Chemotherapeutants against salmon lice *Lepeophtheirus salmonis* – screening of efficacy

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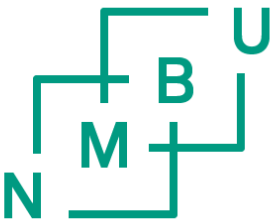
Thesis for the degree of Philosophiae Doctor



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ACRONYMS/TERMINOLOGY

AZA	Azamethiphos
$EC_{50}/_{90}$	Medicine concentration immobilizing 50/90 % of parasites in experiments
EMB	Emamectin benzoate
GABA	Gamma amino butyric acid
H_2O_2	Hydrogen peroxide
Instar	Developmental stage of an invertebrate
IPM	Integrated pest management
IRAC	Insecticide Resistance Committee
Ls	*Lepeophtheirus salmonis*, Latin name of the salmon louse
nAChR	Nicotinic/neuronal acetylcholine receptor
mg/L, mg L^{-1}	Milligrams per litre
ppm	Parts per million (equivalent with mg/L)
Resistance	Genetically based decrease in susceptibility to a pesticide
SNP	Single nucleotide polymorphism; variation of nucleotide at a specific position in the genome

LIST OF PAPERS

I Impact of hydrogen peroxide on hatching ability of egg strings from salmon lice (*Lepeophtheirus salmonis*) in a field treatment and in a laboratory study with ascending concentrations

II First report of reduced sensitivity towards hydrogen peroxide found in the salmon louse *Lepeophtheirus salmonis* in Norway

III A screening of medicinal compounds for their effect on egg strings and nauplii of the salmon louse *Lepeophtheirus salmonis* (Krøyer)

IV A screening of multiple classes of pharmaceutical compounds for effect on preadult salmon lice *Lepeophtheirus salmonis*

SUMMARY

Sea lice constitute a continuous challenge for fish farmers in all salmonid production areas. The salmon louse, *Lepeophtheirus salmonis*, is the most abundant of these in the Northern hemisphere. Through millions of years the parasite has adapted to low host densities resulting in a vast reproductive potential. This feature is not favourable considering the fish densities in modern fish farming, as infective larvae spread rapidly to new hosts. Increased sea lice levels are considered to impact populations of wild salmon in Norway and other countries most negatively. Sea lice on farmed fish may be combatted through the use of several methods, which are subject to constant development. Treatment with medicinal compounds has prevailed due to their previous predictable efficacy, however overuse has resulted in widespread problems with reduced sensitivity and resistance in sea lice populations. This has led to an urgent need for novel medicinal products.

In the work included in the current thesis, several life cycle stages of the parasite were exposed to a broad selection of antiparasitics. Initial screening was performed using exposure periods reflecting field treatment procedures, imitating bath (short-term) and oral (long-term) administration respectively. High concentrations were chosen to ensure identification of effective compounds. The effect on egg strings was minimal for most medicinal products except hydrogen peroxide. Assays on nauplii revealed several effective compounds, some immobilizing the parasite immediately, and some inhibiting the development to later stages. The effect on pre-adult stages has revealed substances possessing commercial potential, and suggesting differential expression between instars of key genes coding for the target proteins. One group of substances, the neonicotinoids, was investigated thoroughly for differential effects between sister compounds. Reduced sensitivity towards a commercially available compound was identified for the first time in Norway. The results indicate that compounds not yet commercially available may have good effect on several salmon louse instars at concentrations not acutely toxic to Atlantic salmon.

SAMMENDRAG

Ektoparasitter, i hovedsak lakselus, utgjør en stor utfordring i lakseoppdrett på både den nordlige og sørlige halvkule. På den nordlige halvkule er *Lepeophtheirus salmonis* den mest utbredte av disse. Parasittene har gjennom millioner av år adaptert til å overleve i populasjoner med meget lav vertstetthet, noe som har resultert i et svært høyt reproduksjonspotensial. Denne egenskapen er uheldig tatt i betraktning den høye vertstettheten i dagens lakseoppdrett der forholdene ligger til rette for at infektive larver raskt kan finne nye verter og reprodusere. Det er dokumentert at økede

lakselusnivåer har en negativ innflytelse på ville laksepopulasjoner i Norge og andre nasjoner. Fjerning av lakselus fra oppdrettsfisk kan gjøres på mange måter, og det avsettes årlig store beløp for å utvikle nye og effektive metoder. Frem til nå har behandling med medisinske preparater vært dominerende på grunn av deres forutsigbart gode effekt, men resistensproblemer har fått fotfeste i lakseluspopulasjoner i alle oppdrettsnasjoner. Dette har ført til et skrikende behov for nye medisinprodukter som kan ta unna gjenblivende resistente lus.

I dette prosjektet ble lakselus i flere parasittstadier eksponert for en serie medisinske substanser. Eksponeringstid ble tilnærmet anvendt behandlingstid i felt, relatert til badebehandling og langtidsbehandling med fôr. Utgangskonsentrasjon ble satt høyt for å få sikker treffeffekt. Svært få substanser affiserte klekking, med unntak av hydrogenperoksid. Forsøk med naupliusstadier ga flere treff, der noen substanser tok livet av naupliene umiddelbart, og noen andre forhindret skallskifte og dermed førte til immobilisering ved et senere tidspunkt. Flere substanser med effekt mot preadulte lakselus ble identifisert, et faktum som tyder på at ekspresjonsnivået for gener ansvarlige for målproteiner varierer mellom stadier. Noen av substansene med effekt mot lakselus har potensiale til å utvikles til kommersielle produkter til bruk lakseoppdrett. En gruppe substanser, neonikotinoider, ble undersøkt i dybden for differensiell effekt mellom søstersubstanser. Nedsatt følsomhet for hydrogenperoksid ble avdekket for første gang i norske oppdrettsanlegg. Utførte forsøk gir håp om å kunne utvikle et produkt som har relativt god effekt på alle lakselusstadier i en dose som ikke er akutt toksisk for laks.

Everything is theoretically impossible, until it is done. (Robert A. Heinlein)

1 INTRODUCTION

1.1 Salmon farming in an international perspective; industrial challenges

In 2014, an approximate 160 million tonnes of fish were farmed and harvested from fisheries globally. It is assumed that farmed fish, of several species, make up 40-45 % of this volume (www.fao.org/3/a-i3720e.pdf, accessed on April 7th 2016). In 1985, the total number was 80 million tonnes, of which farmed fish constituted approximately 7-8 %. Fish farming is thus an extremely rapidly growing industry. Norwegian fish farming was initiated in the late 1960s, as a governmental incentive for economic growth along the coast (Liu Y et al. 2011). However, fish farming has existed for hundreds of years in other parts of the world, especially with long traditions in Asian countries (http://voices.nationalgeographic.com/2013/07/11/sustainable-ancient-aquaculture/, accessed on April 21st 2016). Salmonid farming has proven to be an effective method of producing attractive fish protein, and this industry comprises the largest aquaculture production in Western Europe and South America at present. The main species is the Atlantic salmon, *Salmo salar*, with the primary producing countries being Norway, Chile, Faroe Islands, UK, Ireland, and Canada. Other related farmed species are rainbow trout (*Oncorhyncus mykiss*), and to some degree chinook salmon (*Oncorhyncus tshawytscha*) and coho salmon (*Oncorhyncus kisutsch*) (www.aquaculture.co/files/species-salmon.php, accessed on Jan 28th 2016). Salmon farming is highly industrialized, with technology related to breeding, equipment, and co-habitant species being a wide field of investment both economically and intellectually. A salmon farm can house up to 2 million individuals (maximum ~10.000 tonnes) during the seawater production phase of around 12-24 months. However, the standing biomass of livestock varies considerably within this range, and is more or less regulated in all salmon farming countries. In 2012, 1.3 million tonnes of salmonids were produced in Norway, constituting for 2 % of the total aquaculture production of fish (http://ssb.no/jord-skog-jakt-og-fiskeri/statistikker/fiskeoppdrett/aar-forelopige/2015-06-02, accessed on April 12th 2016, http://www.fao.org/docrep/016/i2727e/i2727e.pdf, accessed on April 13th 2016). Chile, the second largest producing nation, produced around 820.000 tonnes in the same year (http://www.salmonchile.cl/en/produccion.php, accessed on April 4th 2016). The production cycle lasts up to 3 years; where the first year following hatching is spent on land in enclosed, freshwater units. After smoltification, a physiological process preparing the fish for seawater conditions, the fish are transferred to seawater sites.

