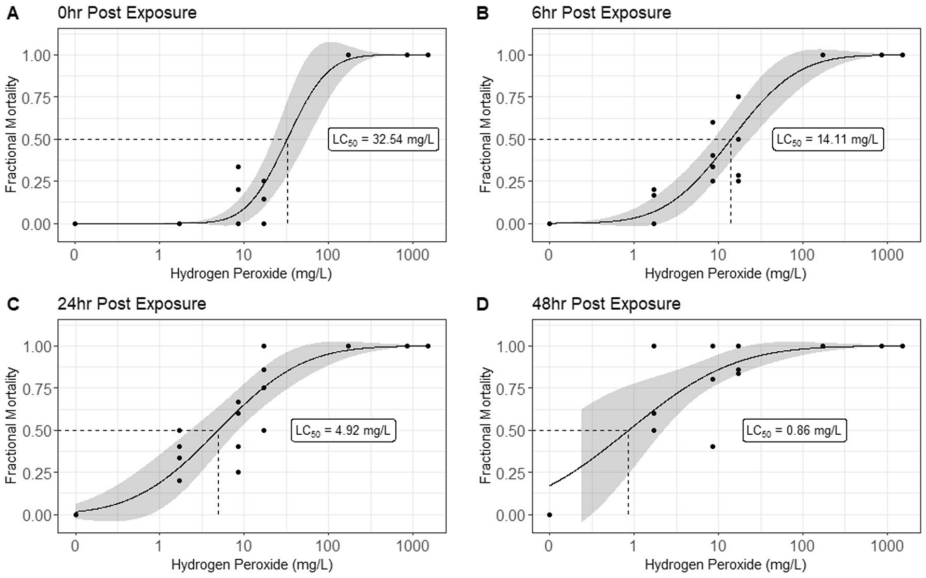

Fig. 1 The toxicity of hydrogen peroxide to *M. norvegica* following 1 h exposure. Dose–response curves showing mortality amongst the northern krill at 0 h, 6 h, 24 h, and 48 h post-exposure to H_2O_2. Each point on the graphs represent an individual replicate tank containing 4 to 6 krill and the line represent the best fit model for the data calculated using a binomial log-probit GLM in R. The shadowed area represents the 95% confidence intervals

have a relatively high tolerance to H_2O_2 exposure and is reflected in low mortality when exposed to concentrations similar to or higher than the recommended treatment dose. This applies to both newly hatched larvae and adult of American lobster (*Homarus americanus*), sand shrimp (*Crangon septemspinosa*), the mysid *Mysid* sp. (Burridge et al. 2014), rock pool shrimp (*Palaemon elegans*) and chameleon shrimp (*Praunus flexuosus*) (Brokke 2015). For some species, low mortality was observed even when a post-exposure period was included in the study. Following an exposure of 1 h and a 95 h post-exposure period, the calculated LC_{50} values were 1673 mg/L for *H. americanus* larvae, > 3750 mg/L for adult American lobster, 3182 mg/L for sand shrimps and 973 mg/L for *Mysid* sp. (Burridge et al. 2014; Van Geest et al. 2014). For rock pool shrimps and chameleon shrimps the acute mortality after 1 h exposure was low indicating LC_{50} values higher than the highest exposure concentration of 1700 mg/L for both species (Brokke 2015). However, a significant mortality occurred during the 24 h post-exposure period, resulting in LC_{50} values of 174.1 mg/L and 77.5 mg/L for rock pool shrimp and chameleon shrimps respectively, classifying these species as highly sensitive. In the study by Bechmann et al. (2019), the Northern shrimp (*Pandalus borealis*) was exposed to 15 mg/L H_2O_2 for 1 h. The very low acute mortality observed immediately after exposure did however increase during the post-exposure period (7 days) but as the total mortality never exceeded 30%, no LC_{50} could be calculated. Damage on the gills was observed in the shrimps exposed to H_2O_2 and suggested as the major cause of the delayed mortality (Bechmann et al. 2019).

In comparison, species like the copepods *Acartia Hudsonica* and *Calanus* spp. have shown higher sensitivity to H_2O_2 exposure, resulting in EC_{50} and LC_{50} values of 2.6–10 mg/L and 30.6 mg/L respectively, following a 24 h post-exposure period (Van Geest et al. 2014; Escobar-Lux et al. 2019). In the case of the European lobster (*Homarus gammarus*) larvae (stage I–IV), a 1 h exposure to 1530 mg/L followed by a 24 h post-exposure period, resulted in mortalities between 75 and 100% (Escobar-Lux et al. 2020) and calculated LC_{50} values of 177 mg/L, 404 mg/L, 676 mg/L and 738 mg/L, for stages I, II, III and IV respectively. For species other than crustaceans, the polychaete *Ophryotrocha* sp. and the sugar kelp *Saccharina latissima* are amongst the more sensitive marine species with LD_{50} values of 64.3 mg/L and 80.7 mg/L following 72 h and 7 days' post-exposure periods, respectively (Fang et al. 2018; Haugland et al. 2019). The LC_{50} values calculated for northern krill are therefore, to our knowledge the most sensitive species examined so far.

This study has shown that a bath treatment with H_2O_2 has a detrimental effect on *M. norvegica.* However, it is important to assess whether these laboratory-based concentrations are likely to pose a significant risk to krill at the proximity of salmonid aquaculture sites. Due to differences in experimental set-ups the variation in half-lives reported for H_2O_2 in seawater in large, with results between 1 and 58 days (Bruno and Raynard 1994; Lyons et al. 2014; Fagereng 2016; Parsons and Samuelsen unpubl. data). Several factors affect both the toxicity and the degradation of H_2O_2, for example the water temperature or the irradiance (Stratford et al. 1984; Treasure et al. 2000)". However, even the shortest degradation time reported (1 day) is significantly longer than the 1 h exposure needed in the present study to cause considerable mortality of the Northern krill. Even though H_2O_2 is extensively used around the world as an anti-sea lice bath treatment, few studies have initiated the use of mathematical models to predict its' dispersal and its' impact on non-target species. One such study from Norway has indicated that the spread of H_2O_2 may be larger than previously thought (Refseth et al. 2017). According to the model, concentrations up to 300 mg/L may occur within a 1 km radius from the farm and 100 mg/L within a radius of 2 km. Furthermore, the model also suggested that a concentration of 100 mg/L can be present in surface waters for several hours after discharge. The presented model simulations therefore suggest that the Northern krill within 2 km of a salmonid farm may be exposed to lethal concentration of H_2O_2.

Parsons et al. (2020) used dispersion models to predict the spreading of pharmaceuticals from salmonid farms in Norway, following bath treatment. Based on the models and LC_{50} values (1 h exposure followed by 24 h post-exposure period) for European lobster larvae (stage I and II) they calculated impact zones around 23 Norwegian fish farms for the pesticides azamethiphos and deltamethrin. This model however, did not take into account the degradation of the compounds due to the presence of organic matter in the water. While the azamethiphos impact zones around farms were relatively small (mean area of 0.04–0.2 km^2), deltamethrin impact zones covered much larger areas (mean area of 21.1–39.0 km^2). The difference in impact zone is due to the difference in toxicity between the two drugs. For azamethiphos the 1 h-LC_{50} values (95% CIs) for stage I and II larvae were 43.1 µg/L (13.0–131.0 µg/L) and 20.5 µg/L (13.2–30.9 mg/L), respectively, representing approximately 2- and fivefold dilutions of the treatment concentration (100 µg/L) used on Norwegian fish farms. For deltamethrin the 1 h-LC_{50} values (with 95% CIs) for stage I and II larvae were estimated to be 2.6 ng/L (0.6–11.0 ng/L) and 2.9 ng/L (1.5–5.7 ng/L), representing approximately 800-fold dilution of the treatment concentration of 2000 ng/L. Considering the sensitivity of krill towards H_2O_2 found in the present study, where the LC_{50} ranged from 52- to 2000-fold dilution with increasing post-exposure period, impact zones like those

calculated for deltamethrin in Parsons et al. (2020) will be most relevant for impact zones for H_2O_2 and krill.