As fish farming has grown from nothing to a multi-billion industry in a span of 50 years, biological and technological challenges are inevitable. Among the greatest problems, for economic, fish welfare, and environmental reasons, are sea lice. Estimates made by Costello (2009) suggest a worldwide cost of

sea lice control at around US$ 480 million in 2009. This figure includes both surveillance and medical treatments, making sea lice the most costly parasite in salmon production. Along with the technological progress in fish farming, the true economic cost of sea lice is getting harder to estimate. Nofima (Norwegian Food, Fisheries and Aquaculture Research Institute) reported an annual figure of 0.60 NOK/kg related to sea lice medicines only in a report published in 2015 (Iversen et al. 2015). Additionally, other expenses such as net cleaning, cleaner fish, mechanical de-lousing, louse laser, plankton shielding, well boat hire, crew costs, surveillance, and lice counts represent a massive cost related to sea lice for fish farmers and government. One can safely assume that Costello's estimate will be exceeded by far in 2016. Other pathogenic agents are also affecting the salmon industry negatively. Viral diseases, such as pancreas disease, infectious salmon anaemia, infectious pancreas necrosis, heart and skeletal muscle inflammation, are difficult to combat and often lead to forced slaughter. The parasite *Paramoeba perurans* is another, quite new pathogen in European salmon farming, whereas the bacterial diseases winter ulcer, furunculosis, vibriosis, and piscirickettsiosis from time to time cause outbreaks, with the latter being predominant in Chile. Accumulated mortality during the seawater phase in Norwegian salmonid farming is reported to be between 10-20 % (Mattilsynet 2011), a very high number under any circumstances.

Farmed salmonids bred with genetic material from wild salmon strains co-exist with the wild salmon in many production areas. This interaction has led to several conflicts between the two populations, with undesired effects on wild salmon stocks occurring when genetic material from escaped farmed salmon are incorporated in the wild salmon genes (McGinnity et al. 2003, Hindar et al. 2006). Sea lice originating from salmon farms are thought to impact wild salmon stocks, but the actual effect is hard to define (Nilsen et al. 2016, Torrissen et al. 2013).

1.2 Salmon lice

1.2.1 History and geographic distribution

"Sea lice" is used for all crustaceans parasitizing fish. Salmon lice is used for *L. salmonis* and sometimes also for *Caligus rogercresseyi*, although in this text for *L. salmonis* only. Lots of species-specific fish lice exist, such as the salmon louse, cod louse (*Caligus curtus*), halibut lice (*Entobdella hippoglossus*), and yellowtail lice (*Caligus spinosus*). Of all parasitic copepod species, only 55 % are restricted to one host, however the mean number of hosts for the remainder is 2.8 (Boxshall, pers. comm.). The salmon louse is thus a moderate parasite regarding host specificity, as it colonizes several salmonid species, in addition to the stickleback (Jones et al. 2006).

L. salmonis, a marine ectoparasitic copepod, was first described by the Danish zoologist Henrik Nikolai Krøyer in 1837. It belongs to the subclass Copepoda which contained around 11.500 species in 2005 (Boxshall 2005), of which some are planktonic, some benthic, and some parasitic. Copepods are thought to have existed for over 100 million years (Boxshall 2005). The parasites reside on fish living in seawater, but may also tolerate shorter periods in brackish water when the hosts migrate to such areas. Salmon has traditionally been a most precious protein source for human beings, captured in fjords, estuaries, and rivers, with social communities being founded on the basis of this resource. In the medieval ages, innovative institutions, such as monasteries, were built near attractive salmon fishing riversides. Before the salmon farming era, observations of sea lice on captured salmonids were considered a sign of quality, as they would indicate a recent arrival to the river from the sea. Since the very beginning in the 1970s, sea lice have been observed in salmon farms (Rae 2002). The impact of the salmon louse on wild salmonid populations has been subject to intense observation, especially over the past few years. Medicinal compounds were the first choice when combatting sea lice on farmed salmonids (Brandal & Egidius 1979). Several medicinal compounds have had periods of intensive use, each seen as the industry's saviour, but decreasing efficacy has become a result of inadequate administration.

Due to preventive measures and strict regulations regarding the maximum allowed number of parasites per fish (https://www.lovdata.no/for/sf/fi/ti-20121205-1140-0.html, accessed on April 12th 2016), wounds and scale loss caused by sea lice are relatively rare in farmed fish nowadays. However, exceptions exist, primarily when multiple pathogens are involved. In general, salmon lice infestations are the most severe, and potentially fatal, for wild salmonid smolts residing in or migrating through waters where the level of infective larvae is high (Tully & Whelan 1993, Birkeland 1996, Costello 2014, and reviewed by Torrissen et al. 2013). Therefore, the salmon louse is considered a major obstacle to a sustainable salmon production, engaging a significant number of employees, both at site and in corporate functions, social communities, and infrastructure. And, although being present in relatively low numbers, sea lice is most likely an unpleasant experience for their hosts. Several species of sea lice parasitize salmonids around the globe. This is also the case in Chile, where their "salmon louse", *C. rogercresseyi*, is causing problems similar to those seen in areas where *L. salmonis* is dominant (Bravo et al. 2013). In the North-Atlantic Ocean, *Caligus elongatus* may from time to time also parasitize farmed salmonids (Wootten et al. 1982). As a parasite colonizing wild fish over thousands of years, the salmon lice genome is constructed of features specialized for this purpose. However, within a short period, several novel traits are developed in order to facilitate survival in the current situation. Higher host density, chemotherapeutants, increasing water temperatures, and tough physical conditions are among the possible factors impacting survival, and preferences for surviving individuals. The current

genome composition is a perfect example of microevolution in the making, representing genomic variations in large scale during a short time frame.

1.2.2 Salmon lice life cycle

The life cycle of the salmon louse consists of initial pelagic stages followed by parasitical stages. Originally, 10 developmental stages were described for *Lepeophtheirus* species (Boxshall 1974). Recent evidence however has shown that *Lepeophtheirus salmonis* has only eight stages in total (Figure 1), separated by the process of shedding the exoskeleton (moulting) (Hamre et al. 2013). Nauplius I, nauplius II, and the infective copepodid are free-living, whereas chalimus I and II, preadult I and II, and adults, are parasitic. The speed of development is dependent on the water temperature, and the period from nauplius I to adult male takes 40 days at 10 °C (Pike & Wadsworth 1999). Adult males and females copulate while situated on the fish. Embryos are successively organized into the egg string while this is folded within the female. The proximal section of the egg string does not contain embryos. When the egg string is completed, it is unfolded externally and attached to the female by a hook (Heuch et al. 2000). The egg strings are thus independent from their origin, and undergo a maturing process either while still attached, or while released. As they mature, the egg strings obtain a darker colour through pigment development in embryos. The embryos within one egg string develop synchronically. The egg string membrane ruptures and larvae are subsequently released, as the whole hatching process is completed within a few hours. Little is published regarding the ultrastructure, molecular composition and permeability of the egg string membrane.

The nauplii and copepodids (0.5-0.7 mm) (Schram 1993) are free floating in the water column, spending innate energy while developing (Johnson & Albright 1991). Nauplii are hovering more or less continuously, while the copepodid has the ability of purposeful movement. Both nauplii and copepodids possess phototactic abilities (Heuch et al. 1995), which in combination with mechanical stimuli such as specific wavelengths caused by salmonids (Heuch & Karlsen 1997), gives the copepodid the ability to locate a host when one passes. When a host is within reach, the copepodid initially grabs it with its clawed antennae (Bron et al. 1993a). Once attached, physiological properties of the salmon mucus stimulate the copepodid to make a choice of whether to remain or to seek another host. Devine et al. (2000) showed that specific cues emitted by fish triggered behavioural responses in adult salmon lice. *L. salmonis* may thus attach to other species for shorter periods, however detach when the desired physical features are not found. When deciding to stay with the host, the copepodid develops a protein filament while moulting to chalimus I (reviewed by Boxaspen 2006). Immune responses are activated

by the host (Skugor et al. 2008, Braden et al. 2012), however, the salmon lice are capable of overcoming these. Atlantic salmon and rainbow trout are among the least resistant salmonid species compared to pacific salmon species, with rainbow trout being the more resistant of the two (Johnson & Albright 1992, Fast et al. 2002).

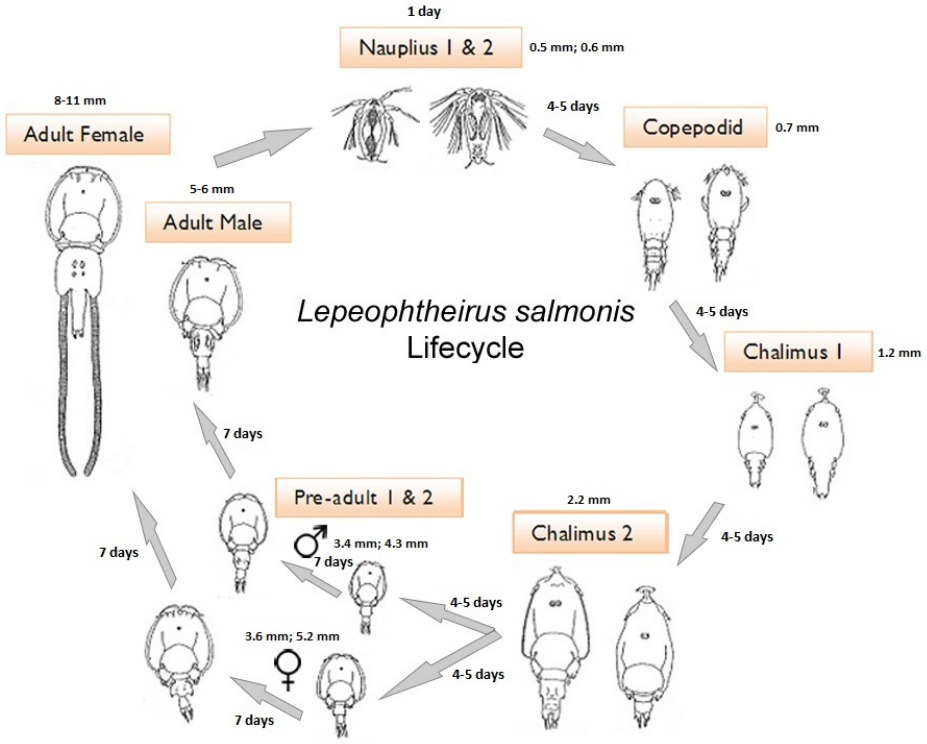

Figure 1: The life cycle of *L. salmonis*. From Schram (1993) and modified by the Marine Institute of Galway, Ireland.

Chalimus larvae are randomly distributed on the host (Tucker et al. 2000) and feed on skin and mucus. Chalimus I instars moult to chalimus II (0.8-2.8 mm, Hamre et al. 2013) and finally to pre-adult I as the protein filament is usually lost. The protein filament seems to be re-constructed within the moult from chalimus I to chalimus II (Gonzalez-Alanis et al. 2001). However, some preadults seem to remain attached for a while when the filament is not sufficiently decomposed. Compared to the *Caligus* species this is unique, as these species keep this filament through the four chalimus stages before moulting directly to adults. Preadult and adult stages are mobile and seek the best location on the host. Most mobile salmon lice have preference for the dorsal side of the fish (own observation, Pike et al. 1993) on the frontal region and behind the adiposal fin, and also the area around the anus and on the head in general are popular places for these instars. The parasites grow considerably during the last three stages, the female being the largest in every stage, with size ranging from 3.4 mm to 11 mm

(Schram 1993). During copulation, a spermatophore is transferred from a male to a female (Ritchie et al. 1996, Hull et al. 1998, Heuch et al. 2000). From one single mating, the female develops a number of successive egg strings, with up to 11 pairs being observed in laboratory experiments (Heuch et al. 2000). Egg strings occur as either singles or pairs, the latter being the most frequent, where one egg string may carry up to 700 embryos (Heuch et al. 2000).

1.2.3 Pathology caused by salmon lice

L. salmonis causes severe damage to salmonids when present in high numbers, and reduced fish welfare caused by salmon lice is in fact currently observed also in fish farms (Mattilsynet 2015). Especially adult parasites may induce wounds, causing anaemia and in some cases osmoregulatory unbalance (Grimnes & Jakobsen 1996). Secondary infections may arise as a side effect of these wounds, and *L. salmonis* is also reported to vector viral diseases such as infectious hematopoietic necrosis (Jakob et al. 2011) and infectious salmon anaemia (Nylund et al. 1993). The stress response caused by even low numbers of salmon lice is also considerable (Nolan et al. 1999). Generally, the impact of salmon lice in both wild and farmed fish is a major threat for fish welfare and for the health profile of the individual fish.

1.2.4 Salmon lice cultivation in the lab

Since the late 1980s, when sea lice problems in the salmon farming industry escalated for the first time, salmon lice have been cultivated in laboratories for research purposes (Hogans & Trudeau 1989). Generally, the cultivation scheme consists of egg string hatching, development to copepodids, artificial infestation of fish, and finally holding of the infected fish in aquariums (Johnson & Albright 1991). The parasites then develop to the desired endpoint, which are often preadults for experimental use, or adults for further cultivation. *L. salmonis* have been the easier species to cultivate, compared to *Caligus* species for instance. A laboratory set-up has been described by Hamre et al. (2009), from which equipment, methods, and procedures form the basis for our laboratory set-up. Sea lice cultivation is of course possible in a number of ways. The penultimate success factors have been flowing seawater at the correct temperature (Tucker et al. 2000), water filtration, applicable equipment, and adequate management.

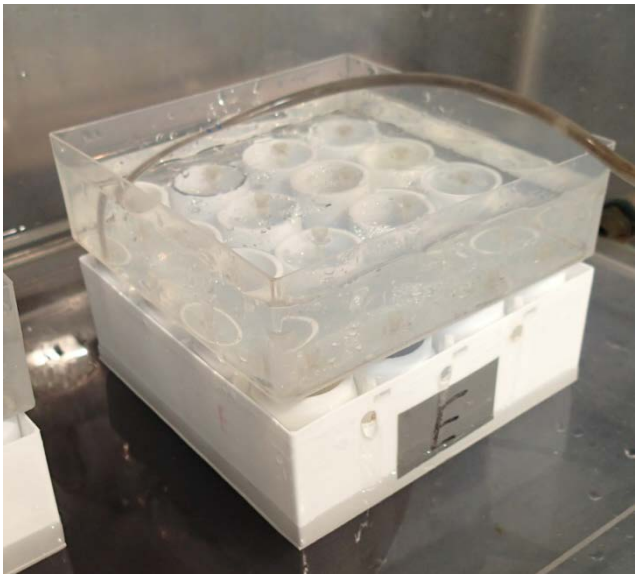

Figure 2: Hatching set-up at Solbergstrand. Photo: Stian Mørch Aaen

1.3 Approaches to combat sea lice

1.3.1 Medicinal interference: Antiparasitic chemotherapeutants

Anti-parasitic agents have a long history. In ancient times, naturally occurring substances killing or repelling insects or other unwanted organisms were used. Sulphur was reportedly applied against insects and mites in Mesopotamia as early as 4500 years ago (Tierney et al. 2015). The predecessor of the pyrethroids, pyrethrin, extracted from *Chrysanthemum cinerariaefolium*, was a well-known insect repellent in ancient China (Glynne-Jones 2001). In modern times, these ancient substances have been chemically modified to synthetically manufactured analogues. At present, hundreds of compounds effective against pest organisms are detected and developed, and numerous ways of organizing these compounds exist. One grouping system has been created by The Insecticide Resistance Committee (http://www.irac-online.org/modes-of-action/, accessed on April 25th 2016), classifying agricultural pesticide agents by their mode of action. Within each insecticide class, substances are sub-grouped when chemically distinct. Cross-resistance is sometimes seen in two or more compounds belonging to different sub-groups. Substances targeting identical structures, i.e. receptors or physiological processes, are separately grouped when their actions differ. Agonists and antagonists of the same receptor are thus not in the same group. The classification is dynamic, and is frequently re-structured according to the latest research findings. Insects and crustaceans are both arthropods, being in the same taxonomic subclass, and thus share several features. Since the salmon louse is a located in seawater, several features favourable when residing in this habitat are present in the species. *L. salmonis* may also share features with other classes of parasitical organisms, such as nematodes or trematodes.

Five groups of compounds are currently in use against sea lice (Table 1); organophosphates (azamethiphos), pyrethroids (cypermethrin and deltamethrin), avermectins (emamectin benzoate), benzoyl urea compounds (diflubenzuron and teflubenzuron), and disinfectants (hydrogen peroxide). Three compound groups act on the nervous system of the parasite, one on the process of exoskeleton synthesis, and one through a hitherto unknown mechanism. Azamethiphos works on the enzyme acetylcholinesterase, catalysing the neurotransmitter acetylcholine to acetate and choline. By inhibiting the enzyme, the compound disrupts the signal process in the neuron, leading to a fatal long-lasting muscle contraction. The effect is quickly induced in salmon lice. Pyrethroids are thought to interfere with opening and closing of voltage-gated sodium channels, resulting in excitation and subsequently paralysis. A third substance working on the nervous system is emamectin benzoate, an avermectin that traditionally was thought to exert their effect on both glutamate-gated and gamma amino butyric acid (GABA)-gated chloride channels (Arena et al. 1995), with higher affinity to the former. Recent evidence however propose that avermectins also may act on other physiological pathways. Elevated calcium level as responses to influx of chlorine ions have been demonstrated to induce P-glycoprotein efflux pumps and thereby possibly modulate the accumulation and effect of avermectins (Lou et al. 2013). Furthermore, genes coding for the nicotinic acetylcholine receptor were upregulated in emamectin-resistant parasites, suggesting this receptor to be an additional target for avermectins (Carmichael et al. 2013). Supplementary detailed insight into the cellular effects of avermectins is likely to occur. These three compound groups are widely recognized as pesticides in many agricultural disciplines, and their relatively low toxicity combined with their rapidly induced effects are highly appreciated. Chitin synthesis inhibitors in the benzoyl urea group have been utilized to some extent against sea lice, the use limited by adverse environmental effects and the fact that adult parasites remain unaffected since they do not produce chitin for another exoskeleton. In recent years, chitin synthesis inhibitors have been re-introduced as anti-sea lice agents because of reduced sensitivity towards other available compounds. The last compound in question is hydrogen peroxide (H_2O_2), formerly known as a disinfectant, and currently with an undefined mode of action against ectoparasites. The compound works quickly on preadult and adult parasites, which however recover to great extent after exposure has ended (Hodneland et al. 1993, Johnson et al. 1993, Treasurer 1997).