Meganyctiphanes norvegica can be found around the North Atlantic, with the Norwegian sea being a major hotspot for its distribution (Melle et al. 2004). Due to their distribution, krill can often be found in waters close to aquaculture sites and therefore be negatively impacted by the dispersal of effluent plumes after treatments. Based on our findings and the information from previous mathematical models, H_2O_2 may cause a larger impact than it was previously believed. Therefore, that some cases of mass mortality of krill observed in past years may have been caused by H_2O_2 exposure, cannot be overlooked.

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The impact of anti-sea lice pesticides, azamethiphos and deltamethrin, on European lobster (*Homarus gammarus*) larvae in the Norwegian marine environment☆

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ABSTRACT

Anti-sea lice pesticides, used in the salmonid aquaculture industry, are a growing environmental concern due to their potential to adversely affect non-target crustaceans. Azamethiphos and deltamethrin are two bath treatment pesticides used on salmon farms in Norway, however, limited information is available on their impact on European lobster (*Homarus gammarus*) larvae in the Norwegian marine environment. Here, we firstly report the lethal (LC_{50}) and effective (EC_{50}) concentrations of azamethiphos and deltamethrin for stage I and stage II larvae, following 1-h exposures. Using a hydrodynamic model, we also modelled the dispersal of both compounds into the marine environment around selected Norwegian farms and mapped the potential impact zones (areas that experience LC_{50} and EC_{50} concentrations) around each farm. Our data shows that azamethiphos and deltamethrin are acutely toxic to both larval stages, with LC_{50} and EC_{50} values below the recommended treatment concentrations. We also show that the azamethiphos impact zones around farms were relatively small (mean area of 0.04–0.2 km^2), however deltamethrin impact zones covered much larger areas (mean area of 21.1–39.0 km^2). These findings suggest that deltamethrin poses a significant risk to European lobster in the Norwegian marine environment while the impact of azamethiphos may be less severe.

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1. Introduction

In the past three decades, global aquaculture production has expanded rapidly, from 5.2 million tonnes in 1981 to 110.2 million million tonnes in 2016 (FAO, 2018). This expansion has led to growing environmental concerns over the industry's impact on water quality, natural ecosystems and human health (Liu et al., 2017; Páez-Osuna, 2001). Norway is the largest producer of farmed Atlantic salmon (*Salmo salar*) in the world, with 1.2 million tonnes produced annually (FAO, 2018). Sea lice (*Lepeophtheirus salmonis*) infestations are common in the salmonid aquaculture industry, reducing the general welfare of the farmed fish and causing significant economic losses to the industry (Pike et al., 1999; Wagner et al., 2008; Burka et al., 1997). Chemotherapeutic drugs and pesticides applied either as in-feed additives or bath treatments are one of several methods for controlling these infestations on salmonid farms. Bath treatments involve surrounding fish cages with a tarpaulin or transferring the fish to well-boats so they are enclosed. The recommended treatment concentration for the pesticide is added, and salmon are held in the bath for the recommended treatment time. Following the treatment, the enclosed water is directly released into the surrounding aquatic environment (Burridge et al., 2010). Azamethiphos and deltamethrin are important bath treatment pesticides used in major regions of salmonid aquaculture worldwide (Burridge et al., 2010; Scottish Environmental Protection Agency (SEPA), 2019; Folkehelseinstituttet, 2019). Azamethiphos, the active ingredient in the commercial formulations Salmosan Vet® and Trident Vet®, is a neurotoxic insecticide, causing acetylcholinesterase (AChE) inhibition which consequently results in paralysis and eventual mortality of the target organism (Baillie et al., 1985). On salmon farms, a

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20–40 min azamethiphos treatment is recommended with a target concentration of 100 μg L^{-1}. Deltamethrin, a synthetic pyrethroid insecticide, is the active ingredient in the commercial formulation AlphaMax®. It interacts with the sodium (Na+) channels of nerve membranes, resulting in depolarisation of nerve endings and over-stimulation of cells and eventual paralysis (Miller and Adams, 1982). A 30-min deltamethrin treatment is recommended with a target concentration of 2 μg L^{-1}. The use of azamethiphos and deltamethrin, along with other delousing pesticides, was widespread on Norwegian fish farms between 2010 and 2015, as a result of increased resistance amongst sea lice to the different pesticide compounds. The current annual consumption of azamethiphos and deltamethrin is relatively low in comparison to previous years, with only 154 kg and 10 kg (active substance) used in 2019, respectively (Folkehelseinstituttet, 2019).

Given the growing evidence showing that anti-sea lice pesticides are toxic to non-target species, particularly crustaceans, their direct release into the marine environment is an increasing cause for concern (Burridge et al., 2010; Urbina et al., 2019). To better assess the impacts of azamethiphos and deltamethrin on non-target species in the Norwegian marine environment, a greater understanding of their toxicity and environmental concentrations around fish farms is required. To date azamethiphos and deltamethrin acute toxicity tests using marine crustaceans have mostly involved 24, 48 and 96 h exposure periods (Burridge et al., 1999; Ernst et al., 2001; Ernst et al., 2014; Oliveira et al., 2012; Adam et al., 2010), which do not reflect the highly acute exposures expected to occur in the marine environment following the release of bath treatment effluents (Ernst et al., 2001; Burridge et al., 2014; Bruno and Raynard, 1994; Tomlin, 1997; Scottish Environmental Protection Agency (SEPA), 2005). Currently, there also is limited information available on the dispersal of azamethiphos and deltamethrin in the marine environment around Norwegian fish farms. Consequently, it is difficult to assess whether threshold concentrations, calculated from laboratory based toxicity tests, are likely to pose a risk to non-target species living in the wild near aquaculture facilities. While mathematical models have been developed for assessing the dispersal of bath treatment compounds from farming systems located in shallow estuarine, semi-enclosed (e.g. sea lochs) and coastal environments in Scotland and Ireland (Falconer and Hartnett, 1993; Gillibrand and Turrell, 1997; Gillibrand and Turrell, 1999; Scottish Environmental Protection Agency (SEPA), 2008), the environmental conditions in Norway's fjords are considerably different. Therefore there is an urgent need to apply a hydrodynamic model to assess the dispersal of bath treatment compounds specifically in the Norwegian marine environment (Rico et al., 2019).

The main aim of this study was to assess the potential impact of azamethiphos and deltamethrin on a native non-target crustacean species in the Norwegian marine environment. Our first objective was to examine the toxicity of both compounds to European lobster *(Homarus gammarus)* larvae following an environmentally relevant exposure period (1 h) and establish threshold concentrations associated with exposure. *H. gammarus* is an important commercial species in many coastal regions of Europe, including Norway, and is often located near salmon aquaculture sites. *H. gammarus* larvae are pelagic and remain in the surface layers and therefore can move with pesticide plumes following the operational release of bath treatment effluents. Consequently, the larvae are potentially more vulnerable to exposure than benthic invertebrates such as adult lobsters. The second objective of this study was to use a hydrodynamic model to simulate the dispersal of azamethiphos and deltamethrin into the marine environment at multiple Norwegian fish farms. Using the simulated dispersal data, we subsequently mapped the areas around each of the farms which experience pesticide concentrations exceeding the lethal and effective threshold concentrations calculated here for *H. gammarus* larvae.