Table 1: Systemic list showing anti-sea lice medicines currently on the market. Abbreviations: ch=chalimus, pa=preadults, a=adults.

Active substance	**Medicinal group**	**General mode of action**	**Products (Norway)**	**Administration**	**Sensitive instars**
Azamethiphos	Organophosphate	Nervous system	Azasure, Salmosan	Bath	pa, a
Cypermethrin & deltamethrin	Pyrethroid	Nervous system	AlphaMax, Betamax	Bath	all
Emamectin benzoate	Avermectin	Nervous system	Slice	Oral	all
Diflubenzuron & teflubenzuron	Benzoyl urea	Exoskeleton change	Ektobann, Releeze	Oral	ch, pa
Hydrogen peroxide	Disinfectant	Unspecific	Hydrogenperoksid, Paramove	Bath	pa, a

The latest medicinal compound to be introduced was emamectin benzoate in 1999 (Roth 2000).

1.3.2 Resistance in sea lice against chemotherapeutants

Reduced treatment efficacies of several anti-sea lice compounds have been reported over the past few years (reviewed by Aaen et al. 2015). In fact, resistance has been documented against the four most utilized medicine groups, azamethiphos, pyrethroids, emamectin benzoate and hydrogen peroxide (paper II), and further resistance discoveries are expected to follow. Generally – resistance towards parasiticides is a common problem in all farming industries, as hundreds of different species worldwide are resistant to one or more substances (Denholm et al. 2002).

Resistance can be diagnosed in several ways. Bioassays are experimental set-ups using live organisms (Robertson et al. 2007), and have been the traditional method for sea lice sensitivity testing towards medicinal compounds (Westcott et al. 2008, Sevatdal et al. 2005a, Helgesen & Horsberg 2013). Bioassays are an important tool for deciding which medicinal product to use when the salmon lice abundance exceeds the treatment threshold. Furthermore, they are ideal for laboratory testing of novel medicinal compounds.

A wide range of resistance mechanisms exist, of which some have been explained in Figure 3. In the case of salmon lice, surveillance of resistant populations is important for determining the most effective chemical compound before a treatment. As bioassays are dependent on live parasites, *in vitro* molecular methods using dead parasites may offer a more convenient alternative. Therefore, knowing the exact resistance mechanism is necessary for development of molecular methods for this purpose. Such molecular methods are increasingly utilized beside the established bioassay methods. However, some resistance mechanisms are hard to identify, and the cause for pyrethroid resistance has in particular been challenging. A point mutation responsible for such resistance in *L. salmonis* was reported in 2005 (Fallang et al. 2005), but has never been re-produced (unpublished data). Further mutations in other species have since been reported, as reviewed by Rinkevich et al. 2013. Another mechanism, involving drug-metabolizing enzymes has also been proposed (Sevatdal et al. 2005b). A mutation in the mitochondrial DNA is also suspected to play a role in pyrethroid resistant salmon lice (http://www.google.com/patents/WO2015018863A1?cl=en, accessed on April 13th 2016). Furthermore, genetic markers associated with pyrethroid resistance have been identified (http://patogen.no/kontakt-oss/aktuelt/licence-to-kill/, accessed on Feb 17th 2016), however, the precise mechanism has yet to be confirmed.

Carmichael et al. (2013) illustrated that the expression of genes coding for two different receptors were connected with reduced sensitivity to emamectin benzoate, whereas Igboeli et al. (2012 & 2014) found some evidence for the involvement of P-glycoprotein efflux pumps linked to EMB resistance. None of these studies, however, concluded that the respective mechanism was the only factor involved in EMB resistance. Parasites with SNP profiles similar to those found in EMB-resistant parasites have been detected across the Atlantic Ocean, indicating that the mechanism(s) are widely dispersed (Besnier et al. 2014).

A mechanism responsible for resistance towards azamethiphos in *L. salmonis* is caused by a mutation in one of the two genes coding for the enzyme acetylcholinesterase in the parasite (Kaur et al. 2015). This mechanism is highly dispersed throughout Norway (Kaur et al. 2016). In addition, several other mechanisms towards organophospates exist in other species (Claudianos et al. 1999, Baek et al. 2005). General resistance mechanisms related to organophosphate resistance, such as up-regulation of detoxifying enzymes, are also found in several species (Oppenoorth & Van Asperen 1960). The last resistance issue in *L. salmonis*, resistance towards hydrogen peroxide (Treasurer et al. 2000, paper II), is emerging, and one mechanism involved seems to be increased activity by the enzyme catalase (Helgesen 2015). Several mechanisms may be involved with resistance to one or more compounds, making the task of developing high-throughput resistance tests more difficult.

A wide range of resistance mechanisms exist in ectoparasites (Figure 3). Several methods to avoid resistance are undertaken, often in combination with general anti-parasitic measures. In agriculture, refugia populations are maintained to slow down the resistance drift. In the case of salmon lice, natural refugia populations are present on wild salmonids, and to some extent exist in the Atlantic Ocean. Recently, resistant parasites were observed also to parasitize wild fish (Grøntvedt et al. 2016). This limits the potential reservoir for wild-type genes. A novel medicinal compound, assuming that no cross-resistance to existing remedies would occur, will under no circumstances re-set the sensitivity status to zero in surviving individuals. Good management practice by the introduction of a novel medicinal compound is of paramount importance. Development of reduced sensitivity is most likely inevitable, but a prolonged phase of full sensitivity should, however, be possible due to the current availability of non-medicinal measures.

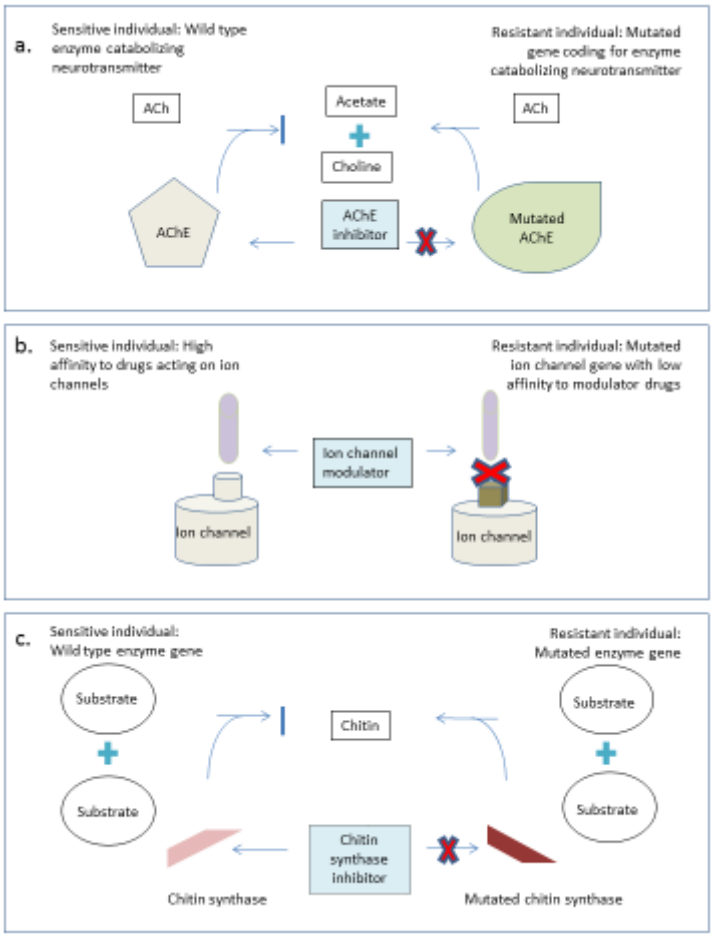

Figure 3: Frequent use of medicinal compounds will select individuals who possess features favouring medicine avoidance for the next generation. Such features, which are subject to molecular studies, are obtained by mutations from the wild type. In example (A) the mutated enzyme will not be targeted by the xenobiotic, and thus provide for effective neuronal transmission. (B) A mutated gene coding for ion channels responsible for neuronal signalling make the xenobiotic incapable of binding to its receptor,

continuing the neuronal impulse in the organism. (C) Xenobiotics interfering with specific metabolic processes in target cells are ineffective in individuals possessing mutated enzymes, where physiological processes may continue. (From Aaen et al. 2015).

More specifically, resistance is a major obstacle for sea lice management, and an important incentive for the work presented in this thesis. Understanding the available compounds' respective modes of action has been adequate in order to generate a basis for further mapping of which targets that exist in salmon lice, and to identify possible related substances outside the IRAC system. Furthermore, to avoid unnecessary work with compounds already considered ineffective because of cross-resistance. To overcome the problems with resistant parasites in the field, several non-medicinal measures have been developed and implemented.

1.3.3 Non-medicinal intervention: examples

Non-medicinal measures (Figure 4) for sea lice reduction are increasingly utilized in the industry. They are important factors for continuous reduction of sea lice, and have to be considered in integrated pest management that also contains medicinal products.

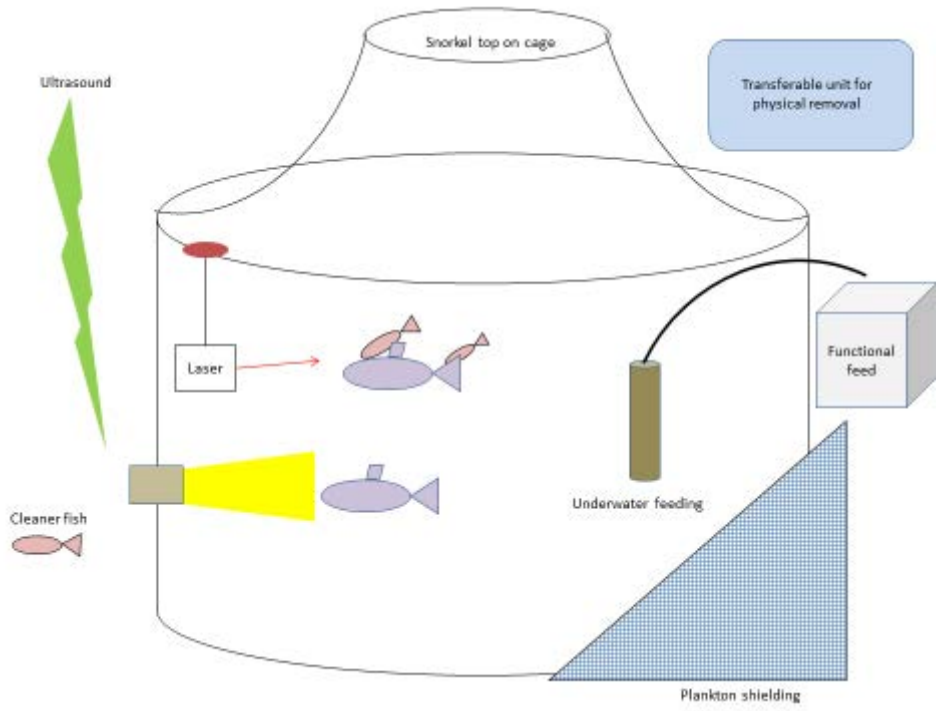

Figure 4: Modern net-pen, with systemic overview of non-medicinal anti-sea lice measures

1.3.3.1 Physical barriers

Since the infective stage of sea lice is planktonic, net-pens may in principle remain louse free following a successful treatment when plankton shielding is present. Protective plankton walls, also called skirts, have been shown to significantly reduce sea lice burdens, with 10 m skirts being the most effective (Næs et al. 2014). However, such skirts require continuous husbandry due to excessive algae growth, which may cause reduced water flow and thereby reduced oxygen concentration. This may be critical at crucial time-points such as bath treatments. The phototactic properties of copepodids induce a typical behaviour of migrating upwards in the water column at day, followed by an opposite movement towards deeper waters at night (Heuch et al. 1995). Although being considered to inhabit the upper layers of the water column (Huse & Holm 1993, Hevrøy et al. 2003, reviewed by Brooks 2005), some infective copepodids may still find their way into the net-pen irrespective of partial physical barriers. Snorkel cages implement the same principle, with fish being temporarily restricted to deeper waters (Stien et al. 2016). Enclosed units is another option for sea lice prevention, offering the additional asset of collecting feed spillover and excrements (Kutti et al. 2007). The cost of pumping water and feed, filtration, and constant oxygen level maintenance is significant in such salmon production, but may be justified by the absence of disease. Offshore cages are also planned, incorporating technology from the oil and gas industry to allow for large dimension net-pen structures. Such a localization would minimize the impact on the ocean floor, facilitate larger fish stocks, and could ultimately reduce the sea lice infection pressure, especially on wild salmonids. Underwater cages, with the ability to reach the water surface by mechanical lifting, have also been suggested. Such cages can be supplied with underwater light, which has been proven effective at manipulating the residence depth of the fish (Frenzl et al. 2014).

1.3.3.2 Optical and acoustic control measures

Laser devices are a novel way of terminating individual sea lice. By using extensive software, the laser is able to recognize preadult and adult lice, and rapidly shoot the louse attached to the fish with the laser beam. Up to two laser units are usually installed in each cage, and they also offer parasite counting, reporting this to a database (http://www.google.ch/patents/US9072281, accessed on April 13th 2016). Opaque water may however disrupt the laser's ability to function optimally. Ultrasound is another way of utilizing technology, by refusing infective larvae access to the cage (Rae 2002).

1.3.3.3 Functional feeds, vaccine, breeding

Functional feeds for manipulating the fish's mucus layer or other immunosystemic properties (Burrells et al. 2001) are available from the main feed producers. Such feeds may contain repellents; molecules that makes the mucus or underlying tissue less attractive to the parasites, or other substances that protect the mucus layer. Breeding of salmon strains that are either more resistant or less attractive to sea lice is also in process (Holm et al. 2015). Massive resources have been granted for developing an anti-salmon lice vaccine (Raynard et al. 2002). Currently, very few vaccines against ectoparasites exist (Willadsen 1999). Several interesting target proteins are identified, but the process is time-consuming.

1.3.3.4 Biological de-lousing: cleaner fish and freshwater

Several species of crustacean predators have been investigated due to their ability and preference for eating sea lice grazing on farmed salmonids (Deady et al. 1995). Indeed, some species have been very effective, exposing an elegant way of removing preadults and adults. The idea of cleaner fish was in fact introduced more than 25 years ago (Bjordal 1989). There are a few natural obstacles to this method, among others the availability of sea lice. Low lice levels makes the fish graze elsewhere, for example on nets where algae and other organisms are flourishing, but also too large lice burdens are problematic, as the cleaner fish's appetite is not infinite. The general welfare of cleaner fish has also been questioned (https://www.imr.no/filarkiv/2010/04/hvorfor_dor_leppefisken_i_merdene_akvakultur_.pdf/nb-no, accessed on April 14th 2016), together with intensive overfishing in vulnerable areas. Cleaner fish are efficient co-habitants (Bjordal 1989), and currently present in many salmon farms. Freshwater treatment is another option (Bricknell et al. 2006), though a costly and effort-requiring one, but gentle on both the fish and environment. However, as the salmon louse is capable of surviving in brackish water for a short period (Johnson & Albright 1991), they may in theory be capable of adjusting to lower salinities, which eventually will lead to reduced efficacy of freshwater treatments.

1.3.3.5 Physical removal

Salmon lice are vulnerable to temperate water, and medium sized, removable installations such as the Thermolicer® (http://www.fhf.no/prosjektdetaljer/?projectNumber=901010, accessed on February 11th 2016) exploit this weakness. External handling is stressful to the fish, implying that the fish welfare is sometimes compromised in order to reduce the sea lice burden. Mechanical flushing is another, physical way of removing the parasites (Nilsen et al. 2010). This procedure requires considerable handling, which potentially coincides with stress, fear, skin erosions, and mortalities

(http://ilaks.no/store-tap-i-forbindelse-med-avlusning/?_ga=1.189452584.249538662.1452086143, accessed on April 20th 2016).

1.3.3.6 Fallowing and geographical zones

A general measure to minimize the dispersal of all pathogenic agents, has been post-slaughter fallowing of farms (Bron et al. 1993b). Fallowing is often combined with synchronized seawater outset of the livestock within geographical zones.

1.4 Rationale

Clearly, all of these measures together create a strong platform for reducing the sea lice abundance in fish farms. The environmental conditions, for example temperature, water currents, energy supply or well boat treatments, may not always cooperate with the responsible personnel. However, extensive use of non-medicinal measures has to date not reduced the sea lice abundance sufficiently, and are not yet proven to maintain sea lice levels below the thresholds set by the authorities. A combination of medicinal interference and non-medicinal measures is likely to remain the common strategy in the near future. The contribution from the work presented in this thesis may be beneficial for development of a novel medicinal product against sea lice.