2. Material and methods

2.1. Animal collection and maintenance

This experiment was approved by the Norwegian Food Safety Authority (ID 15510) and has been carried out according to The Code of Ethics of the World Medical Association for animal experiments (The Norwegian Ministry of, 2010; The Norwegian Ministry of, 2015). Six ovigerous *H. gammarus* females were purchased from a local lobster dealer on 22 May 2018 and transferred to the Institute of Marine Research (IMR) field station at Austevoll, located outside Bergen (N60°05′15.36″, E5°15′54″). They were initially kept in holding tanks (1.5 m × 1.5 m x 1 m) supplied with filtrated seawater from 160 m depth at a flow of 30 L min^{-1} (salinity of 34.7 ppt and temperature of 8 °C). Females were fed frozen shrimp twice per week and the temperature was kept low to postpone and control hatching. Experiments were conducted at the same location in August–November 2018. The ovigerous females were transferred to holding tanks with 16 °C to stimulate hatching.

When spawning occurred larvae were removed from the hatching tanks every morning and transferred to 40 L fibreglass incubators (plankton Kreisler tanks) (Hughes et al., 1974), which were supplied with oxygenated seawater (15–16 °C) at a rate of 8–10 L min^{-1} and kept in a 16:8 h light: dark cycle. Maximum density for each incubator was set to 50 larvae L^{-1}. The incubators were treated for the bacteria *Leucatrix minor* with chloramine-T (every third day at 0.02 g L^{-1} for 1 h). Larvae were fed frozen artemia twice a day and checked daily to determine the stage of development. The larvae were staged I-II according to Sars (1874). Briefly, stage I larvae are characterised by the lack of pleopods while stage II larvae had developed pleopods. At the selected water temperature, the approximate number of days required to pass through the stage I to stage II larval stages were 4 and 5 days, respectively. The larvae used in each lethality test were of the same stage and approximate age. The mean carapace length for stage I and stage II larvae was 2.3 mm ± 0.1 and 3.3 mm ± 0.1, respectively.

2.2. Acute toxicity tests

H. gammarus larvae (5 larvae per tank, 3–4 replicates per concentration) were exposed to a range of concentrations of azamethiphos (1–1000 μg L^{-1}) and deltamethrin (0.01–200 ng L^{-1}) in 700 mL of test solution for 1 h to generate cumulative mortality curves. Each assay was repeated twice (approx. 40 larvae per concentration). The chosen concentrations were based on LC_{50} values estimated for *H. americanus* lobster larvae (Burridge et al., 2014). Azamethiphos (Trident Vet 500 mg g^{-1} powder) was purchased from Neptune Pharma Ltd. (London, UK) and deltamethrin (AlphaMax 10 mg ml^{-1}) from Pharmaq A/S, (Overhalla, Norway). Stock solutions (1 μg L^{-1} and 10 mg L^{-1}, respectively) of azamethiphos and deltamethrin were prepared using the stock formulations and filtered seawater (0.2 μm). Test concentrations were prepared by serial dilutions of stock solutions. All experimental units and equipment for preparing stock solutions were made of glassware (as deltamethrin is known to readily bind to the walls of plastic test vessels). After each exposure, the larvae were placed in 1 L recovery units supplied with fresh seawater. The number of mortalities and immobile larvae were recorded at 0 h and 24 h post-exposure in each tank. Lobsters were considered immobile if they sank to the bottom of the tank, i.e. normal swimming behaviour was absent and considered dead when there was no movement of pleopods even after gentle prodding. Larvae were fed

compound fish feed (Otohime C, Marubeni Nisshin Feed Company, Japan) during the 24 h recovery period. Water temperatures ranged between 13.5 and 17.7 °C. Lethal and total effect dose-response curves were generated for each individual assay (as each pesticide assay was repeated twice) as well as the data combined (i.e. assay one and two were combined). For each of the dose-response curves, LC_x and EC_x values, based on mortality and total effect (mortality + immobility) after the 24 h recovery period, were calculated, respectively.

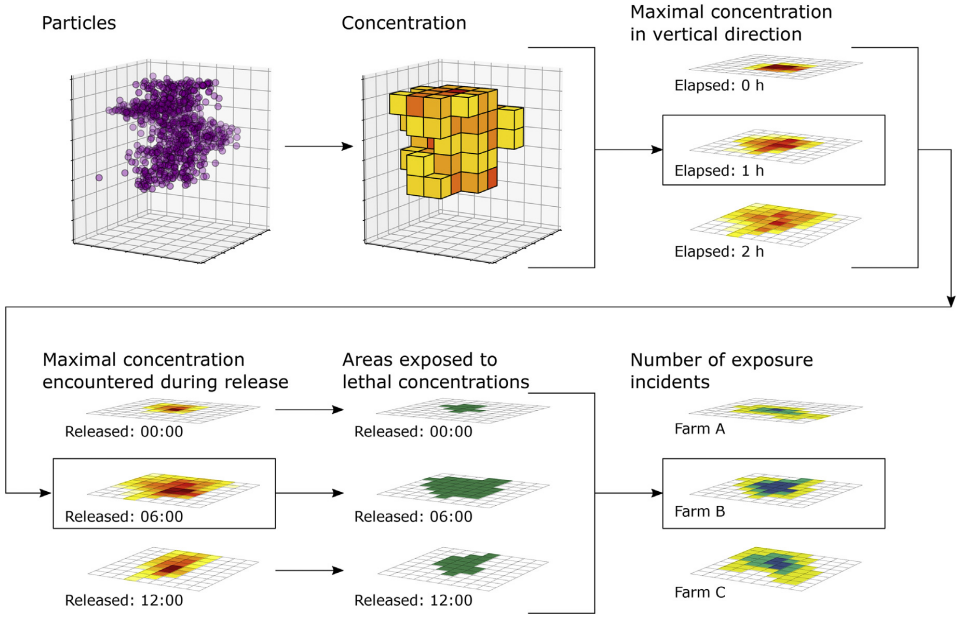
2.3. Modelling pesticide dispersal and impact zones

The dispersal of azamethiphos and deltamethrin into the marine environment around Norwegian fish farms was simulated for a 24 h period post bath treatment effluent release using a hydrodynamical model. The dispersal data was subsequently used to map the potential impact zones around fish farms. Impact zones are defined as areas around fish farms which are exposed to the lethal and effective concentrations of azamethiphos and deltamethrin (as per the 1 h toxicity tests carried out with *H. gammarus* larvae in the present study) at any point during the 24 h simulation. A schematic of the procedure used for computing impact zones is outlined in Scheme 1.

From the BarentsWatch database, we selected a sample of 23 Norwegian fish farms (referred to here as farms A-W) that carried out delousing bath treatments with azamethiphos or deltamethrin in the period 2017–2018 (BarentsWatch, 2019) (Fig. S1 of the Supporting Information). Particle tracking software LADiM (Myksvoll et al., 2018) was used to simulate the release and dispersal of both pesticides from each farm. Ocean current data, based on the NorFjords hydrodynamic model, was entered into the particle tracking software. The NorFjords model, an implementation of the Regional Ocean Model System (ROMS) (Shchepetkin and McWilliams, 2005; Haidvogel et al., 2008) has a resolution of 160 m × 160 m and 35 vertical levels, and includes recorded input data from atmosphere, tides and rivers (Isachsen, 2014; Storesund et al., 2017). The model is based on the NorKyst800 model (Albretsen et al., 2011) which has a horizontal resolution of 800 m × 800 m. The release sites span a wide geographical region, with latitudes in the range 59.5 °N - 70.5 °N (Fig. S1). Various types of hydrodynamic regimes are represented including sheltered locations with modest tidal currents (Farms A, R, V), larger fjords with more pronounced tidal activity (Farms D-H, J-M, Q, S, W), open-ended fjords with a dominant current direction (Farms B, C, I, O, T, U) and exposed locations that are highly influenced by the Norwegian coastal current (Farms N, P). Chemical plumes released in sheltered areas tend to disperse and move slowly. In exposed regions, turbulent currents dissolve released plumes quickly and disperse contaminants over a large area in a short time.