One way of elucidating physiological pathways of microorganisms is to screen them with compounds with known mode of actions. As parasiticides and seawater compatibility is a reasonably unexplored field, this has been one of the main issues of the work presented in this thesis.

Through this project, we aimed at developing protocols to approach a laboratory screening of existing and tentative anti-sea lice compounds across genders and instars. In addition, in order to explore the direct effect of each compound, we targeted to elucidate medicinal effect in a wider context of developmental impact. A wide range of projects involving sea lice are on-going, and uniform protocols for screening of medicinal compounds, anti-attachment molecules, or vaccines will be useful. Protocols for evaluating effect on hatching and preadults are generally well established, whereas moulting assays and infestation protocols are not. Novel sea lice medicines are desired for two reasons: Overcoming the selection of resistant parasites, and easing the sea lice problem in general.

2 AIMS

The main aim was to identify medicinal compounds with a lethal or immobilizing effect on salmon lice.
Part-objectives

- Development of protocols for sensitivity screening of antiparasitics on different developmental stages of salmon lice.
- Screening of a series of model compounds with different modes of actions on egg strings, nauplii and preadults of salmon lice to identify drug-sensitive targets in the parasite.
- Elucidation of variations in efficacy and ligand binding properties between different compounds with similar mode of action and displaying high efficacy towards salmon lice.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Salmon lice

Our laboratory contains several sea lice strains. Each strain is given a sensitivity status after being assayed against the available anti-sea lice products. One strain, originating from the Norwegian municipality of Alta (Finnmark county) is sensitive to all compounds used up until 2015. The F0 generation of this specific strain (Ls A) was collected in 2011, and >10 generations have been bred for research purposes. Another fully sensitive strain (Ls G) originating from Gulen (Sogn & Fjordane county) (Hamre et al. 2009) was obtained by the University of Bergen, and used in some experiments. This strain was established in 2006, indicating that a relatively high number of generations have been developed. The strains were chosen in order to provide baseline sensitivity towards medicinal compounds in the species. In the experiments described in paper I, a strain of unknown sensitivity status collected in the field was utilized. The experiments in paper II included several sea lice strains from the field, all collected in mid-Norway (Nord-Trøndelag county). For experimental work, preadult stages were chosen ahead of adults, as the former are relatively more abundant than adults in the laboratory. Egg strings and nauplii were used for the other screenings.

3.1.2 Fish – Atlantic salmon

The fish used for cultivating sea lice were purchased from Sørsmolt via NIVA, and were kept in tanks of 100 to 1000 L. Flowing seawater was supplied continuously. For anaesthesia, 100 mg/L metacaine was used for 1-4 minutes. Fish and salmon lice were kept at the wet-lab facility of NIVA Solbergstrand,

Oslofjord, 59°36'57"N, 10°39'7"E. Feeding and experiments were approved by the Norwegian Animal Research Authority NARA (www.fdu.no).

3.1.3 Water

For fish housing, salmon lice hatching and fish anaesthesia, seawater rinsed through a sand filter (1-6 mm particles) was utilized. Additional filtration through a 150 µm plankton mesh was used when dissolving pharmaceutical compounds and for medicine exposure. The seawater was obtained from a depth of 60 m near the lab facility of Solbergstrand.

3.1.3 Medicinal compounds

Medicinal compounds (Table 2) were purchased from Sigma Aldrich, Nerliens Meszansky, and VWR. One compound was kindly supplied by Elanco, Switzerland, and one by Bayer, Norway. Hydrogen peroxide for both studies in paper I was supplied by Solway Chemicals, United Kingdom. For the experiments in paper II, H_2O_2 were supplied by Solvay Chemicals and Akzo Nobel, Sweden. All relevant groups of insecticides were incorporated, some substances were however not available for purchase. The IRAC system of classifying medicinal compounds was chosen as a template since insects and crustaceans are relatively closely related, and that most insecticides available worldwide are included in this scheme. In addition to these compounds, other anti-parasitic compounds that might interfere with salmon lice instars or life cycle were also included.

Table 2. List of substances utilized in the studies, grouped according to the IRAC mode of action classification.

IRAC group	Substance	General mode of action	Mode of action	Substance group	Project candidate
1A	Propoxur	Nervous system	Acetylcholinesterase inhibitor	Organophosphates	Test substance
1B	Azamethiphos	Nervous system	Acetylcholinesterase inhibitor	Organophosphates	Model substance
2B	Pyriprole	Nervous system	GABA-gated chloride channel blocker	Phenylpyrazoles	Test substance
3	Cypermethrin	Nervous system	Sodium channel modulator	Pyrethroids	Model substance
4A	Imidacloprid	Nervous system	Nicotinic acetylcholine	Neonicotinoids	Test substance

			receptor competitive modulator		
4A	Nitenpyram	Nervous system	Nicotinic acetylcholine receptor competitive modulator	Neonicotinoids	Test substance
5	Spinetoram	Nervous system	Nicotinic acetylcholine receptor allosteric modulator	Macrocyclic lactone	Test substance
5	Spinosad	Nervous system	Nicotinic acetylcholine receptor allosteric modulator	Macrocyclic lactone	Test substance
6	Emamectin benzoate	Nervous system	Glutamate-gated chloride channel allosteric modulator	Macrocyclic lactone	Model substance
7B	Fenoxycarb	Hormone mimic	Juvenile hormone mimic	Carbamate	Test substance
7C	Pyriproxyfen	Hormone mimic	Juvenile hormone mimic	Unclassified	Test substance
9	Pymetrozine	Perception	Modulator of chordotonal organs	Pyridines	Test substance
13	Sulfluramid	Energy supply	Uncoupler of oxidative phosphorylation	Fluorinated sulfonamides	Test substance
14	Cartap	Nervous system	Nicotinic acetylcholine receptor channel blocker	Unclassified	Test substance
15	Diflubenzuron	Moulting process	Inhibitors of chitin synthesis, type 0	Benzoylureas	Model substance
17	Cyromazine	Moulting process	Moulting disruptor	Triazines	Test substance
18	Tebufenozide	Moulting process	Ecdysone receptor agonist	Diacylhydrazines	Test substance
19	Amitraz	Nervous system	Octopamine receptor agonist	Amidines	Test substance
22	Metaflumizone	Nervous system	Voltage-dependent sodium channel blocker	Semicarbazones	Test substance
23	Spiromesifen	Energy supply	Inhibitor of acetyl CoA carboxylase	Tetronic acids	Test substance
28	Chlorantraniliprole	Nervous system	Ryanodine receptor modulator	Anthranilic diazines	Test substance
29	Flonicamid	Perception	Modulator of chordotonal organs	Pyridines	Test substance
N.a.	Praziquantel	Unknown	Calsium channel modulator	Unclassified	Test substance
N.a.	Thiabendazole	Hemostasis	β-tubulin inhibitor	Benzimidazoles	Test substance

N.a.	Azadirachtin	Feeding blocker	Unknown	Limonoid	Test substance
N.a.	Pyridalyl	Unknown	Unknown	Pyridalyls	Test substance
N.a.	Hydrogen peroxide	Unknown	Unknown	Disinfectant	Test substance

N.a.: Not applicable

3.1.4 Dissolvents

All pharmaceutical compounds except H_2O_2 were dissolved in a mixture of 50/50 dimethyl sulfoxide (DMSO) and emulsion. DMSO was purchased from Sigma Aldrich, and was stored and utilized at room temperature. The emulsion was made following a recipe by Muan et al. (1985), and heated to 40 °C prior to use. After weighing the medicines, they were dissolved in DMSO and thereafter in the emulsion, before being vortexed for up to 60 minutes, dependent on the compound's solubility. A small volume of seawater was added to the solution, followed by quick vortexing for 1 minute prior to the final mixing with seawater.

3.2 Methods

3.2.1 Hatching assays with egg strings

An experimental set-up for evaluating hatching of egg strings after exposure to medicinal compounds was developed. Egg strings were cut in two, with one half serving as control and the other half being exposed to a medicinal compound. The test material did not allow egg strings to be grouped according to their maturity status, because a vast majority was developed to a similar stage. A gentle shaking of containers with egg strings and medicinal compounds was necessary to get consistent results. Medicine exposures of 30 minutes (36 ± 3 in the hydrogen peroxide assay, paper I) were performed, followed by washing in clean seawater before incubation. Hatching of the egg strings and subsequent viability of nauplii and copepodids was registered for up to 20 days.

3.2.2 Survival assays with nauplii

The egg strings were handled similarly to those used in the hatching assays. The nauplii were exposed to either a medicinal test substance dissolved in 50/50 DMSO/emulsion, or a control solution of seawater and DMSO/emulsion for 30 minutes, 24-48 hours after hatching. They were then put back into the incubator and supplied with constant waterflow for up to 12 days.