The released pesticide was represented by 100,000 particles, initially dispersed within a volume of 50 m × 50 m x 10m, which is roughly equal to the size of a typical large Norwegian fish cage (Fiskeridirektoratets og Mattilsynets anbefalinger, 2010). The particles were tracked for 24 h. A rectangular grid (100 m × 100 m in the horizontal direction, 1 m in the vertical direction) was constructed around the cage, and the *particle density* was calculated from the grid block volume and the number of particles within each block. From this we computed the maximal particle density in the vertical direction and stored the result on a 2D grid, for each time step. The data was combined into a dilution map, where each point represents the largest particle density encountered during the simulation (Fig. S2).

The BarentsWatch database does not specify the date or time of bath treatments, only the week in which the treatment was performed. Because of this, and to study the effect of varying weather and tide conditions on pesticide dispersal, we simulated a pesticide release at 00:00, 06:00, 12:00 and 18:00 for each day the treatment was performed for all 23 locations under consideration. This



Scheme 1. Schematic representation of the procedure for computing bath treatment impact zones.

resulted in a total of 28 releases per location, and therefore 28 dilution maps per farm. The releases were not cumulative; the location was assumed pristine before each release. In order to create maps of the impact zones around each fish farm, each of the dilution maps were then related to the EC_{50} and LC_{50} values for the combined data (i.e. the data from the two repeat assays combined) reported here. For instance, if the LC_{50} value of a pesticide corresponds to N % of the recommended treatment concentration, the LC_{50} impact zone for that drug is the portion of the dilution map that exceeds N % of the initial particle density. The impact zones for each farm were subsequently overlaid and the resulting maps show the proportion of releases that result in areas around the farms experiencing lethal or effective concentrations of the pesticides.

The impact zones vary in shape and size depending on the pesticide, location and time of release. In order to summarize the data, we computed radial and areal extent of the impact zone for each of the simulated releases. The radial extent is defined as the largest distance from the fish farm to the edge of the impact zone, while the areal extent is simply the area of the impact zone.

2.4. Statistical analyses

All statistical analyses were conducted in R Studio (3.4.3) (RStudio Team, 2016). LC_{50} and EC_{50} values, and their 95% confidence intervals (CI), for each pesticide were calculated using generalised linear models (GLM) within the *ecotox* R package (Hlinaet al., 2019), with binomial error structures and probit links according to Finney (1971). Pesticide concentrations were log transformed ($\log_{10}$) to linearise the data. Dose-response data were plotted using the *ggplot2* R package (Wickham, 2009). The dose-response curves for the repeated assays were compared statistically using ratio tests within the *ecotox* R package, as well as the confidence interval overlap method. Dispersal models were performed in Python and exposure areas were plotted using the package *holoviews* with the backend *matplotlib* (Hunter, 2007).

3. Results and discussion

3.1. Acute toxicity of azamethiphos and deltamethrin

A summary of the azamethiphos and deltamethrin LC_{50} and EC_{50} values, and their corresponding 95% CIs, for each of the repeated assays as well as the combined data are provided in Table S1 and Table S2. One hour exposures to azamethiphos were acutely toxic to both stage I and stage II *H. gammarus* larvae, with both mortality and immobility increasing in a dose dependent manner (Table S3). For both stage I and stage II larvae, there was a significant difference in the mortality dose-response curves for each of the azamethiphos assays performed and the associated lethal threshold concentrations (Fig. S3; Ratio Test, $p < 0.001$). For stage I and stage II larvae, the 1h-LC_{50} values for azamethiphos ranged from 23.8 to 75.7 μg L^{-1} and 8.5–75.7 μg L^{-1}, respectively. As the two assays were performed with larvae hatched from two different females, the differences in mortality levels between assays may be suggestive of differences in inherited tolerance. Interestingly, however, there was no significant difference in the total effect dose-response curves for the two repeated assays, as well as the estimated effective threshold concentrations (Ratio Test, $p > 0.05$), which suggests that the overall effect of azamethiphos between different populations may not be drastically dissimilar. When the data from the two assays were combined, the 1h-LC_{50} values (95% CIs) for stage I and II larvae were 43.1 μg L^{-1} (13.0–131.0 μg L^{-1}) and 20.5 μg L^{-1} (13.2–30.9 μg L^{-1}), respectively, representing approximately 2- and 5-fold dilutions of the treatment concentrations used on Norwegian fish farms (Fig. 1). Our results are in line with a recent study which found that azamethiphos (10–500 μg L^{-1}) induced significant mortalities in crab larvae (*Metacarcinus edwardsii*), following short term exposures (30-min) (Gebauer et al., 2017). In contrast, 1-h exposures to azamethiphos, at similar concentrations to those tested here, did not lead to a significant increase in mortalities amongst exposed *H. americanus* lobster larvae (stage I and III) and several shrimp species (*M. stenolepsis*, *C. septemspinosa*, *P. flexuosus, P. elegans)* (Ernst et al., 2014; Burridge et al., 2014). In addition, limited mortalities were observed amongst northern shrimp (*Pandalus borealis*) exposed to azamethiphos for a 2 h period, however, it should be noted that exposure concentrations in these studies were relatively low (100–200 ng L^{-1}) (Bechmann et al., 2020; Frantzen et al., 2019). It is interesting to observe here that stage II larvae were slightly more sensitive to azamethiphos exposure than stage I larvae. It has been hypothesised that stage-specific differences in crustacean sensitivity to pesticides is a result of differences in metabolism, moulting frequency, detoxification mechanisms and allometric differences (i.e., surface area to volume), with adult life stages often less sensitive than earlier life stages (Medina et al., 2002; Willis and Ling, 2004). Similar to our findings, however, higher sensitivity in later life stages has more recently been observed in several crustaceans including copepods (*Acartia hudsonica)* and krill (*Calanus* spp.) (Van Geest et al., 2014a; Escobar-Lux et al., 2019), though no plausible explanation has yet to be determined.

One hour exposures to deltamethrin were considerably more toxic than azamethiphos to both stage I and stage II *H. gammarus* larvae, with both mortality and immobility increasing in a dose-dependent manner (Fig. 1, Table S4). There was no significant difference in the lethal and total effect dose-response curves, as well as the estimated threshold concentrations for the two repeated deltamethrin assays (Ratio Test, $p > 0.05$; Fig. S4). For the combined data, the 1h-LC_{50} values (with 95% CIs) for stage I and II larvae were estimated to be 2.6 ng L^{-1} (0.6–11.0 ng L^{-1}) and 2.9 ng L^{-1} (1.5–5.7 ng L^{-1}), representing approximately 800-fold dilution of the treatment concentration. These results are consistent with those reported for stage I *H. americanus* lobster larvae (3.4 ng L^{-1}), though reduced sensitivity was also observed in adults (19 ng L^{-1}) and stage III larvae (36.5 ng L^{-1}) (Burridge et al., 2014; Fairchild et al., 2010). Lobster species appear to be more sensitive to deltamethrin compared to many other taxonomic groups, with higher 1h-LC_{50} values reported for shrimp (105.1–142 ng L^{-1}), *mysid* (13.9 ng L^{-1}), amphipod (13.1–70 ng L^{-1}) and crab larvae (1300 ng L^{-1}) (Burridge et al., 2014; Fairchild et al., 2010; Van Geest et al., 2014b; Parsons et al.). In two recent studies examining the toxicity of deltamethrin to *P. borealis*, high levels of mortality were observed amongst individuals exposed to low concentrations of deltamethrin (0.2–6 ng L^{-1}) for short time period (2 h), however LC_{50} values were not estimated, therefore, direct comparisons with these studies cannot be made (Bechmann et al., 2020; Frantzen et al., 2019). These results demonstrate that there are species-specific and life-stage specific differences in sensitivity to azamethiphos and deltamethrin amongst crustaceans. *H. gammarus* larvae appear to be one of the most sensitive crustacean species tested to date and therefore the present results should be included in any future ecological risk assessments investigating the risks of pesticides to the marine environment (Vaal et al., 2000).

Here, we reported EC_{50} values based on the combination of lethality and immobility, which previously has been shown to be a highly sensitive and potentially more environmentally relevant endpoint for assessing neurotoxic compound (Fairchild et al., 2010; Van Geest et al., 2014b). Indeed, we found that EC_{50} values for both azamethiphos and deltamethrin were substantially more sensitive than LC_{50} values based on mortality. The EC_{50} threshold values for azamethiphos were 15.5 μg L^{-1} (9.3–24.5 μg L^{-1}) and 9.2 μg L^{-1}

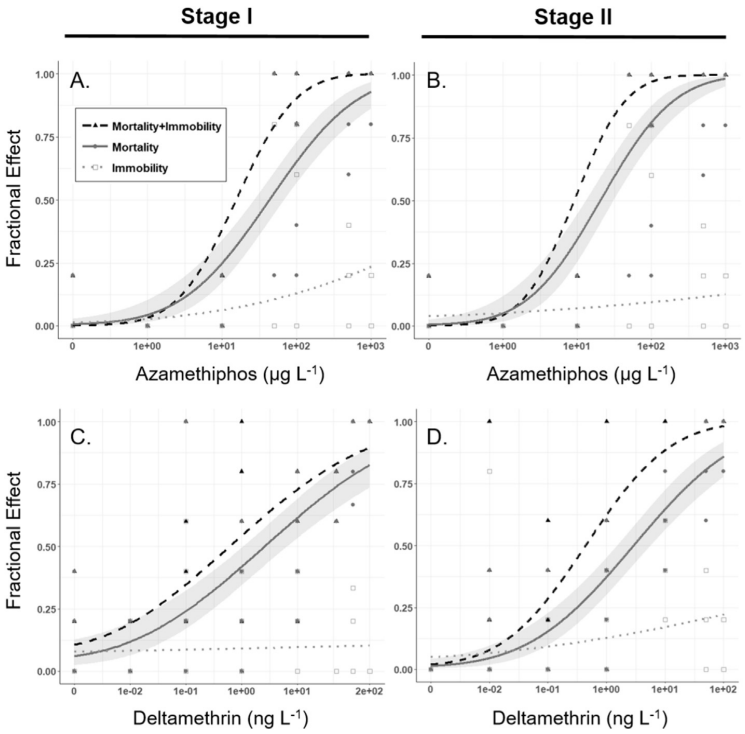


Fig. 1. The toxicity of azamethiphos and deltamethrin to stage I and stage II *H. gammarus* larvae following a 1 h exposure and a 24 h recovery period. Dose-response curves showing (▲) total effect (mortality + immobility, (●) mortality and (■) immobility amongst larvae exposed to nominal concentrations of (A–B) azamethiphos (1–1000 µg L^{-1}) and (C–D) deltamethrin (0.01–200 ng L^{-1}). Each point on the graphs represents an individual replicate glass dish containing 5 larvae and the lines represent the best fit model for the data, calculated using a binomial log-probit GLM in R (model output summarised in Tables S1 and S2).

(5.7–14.1 µg L^{-1}) for stage I and II larvae, respectively, which are 2.2- and 2.7-fold lower than the respective calculated LC_{50} values and approximately 10-fold lower than the recommended treatment concentrations. For deltamethrin, the 1h-EC_{50} values for stage I and II larvae were estimated to be 0.6 ng L^{-1} (0.2–2.1 ng L^{-1}) and 0.4 ng L^{-1} (0.2–1.1 ng L^{-1}), respectively, which are 4.3- and 7.3-fold lower than the respective calculated LC_{50} values and approximately 4000-fold lower than the recommended treatment concentrations. Given that immobile larvae are incapable of maintaining their position in the water column, unable to avoid predators and unable to feed, these larvae are considered to be ecologically dead (Van Geest et al., 2014b) and therefore the data presented here suggested that both azamethiphos and deltamethrin are considerably more toxic than previous published studies have suggested.

Given that the exposure period in this study was extremely short, the larvae were monitored both immediately after the 1 h exposure period and 24 h post exposure, allowing us to assess whether immobilised larvae could recover. In both azamethiphos and deltamethrin assays, larvae that were immobilised at 0 h post exposure typically did not recover by 24 h post exposure and consequently died (Tables S3 and S4). This lack of recovery and delayed mortality may be explained by the mode of action of the two toxicants. Azamethiphos covalently binds to AchE via phosphorylation and while the enzyme remains phosphorylated, its activity is inhibited. Consequently, ACh accumulates in cholinergic synapses, leading to unregulated excitation at neuromuscular junctions of skeletal muscle, preganglionic neurotransmitters and postganglionic nerve endings of the autonomic nervous system, and neurotransmitters in the brain or CNS. The phosphorylated AchE is typically very stable and may persist for days or weeks. The AchE activity is only slowly reactivated by spontaneous hydrolysis of the phosphate ester and recovery usually depends on new enzyme synthesis (Fulton and Key, 2001). Studies in fish, birds, mammals and invertebrates have shown a direct relationship between levels of AChE inhibition in the brain and subsequent mortality (Russom et al., 2014), which may explain the delayed mortality observed here at 24 h post exposure. While there are differences in sensitivity between species and life stage to various AChE inhibiting chemicals, it is evident that upon reaching a critical inhibition threshold, mortality is highly likely. Mortality may arise as a result of adverse physiological responses at the organ level such as altered respiratory activity, altered heart rates, altered blood pressure levels and seizures (Russom et al., 2014). Deltamethrin, on the other hand, inhibits the activity of voltage-gated sodium channels, resulting in depolarisation and prolonged permeability of the nerve to sodium. This consequently produces a series of repetitive nerve signals in sensory organs, sensory nerves, and muscles resulting in eventual paralysis (Soderlund, 2012). As a type II pyrethroid pesticide, deltamethrin contains an α-cyano group that induces long-lasting inhibition of the sodium channel activation gate which again likely explains the lack of recovery observed amongst lobster larvae in the present study.