3.2.3 Bioassays with preadults

Preliminary screening bioassays were performed with one discriminating dose (Helgesen & Horsberg 2013) for exploring baseline sensitivity towards the compound, with results given as % salmon lice immobilized. Two experiment series was conducted: 30-min and 24-h exposures, with parasites kept in glass bottles with constant aeration. Subsequent dilution experiments involving only the effective compounds was performed with 3-5-fold decreasing concentrations in order to obtain EC_{50} values (the concentration immobilizing 50 % of the exposed parasites). The 30-min exposures were performed to mimic short-term exposure treatments, such as bath administration, and 24-h assays were performed to mimic long-term treatments, such as oral administration or injections. The parasites were incubated at 12 °C with constant aeration before registration of viability status (in %) was performed 20-24 hours after exposure initiation. The compounds inducing more than 80 % immobility in each assay were selected for further studies, as their EC_{50} value was sought.

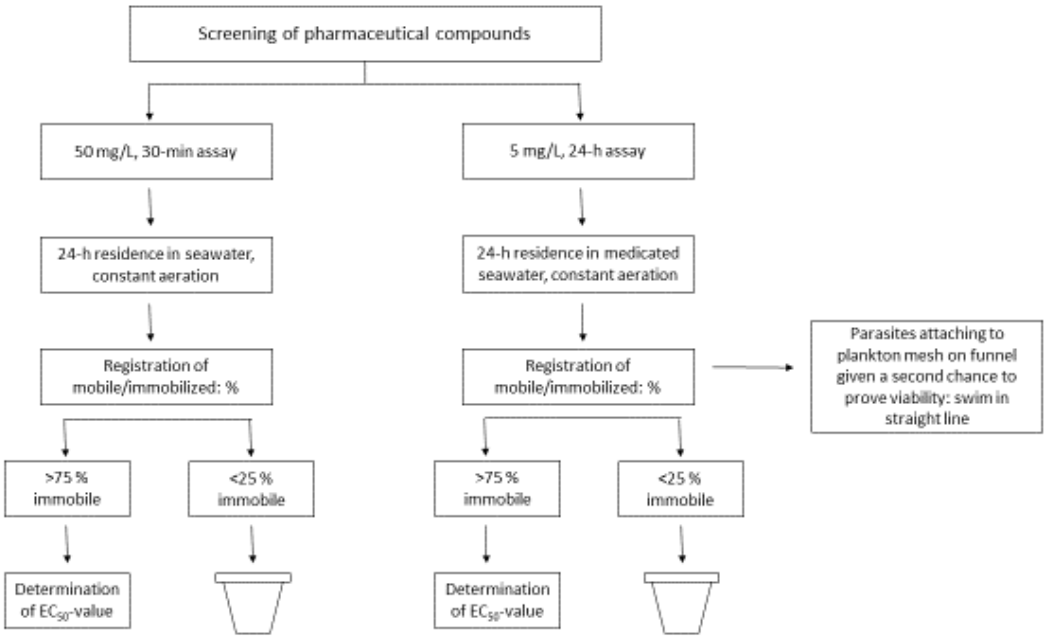

Figure 5: Flow chart for bioassay experiments.

In both experiment series, registration was conducted 24-h after the exposure began (Figure 5). Following short-term exposure, the contents were poured out through a funnel, immobilized parasites were collected, and returned to the bottle, before re-filling the bottle with 1000 mL fresh seawater. Constant aeration was provided and maintained until registration of mobility was performed. In the 24-h assay, the results were recorded directly after exposure. Parasites attached to the bottle wall

were registered as viable, whereas those collected in the funnel were registered as immobilized. The immobilized parasites attaching to the funnel plankton mesh and seeming alive were given a second chance to swim in a direct line in a Petri dish before being classified. Each parasite was classified according to gender and instar. The 30-min bioassays connected to paper II were performed as field bioassays, where polystyrene boxes traditionally have been utilized. The results were recorded immediately after exposure in these experiments, due to the compound's distinct nature and subsequent parasite recovery.

3.2.4 Statistical analysis

The Wilcoxon ranked-sum test was used to compare hatching rate and development of nauplii in the hatching experiments. This non-parametric test was chosen to detect significant differences when there were a high number of 0-values (no hatching) in the dataset. The Area Under the Curve (AUC) was calculated for comparison of larval development over time, as a function of number of larvae multiplied with the number of hours post hatch. In the surviving assay, a Kaplan-Meyer survival test was performed, adjusted for control mortality with the Schneider-Orelli formula:

$$Adjusted\ mortality = \frac{(mortality\ exposed - mortality\ control) * 100}{100 - mortality\ control}$$

Probit analyses were selected for calculation of $EC_{50/90}$ (mg/L) values in the experiments with preadults. The EC_{50} value describes the concentration immobilizing 50 % of the parasites in the experiment. The probit analyses were performed with JMP (SAS Institute Inc.).

4 SUMMARY OF PAPERS

Paper I – Impact of hydrogen peroxide on hatching ability of egg strings from salmon lice (*Lepeophtheirus salmonis*) in a field treatment and in a laboratory study with ascending concentrations.

Aaen SM, Aunsmo A & Horsberg TE

Aquaculture 2014, 422-423, 167-171

Abstract:

Well boat treatments with hydrogen peroxide are used to control sea lice infections on farmed salmonids in many salmon producing countries. A study was performed to investigate the hatching ability of salmon lice egg strings following exposure to hydrogen peroxide in both a field treatment and a laboratory experiment. Egg strings were collected prior to and following exposure to the test compound and subsequently hatched in a laboratory. In the laboratory experiment, the inhibiting concentration of hydrogen peroxide on egg string hatching was investigated by exposures to various concentrations. No hatching was observed after the field treatment with 1750 mg L^{-1} for 31-32 minutes at 6.4 °C. Even low concentrations proved to affect the hatching when egg strings were exposed for 36±3 minutes at 8.0 °C. No infective larvae were observed after exposure to concentrations above 470 mg L^{-1} in the laboratory study. Thus, the current practice of discharging residual well boat hydrogen peroxide treatment water three kilometers from the treatment site seems unnecessary and reduces on-site treatment capacity.

Paper II – First report of reduced sensitivity towards hydrogen peroxide found in the salmon louse *Lepeophtheirus salmonis* in Norway

Helgesen KO, Romstad H, Aaen SM & Horsberg TE

Aquaculture Reports 2015, 1, 37-42

Abstract:

Reduced sensitivity towards chemotherapeutants in the salmon louse *Lepeophtheirus salmonis* (Krøyer) is an increasing problem for the fish farming industry. Most fish farmers are dependent on chemical treatments in order to maintain salmon lice numbers below permitted levels. However parasites showing reduced sensitivity contribute to complicating this task. Hydrogen peroxide (H_2O_2)

is used as a delousing agent in bath treatments and until recently failures due to reduced H_2O_2-sensitivity have not been documented in Norway. The aim of the current study was to develop a bioassay protocol suitable for testing H_2O_2-sensitivity in *L. salmonis*. If failed treatments were found to be caused by parasite insensitivity to H_2O_2 the possibility of this reduced sensitivity being hereditary was looked into. The results show that bioassays permit differentiation between strains of salmon lice with regards to H_2O_2-sensitivity, coinciding with treatment efficacies. Up to ten times variance in sensitivity between two strains was recorded. The progeny of the least sensitive salmon lice also showed reduced sensitivity to H_2O_2 in a bioassay, which indicates that reduced sensitivity towards H_2O_2 is hereditary. The current study presents the first case report of reduced sensitivity towards H_2O_2 in salmon lice in Norway. This change in sensitivity imposes a threat to the Norwegian fish farming industry and should be monitored closely.

Paper III – A screening of medicinal compounds for their effect on egg strings and nauplii of the salmon louse *Lepeophtheirus salmonis* (Krøyer)

Aaen SM, Hamre LA & Horsberg TE

Journal of fish diseases 2016. Doi:10.1111/jfd.12462

Abstract:

Egg strings and nauplii of the salmon louse *Lepeophtheirus salmonis* were exposed to a variety of medicinal compounds at 50 mgL-1 for 30 minutes in two experiment series. This medicine concentration was selected as a starting point for a screening series. Hatching of egg strings and development to copepodid larvae was monitored in one experiment, the survival and development of nauplii was monitored in the other. Two compounds, emamectin benzoate and cypermethrin, inhibited hatching effectively. Several compounds affected nauplii, either directly or through inhibiting development to the infective stage. 50 mgL-1 of azamethiphos, cypermethrin, emamectin benzoate and propoxur was lethal to >70% of the larvae. Diflubenzuron, fenoxycarb, pymetrozine, pyriprole, and tebufenozide diminished the ability of nauplii developing to copepodids.