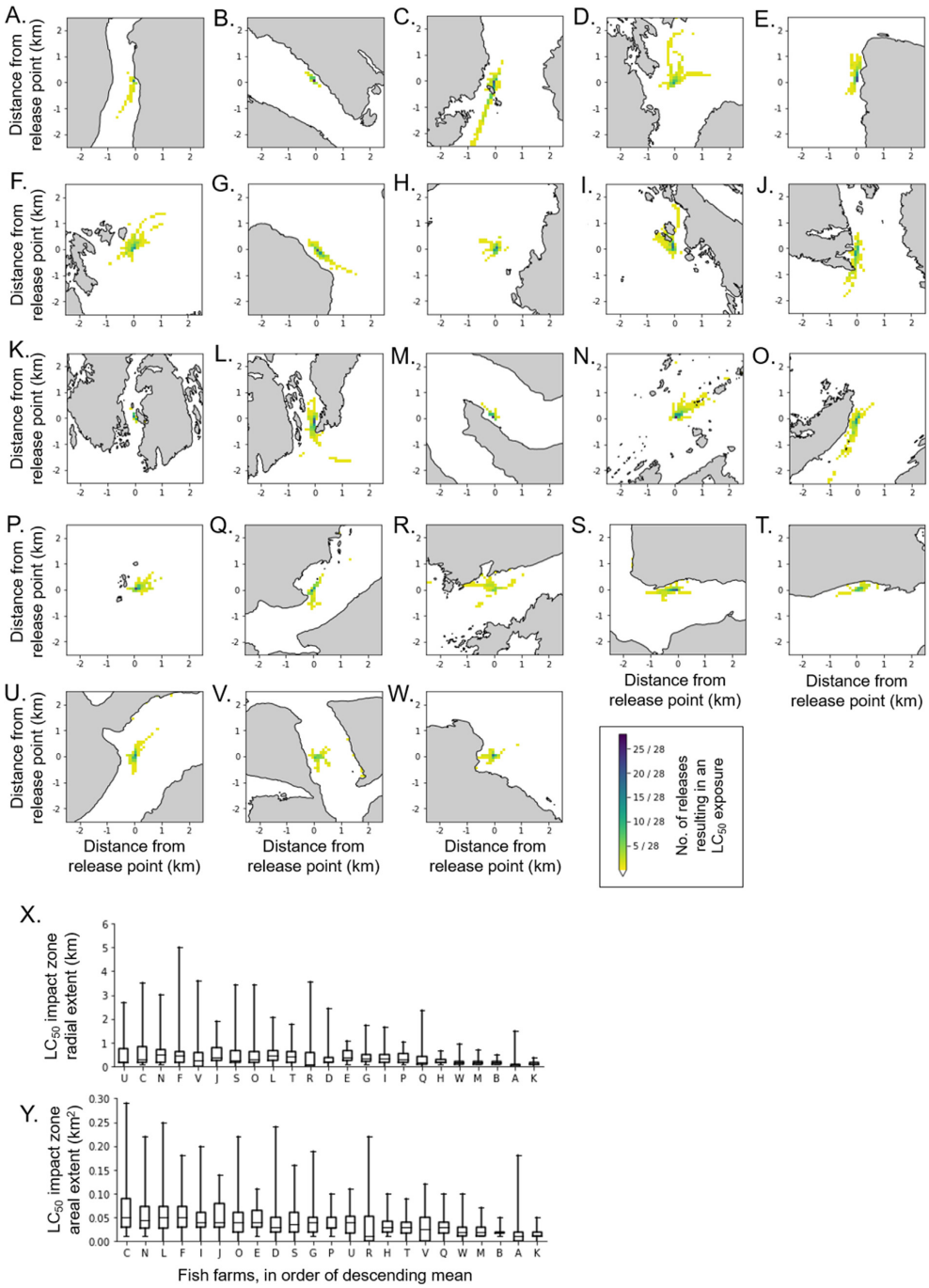


Fig. 2. Azamethiphos LC_{50} impact zones. (A–W) Maps illustrating the areas around 23 Norwegian fish farms which, based on multiple dispersal simulations (covering a range of tide and weather conditions), experienced lethal concentrations of azamethiphos (20 μg L^{-1}) in the 24 h after the simulated release of a bath treatment effluent from a standard size salmon pen which had been treated at the recommended dose of 100 μg L^{-1}. The colour legend displays the number of releases which have resulted in an area experiencing a lethal concentration of azamethiphos. The dark blue colour indicates an area which has experienced lethal concentration of azamethiphos in a high proportion of simulated releases (i.e.

Table 1
Summary of the total areal and radial extent of the azamethiphos and deltamethrin LC_{50} and EC_{50} impact zones for the Norwegian fish farms. Minimum, maximum and mean (±SD) values are shown.

Bath Treatment Pesticide	Lethal and Effective Threshold	Areal Extent of Impact Zones (km^2)			Radial Extent of Impact Zones (km)		
		Min	Max	Mean	Min	Max	Mean
Azamethiphos	LC_{50}	0.0	0.3	0.04 (±0.04)	0.0	5.0	0.04 (±0.04)
	EC_{50}	0.01	1.3	0.2 (±0.2)	0.1	18.5	1.3 (±1.4)
Deltamethrin	LC_{50}	0.1	87.9	21.1 (±13.9)	0.2	28.2	10.6 (±5.6)
	EC_{50}	0.1	144.2	39.0 (±26.5)	0.2	28.2	12.2 (±6.0)

Table 2
Summary of the areal and radial extent of the azamethiphos and deltamethrin LC_{50} impact zones around the selected fish farms. Minimum, maximum and mean (±SD) values are shown.

Farm	Azamethiphos						Deltamethrin					
	Areal extent (km^2)			Radial extent (km)			Areal extent (km^2)			Radial extent (km)		
	Min.	Max.	Mean	Min.	Max.	Mean	Min.	Max.	Mean	Min.	Max.	Mean
A	0.00	0.2	0.02 ± 0.03	0.0	1.5	0.1 ± 0.3	3.5	29.4	8.6 ± 6.2	3.2	23.9	7.9 ± 5.6
B	0.01	0.1	0.02 ± 0.01	0.1	0.5	0.2 ± 0.1	8.5	37.4	20.8 ± 7.5	7.6	20.1	14.1 ± 3.4
C	0.01	0.3	0.07 ± 0.07	0.1	3.5	0.7 ± 0.9	16.9	87.9	42.2 ± 15.0	7.9	28.2	19.9 ± 6.5
D	0.00	0.2	0.05 ± 0.06	0.0	2.5	0.5 ± 0.6	8.4	26.5	15.7 ± 4.9	3.6	13.0	8.0 ± 2.9
E	0.01	0.1	0.05 ± 0.03	0.1	1.1	0.5 ± 0.3	16.6	52.0	31.0 ± 7.3	7.1	17.6	11.7 ± 2.7
F	0.00	0.2	0.06 ± 0.04	0.0	5.0	0.6 ± 1.0	12.5	59.0	23.5 ± 11.1	4.7	18.0	10.0 ± 3.9
G	0.01	0.2	0.04 ± 0.03	0.1	1.7	0.4 ± 0.4	14.6	57.3	30.0 ± 10.7	6.2	25.2	12.9 ± 5.1
H	0.01	0.1	0.03 ± 0.02	0.1	0.7	0.2 ± 0.2	16.0	79.7	36.9 ± 15.3	3.9	20.7	10.5 ± 4.2
I	0.00	0.2	0.06 ± 0.05	0.0	1.7	0.4 ± 0.4	0.1	51.9	28.7 ± 10.9	0.2	23.2	14.6 ± 5.5
J	0.00	0.1	0.05 ± 0.04	0.0	1.9	0.6 ± 0.5	7.8	79.6	31.7 ± 17.4	4.7	23.1	15.0 ± 5.5
K	0.00	0.1	0.02 ± 0.01	0.0	0.4	0.1 ± 0.1	3.8	24.5	8.0 ± 5.0	3.4	15.6	5.9 ± 2.7
L	0.00	0.3	0.06 ± 0.05	0.0	2.1	0.5 ± 0.4	7.4	43.9	25.1 ± 10.9	5.2	22.0	11.2 ± 4.5
M	0.01	0.1	0.02 ± 0.01	0.1	0.7	0.2 ± 0.1	10.3	36.8	21.2 ± 7.7	4.9	20.2	12.2 ± 5.0
N	0.01	0.2	0.06 ± 0.05	0.1	3.0	0.7 ± 0.7	16.1	52.8	30.6 ± 10.9	5.4	20.5	12.0 ± 4.6
O	0.00	0.2	0.05 ± 0.05	0.0	3.4	0.6 ± 0.3	6.2	38.5	16.1 ± 7.4	3.9	22.2	9.3 ± 4.7
P	0.01	0.1	0.04 ± 0.02	0.1	1.1	0.4 ± 0.3	12.7	51.7	27.1 ± 10.3	3.2	21.8	13.6 ± 6.4
Q	0.00	0.1	0.03 ± 0.03	0.0	2.4	0.4 ± 0.5	4.0	28.0	14.4 ± 5.2	2.5	18.0	11.7 ± 3.7
R	0.00	0.2	0.03 ± 0.05	0.0	3.6	0.5 ± 0.9	2.4	20.4	6.4 ± 3.8	1.8	8.2	4.4 ± 1.7
S	0.00	0.2	0.05 ± 0.04	0.0	3.5	0.6 ± 0.8	6.3	56.4	22.6 ± 14.9	3.9	24.3	12.2 ± 5.4
T	0.00	0.1	0.03 ± 0.02	0.0	1.8	0.5 ± 0.5	0.5	21.3	9.3 ± 5.7	1.6	10.7	6.5 ± 2.8
U	0.00	0.1	0.04 ± 0.03	0.0	2.7	0.7 ± 1.0	7.1	36.1	19.0 ± 7.0	3.2	18.4	10.0 ± 4.2
V	0.00	0.1	0.03 ± 0.03	0.0	3.6	0.6 ± 0.9	1.4	7.0	4.1 ± 1.7	1.5	6.7	4.6 ± 1.7
W	0.00	0.1	0.03 ± 0.02	0.0	1.0	0.2 ± 0.2	2.9	33.6	13.2 ± 7.2	2.3	14.6	6.8 ± 2.9

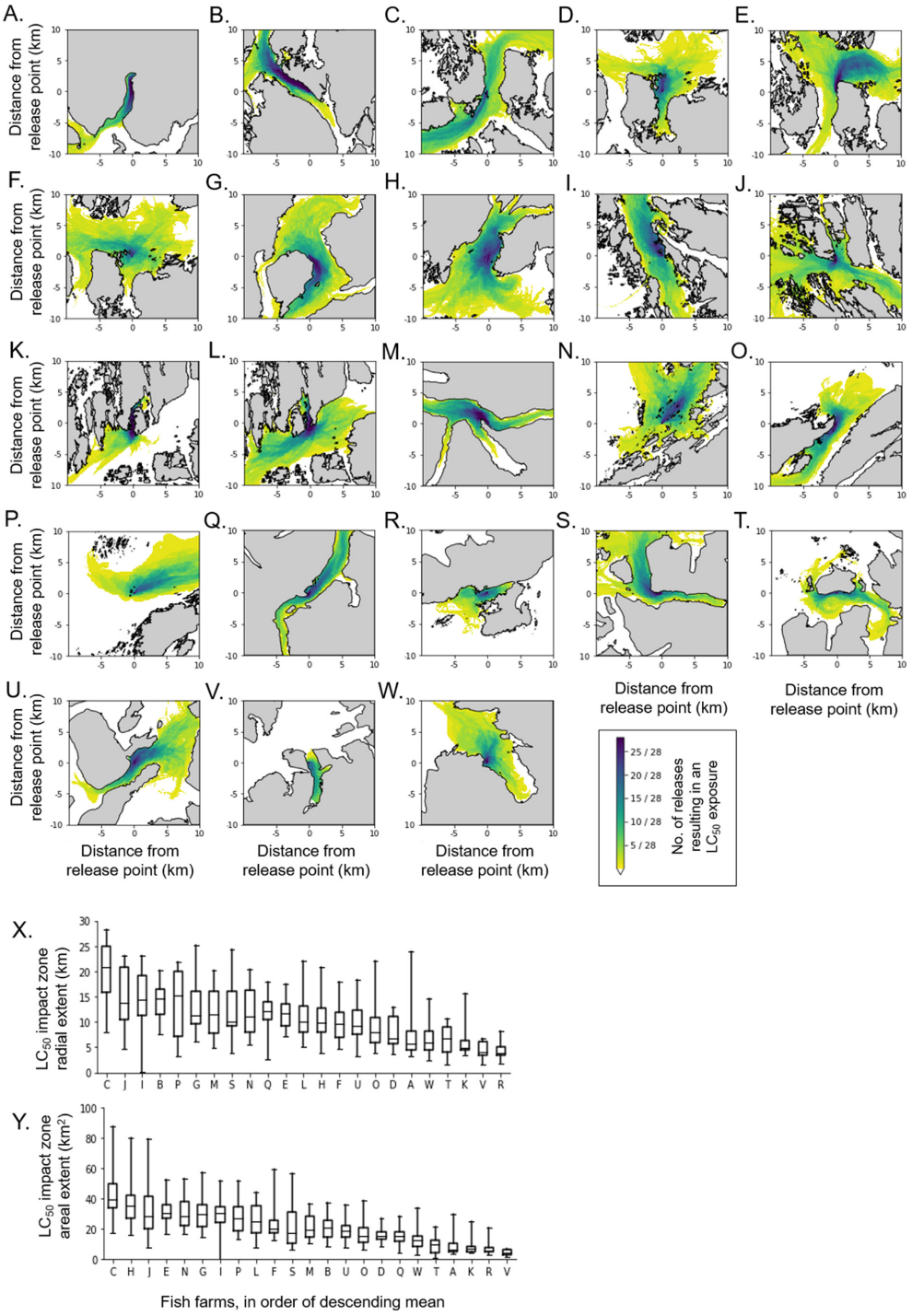
It is important to note that water chemistry was beyond the scope of the current study and therefore dose-response curves and the associated lethal and effective threshold concentrations were generated based on nominal concentrations and not measured concentrations. Previous studies, however, recovered and measured azamethiphos at concentrations consistent with the nominal concentrations (Burridge et al., 2014; Bechmann et al., 2020), which would suggest that the lobster larvae here were exposed to concentrations similar to the nominal concentrations. In contrast, in several studies deltamethrin was either not detected, below the limit of detection or measured at much lower concentrations than the nominal concentrations (Ernst et al., 2014; Burridge et al., 2014; Bechmann et al., 2020). Given that the larvae were severely affected after the 1 h exposure period in the present study, this suggests that deltamethrin was in fact present in the treatment water, however it should be considered that the threshold concentrations estimated here may underestimate the toxicity of deltamethrin.

3.2. Azamethiphos and deltamethrin impact zones

When all farms were considered together, the areas at risk of exposure to lethal concentrations of azamethiphos (corresponding to a dilution limit of 20%) were relatively small (Fig. 2, Table 1). For example, the mean (±SD) areal and radial extent of the azamethiphos LC_{50} impact zones were 0.04 (±0.04) km^2 and 0.4 (±0.6) km, respectively. The areas at risk of exposure to effective azamethiphos concentrations (corresponding to a dilution limit of 10%) were only slightly larger (Fig. S5, Table 1), with the mean (±SD) areal and radial extent of the EC_{50} impact zones calculated to be 0.2 (±0.2) km^2 and 1.3 (±1.4) km, respectively. A summary of the areal and radial extent of the lethal and effective azamethiphos impact zones for each farm is presented in Table 2 and Table S5, respectively.

While field measurements were beyond the scope of this study, previous studies have also shown the dispersal of azamethiphos from fish farms to be limited (Ernst et al., 2014; Langford et al., 2015). Very low concentrations of azamethiphos (26 ng L^{-1}), well below the lethal concentrations reported here for *H. gammarus*, were measured at the edge of a Norwegian fish farm 1 week following a bath treatment procedure and concentrations were reported to decrease with increasing distance from the farm (0.5 ng L^{-1} at 1000 m). It should be noted, however, that the long period between treatment and sampling may explain the low concentrations observed (Langford et al., 2015). The dispersal of azamethiphos from a Canadian fish farm was also limited in the 2–3 h after bath treatment releases. While relatively high

under various environmental conditions) whereas the yellow colour indicate areas which have experienced lethal concentrations of azamethiphos in a low proportion of simulated releases (i.e. under only very specific weather conditions). (X–Y) Boxplots showing the variation in the extent (areal and radial) of the LC_{50} impact zones at each farm (28 simulated releases were performed per farm). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)


A.
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T.
U.
V.
W.
Distance from release point (km)
25 / 28
20 / 28
15 / 28
10 / 28
5 / 28
No. of releases resulting in an LC50 exposure
X.
LC50 impact zone radial extent (km)
C J I B P G M S N Q E L H F U O D A W T K V R
Y.
LC50 impact zone areal extent (km2)
C H J E N G I P L F S M B U O D Q W T A K R V
Fish farms, in order of descending mean

concentrations of azamethiphos (25 μg L^{-1}), similar to the lethal concentrations observed in the present study, were measured in water sampled very close (within 1 m) to the edge of the pen, concentrations decreased significantly with increasing distance from the farm (approx. 1 μg L^{-1} was detected 1000 m from the cage) (Ernst et al., 2014). While our results are generally in line with these field based studies, in that areas likely to be exposed to high concentrations of azamethiphos appear to be small, the field studies may underestimate the size of the impacted areas. For instance, our results show that, on average, lethal concentrations of azamethiphos dispersed 400 m from a fish farm, in comparison to the field studies in which similar concentrations were only detected 1 m from a farm. This discrepancy is likely a result of the fact that concentrations measured don't necessarily reflect the maximum concentration that might occur in any given area. Water samples are taken at a single point in time and location, and any slight deviations from these may lead to a very different measurement. The discrepancy could also be a result of differences in the topography, geography, geology and ocean currents between Norwegian and Canadian farm sites. Indeed, we also found that the extent (both areal and radial) of the zones varied greatly between the Norwegian farms selected for this study. For example, the mean radial extent of the azamethiphos LC_{50} and EC_{50} impact zones varied between 0.1-0.7 km and 0.4–2.9 km across the selected farms, respectively. In addition, the extent of the impact zones varied substantially within the selected farms. For example, on Farm C the radial extent of the azamethiphos LC_{50} and EC_{50} impact zone varied between 0.1-3.5 km and 0.3–18.5 km, respectively (Table 1). This between-farm and in-farm variation in the extent of the impact zones suggests that the degree to which azamethiphos will negatively affect non-target species in the wild is likely to vary substantially between geographical regions and under different environmental conditions (e.g. ocean currents and weather).

Compared to azamethiphos, the deltamethrin LC_{50} impact zones (corresponding to a dilution limit of 0.1%) were extensive (Fig. 3, Table 1). When all farms were considered together, the mean (±SD) areal and radial extent of the deltamethrin LC_{50} impact zones were 21.1 (±13.9) km^2 and 10.6 (±5.6) km, respectively. Our results also show that even larger areas are at risk of exposure to effective concentrations of deltamethrin (Fig. S6, Table 1), with the mean (±SD) areal and radial extent of the EC_{50} impact zones (corresponding to a dilution limit of 0.02%) reaching 39.0 (±26.5) km^2 and 12.2 (±6.0) km, respectively. The areal and radial extent of the lethal and effective deltamethrin impact zones for each farm is presented in Table 2 and Table S5, respectively. Earlier field measurement studies from Canada have suggested that low levels of deltamethrin may disperse into large areas around fish farms, however our results suggest that the deltamethrin impact zones could be far larger than previously predicted. For example, low concentrations of deltamethrin (approx. 1 ng L^{-1}), similar to the lethal and effective concentrations observed here, were measured in water sampled 1000 m from a Canadian fish farm after bath treatment release (Ernst et al., 2014). Our results on the other hand indicate that these low levels of deltamethrin could disperse to a distance approximately 10x greater than that sampled in the Canadian study. Since the extent of the deltamethrin impact zones, like the azamethiphos impact zones, varied considerably both between and within farm sites, the impact on non-target species will depend on the specific geographical region and the weather conditions occurring at the time of treatment.

It is important to discuss our findings in relation to the underlying assumptions and limitations of the hydrodynamic model. It should be stressed that several of the assumptions assigned to the model could result in worst case scenario impact zones. For example, the model assumes that deltamethrin remains in the water and does not adsorb to organic matter for 24 h post release. Deltamethrin, however, has a high Log K_{ow} value (5.43) and therefore is likely to partition to the particulate phase soon after bath treatment releases (International Programme on Chemical Safety & World Health Organization, 1990; Muir et al., 1985). Particle-bound deltamethrin has a greater tendency to sequester to sediments and therefore some of the pesticide would adhere to the seafloor rather than transported over large distances. Consequently, the current model may overestimate the dispersal of deltamethrin and the extent of the potential impact zones. In addition, the model does not consider the length of time of pesticide exposure, but simply maps the areas around the farms that experience lethal/effective concentrations of the pesticides at any point during the 24 h simulation. The threshold concentrations selected here were based on a 1 h toxicity test but some areas around farms may experience these concentrations for much shorter periods of time. If this is the case, the impacts on non-target species in these areas may be less than the model predicts.

While the previous assumptions may result in the overestimation of the extent of the impact zones, other assumptions of the model may lead to an underestimation of their extent. For example, we have assumed the ocean to be pristine after each release, therefore there is no residual levels of the compound left in the water by the time the next release occurs. In reality, delousing operations with azamethiphos and deltamethrin can involve the concurrent and sequential applications of many pens within a single fjord. These treatment methods may result in cumulative loading of the pesticides and subsequently higher concentrations and larger impact zones around the farms. Future studies are necessary to further advance the model described here by incorporating absorption coefficients and allowing for multiple bath treatment releases, which would better reflect dispersal situations in the Norwegian marine environment. Finally, future work that robustly models the interactions between lobster larvae and contaminated plumes of water that are released from aquaculture sites would greatly increase our understanding of the impact of bath treatment pesticides on wild lobster populations. The hydrodynamic model described in this paper does, however, provides a first order estimate of the dispersal of both azamethiphos and deltamethrin into the Norwegian marine environment and their potential risk to wild lobster larvae after bath treatment releases from fish farms. Our results clearly demonstrate that large areas around aquaculture facilities are exposed to lethal and effective concentrations of deltamethrin following anti-sea lice treatments, and therefore this compound is likely to have widespread adverse effects on sensitive non-target crustacean species living in areas close to farms delousing with this compound. It is important to highlight, however, that the consumption of deltamethrin on Norwegian fish farms has reduced dramatically in recent years, with

Fig. 3. Deltamethrin LC_{50} impact zones. (A–W) Maps illustrating the areas around 23 Norwegian fish farms which, based on multiple dispersal simulations (covering a range of tide and weather conditions), experienced lethal concentrations of deltamethrin (2 ng L^{-1}) in the 24 h after the simulated release of a bath treatment effluent from a standard size salmon pen which had been treated at the recommended dose of 2 μg L^{-1}. The colour legend displays the number of releases which have resulted in an area experiencing a lethal concentration of deltamethrin. The dark blue colour indicates an area which has experienced lethal concentration of deltamethrin in a high proportion of simulated releases (i.e. under various environmental conditions) whereas the yellow colour indicate areas which have experienced lethal concentrations of deltamethrin in a low proportion of simulated releases (i.e. under only very specific weather conditions). (X–Y) Boxplots showing the variation in the extent (areal and radial) of the LC_{50} impact zones at each farm (28 simulated releases were performed per farm). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

only 10 kg of the active substance used in 2019 (Folkehelseinstituttet, 2019). Therefore, the use of deltamethrin may have population level effects on *H. gammarus* but only in very specific regions where the consumption is highest. In contrast to deltamethrin, the areas exposed to lethal and effective concentrations of azamethiphos are relatively small and therefore the impact of this compound will likely be less severe. These findings should be considered by legislators both in Norway and in other salmonid aquaculture regions around the world when carrying out future environmental risk assessments of these compounds and in assessing the potential risks associated with the expansion of aquaculture into new sites and increasing production at existing sites.

4. Conclusion

It is clear from the present study that deltamethrin is extremely toxic to *H. gammarus* larvae, in line with various other studies on non-target marine crustaceans. For the first time, we have demonstrated that azamethiphos is also acutely toxic to *H. gammarus* larvae following short 1 h exposures. The hydrodynamic model described in this paper assesses the dispersal of both azamethiphos and deltamethrin into the Norwegian marine environment and their potential risk to wild lobster larvae after bath treatment releases from fish farms. Our results clearly demonstrate that large areas around aquaculture facilities are exposed to lethal and effective concentrations of deltamethrin following anti-sea lice treatments, and therefore this compound is likely to have widespread adverse effects on sensitive non-target crustacean species living in these areas. On the other hand, the areas exposed to lethal and effective concentrations of azamethiphos are relatively small in comparison and therefore the impact of this compound is likely to be less severe.

Author contribution

The project was conceived and designed by all authors. AP, OS, REL, ALA carried out the exposure studies and AP performed data analysis. PNS performed mathematical modelling analyses. AP and PNS wrote the manuscript, with input from OS, REL and ALA.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.114725.

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