Paper IV – A screening of multiple classes of pharmaceutical compounds for effect on preadult salmon lice *Lepeophtheirus salmonis*

Aaen SM & Horsberg TE

Journal of fish diseases 2016. Doi:10.1111/jfd.12463

Abstract:

The salmon louse, *Lepeophtheirus salmonis* Krøyer, is the major obstacle facing a sustainable future for farmers of salmonids in the North Atlantic Ocean. Medicinal compounds have been the most utilized tool to prevent salmon lice infestation; however, the active compounds have become less effective or considered environmentally unfriendly in the past years. Novel medicinal compounds are thus highly desired. In two experiment series, 26 medicinal compounds were screened for their efficacy against salmon lice, in a 30-minute exposure and 24-hour exposure respectively. Pyriprole, imidacloprid, cartap, and spinetoram were effective at 50 mgL-1 in the short-time exposure. In the 24-hour exposure, pyriprole, propoxur, cartap, imidacloprid, fenoxycarb, pyriproxyfen, nitenpyram, spinetoram, spiromesifen and diflubenzuron induced a high level of immobilization at 5 mgL-1. The EC_{50} values of the effective compounds were calculated in further titration studies for both exposure periods. Several physiological and biochemical pathways were discovered as possible targets for medicinal intervention against the salmon louse.

5 DISCUSSION

5.1 Novel methods for medicine screening

Several approaches may be used to identify new, effective compounds. Most commonly, the pharmaceutical industry establish a relevant biological test system (e.g. bioassay) where new compounds are tested for efficacy.

In the current studies, the preliminary target was to establish a controlled infection of the fish with a defined number of copepodids. A series of attempts was undertaken. One method was to anesthetize the fish, and placing it upside down with a sedative administered over the gills via a hose. Keeping the copepodids in a tube placed on the belly of the fish would in theory make it possible to count the number of attached individuals after exposure. However, no parasite attached using this method. In another experiment, copepodids were counted before and after the infestation period of 30 minutes. The method was too labour intensive and the results were not consistent enough to justify the effort. Anesthetized fish kept in a plankton bag with a hole exposing the belly area to water containing a specific number of salmon lice was another method tested. The water surrounding the anesthetized fish was carefully stirred in order to create water movement, and the number of copepodids residing in the water was counted before and after infestation.

These experiments supported the evidence presented by Heuch & Karlsen (1997), proving that oscillations projected by salmonids are essential for the copepodid's ability to approach the host. The previously used method of infecting a number of fish with a decent number of copepodids is a suitable way of doing this. Screening experiments using chalimus instars were considered, but were aborted after pilot screenings of model substances (Table 3) since they required a substantial amount of test fish. The results from our experiments however represent a platform which can be used as template for further research with this instar.

5.2 Industrial innovation in aquaculture and pharmaceutical companies

The work presented in this thesis has been performed under the Sea Lice Research Centre umbrella, which consist of several academic and commercial partners. From the commercial side, fish farming companies, pharmaceutical companies, and service providing laboratories have been involved. This has undoubtedly strengthened the project considerably, as collaboration between different disciplines have steadily evolved. A scenario of a 100 % effective non-medicinal invention, with no adverse effects

on fish and environment, is of course the ultimate goal, although a not realistic solution coming out of SLRC at this point of time. Until such a tool exist, fish farmers will probably be dependent on effective medicinal compounds. The existing anti-salmon lice measures have been reasonably efficient, and without them, the salmon lice abundance would be considerably higher than it is today. In a wider perspective, the fish farming companies play an important role in both small and large communities. They create economic and social opportunities, and substantial ripple effects along coastlines in many countries. This is a valuable asset in a time-period of which urban migration, and digital instead of physical economic growth, is a tendency. In order to attract investment, the fish farming companies need to expand at a certain rate. However, in the period from 2016 to 2020, no further growth in Norwegian salmon farming companies is expected (http://fiskeribladetfiskaren.no/nyheter/?artikkel=44012, accessed on April 13th 2016). The same goes for the medicinal companies, who constantly need to develop and maintain their medicine portfolio. The current experiments should therefore be highly relevant for both research and industrial institutions.

5.3 Administration routes of medicinal compounds to fish

Medicinal products and vaccines for aquatic organisms are currently administered via three methods: Through immersion (bath), injection or feed (Grant 2002). Bath treatments, or topical treatments, were introduced in the late 1970s (Brandal & Egidius 1979) when fish were residing in much smaller cages than today. The organophosphate metriphonate (Neguvon®) was until then administered by feed, however adverse effects such as blindness and increased mortality created a need for other administration routes. With this novel method, the medicinal compound was poured onto the water surface, with the active ingredient being dispersed by current and fish movements. Today's bath treatment procedure is much more comprehensive, regarding personnel, equipment, and time consumption. Monitoring of the oxygen level is an important parameter for maintaining the fish welfare (Hughes 1973). Treatments are also dependent on environmental conditions such as water currents, wind, and temperature. Sub-optimal conditions may result in reduced fish welfare, fish mortality, sub-therapeutic medicine concentrations, injuries to equipment or staff, or delays, which could be financially costly (Stone et al. 2000). In some cases, bath treatments have been performed in well boats (Heuch et al. 2011, paper I), enforcing a major operation of transferring the fish back and forth from the cage. There is also continuous speculation regarding the impact on local wild life due to bath treatments (Goulson 2013, Tucca et al. 2014). Even with these drawbacks, bath treatments are currently the most common method of reducing the sea lice burdens using medicines on fish farms.

Oral treatments, administered through medicinal compounds in the feed, also have strengths and weaknesses. Such treatments are less time consuming and represent lower toxicity to both fish and personnel. In best-case scenarios, most of the chemical compound is administered to the fish itself. However, when the bioavailability is low, or due to low fish appetite, the possibility of therapeutic failure is high. Fish not eating sufficient amounts may also present subclinical doses of the medicine to the parasite, which is thought to select for resistance (Grant 2002). Concerns have also been raised regarding additional consumers of the medicated feed, such as shrimp and lobster (Burridge & Haya 1993, Burridge et al. 2000, Burridge & Van Geest 2014).

All salmonid fish are injected with multi-component vaccines prior to sea transfer. This is conducted by professional vaccine-teams, and includes anesthetizing each individual. No anti-sea lice compound has however been applied against sea lice via injection, for several reasons: Sea lice infestations requiring medical treatment normally occur several months after being transferred to sea, so an injection during smoltification would result in a long period of medicinal treatment in which the pathogenic agent is absent. Physical handling of seawater stage fish is, although being increasingly utilized due to necessity, usually avoided to minimize stress. Injections of irritating compounds can also reduce the fish welfare, manifested as adherences in the gut, focal inflammations, and muscle damage (Midtlyng et al. 1996), and should be kept to a minimum. Anyhow, such adverse effects are probably less of a problem if the injection can secure an effective anti-sea lice treatment. The last anti-sea lice compound to be introduced, emamectin benzoate, is formulated as in-feed treatment. This trend of distributing pharmaceutical compounds in feed or injections is probably perennial, as the production units have been incremental since the start of salmon farming.

5.4 Mixing and bioavailability of medicinal products in seawater

For a screening set-up, an important task has been to dissolve all of the medicinal compounds in seawater. DMSO, a common medium for such purposes was initially used as the only dissolvent. However, some compounds proved insoluble in this medium alone while others precipitated immediately when mixed with seawater. In order to compare all exposure results with each other, as well as to secure reproductive experiments, it was important to dissolve all compounds similarly. The developed emulsion worked well with almost all substances. Combined with gentle shaking, it kept all substances with the exception of spinetoram, well dispersed throughout the exposure period. The blank emulsion was tested in pilot studies and in all control experiments, and did not affect the parasite at all in the concentrations applied. In the egg string studies, a constant movement of the water masses

was essential to obtain repeatable results. In the case of hydrogen peroxide, its distinct nature required specific procedures to be performed when doing experiments with the compound.

5.5 Biochemical targets for anti-sea lice compounds in *L. salmonis*

For many of the compounds used in the present study, the mode of action is well characterized. Novel research has however revealed shortcomings in the knowledge regarding the way some compounds act on their target molecules. Some may act on more than one target, with different affinities and expressions deciding the interaction site. In the following, the test compounds have been grouped according to their presumed main mechanism.

5.5.1 Acetylcholinesterase; nervous system

Organophosphates and carbamates are traditional compound groups inhibiting acetylcholinesterase, an important neurotransmitter present in the synaptic cleft. Several organophosphates have been used against salmon lice infestations since 1974 (Grave & Horsberg 2000). Azamethiphos, an organophosphate, was included as a model substance for our bioassays, and also for investigation of the compound's effect on egg strings and nauplii. Propoxur, a carbamate, was one of the test compounds. Both had good efficacy on nauplii, with correlated accumulated mortality of 70 % for propoxur and 100 % for azamethiphos. Since the therapeutic concentration for azamethiphos is 0.1 mg/L, the test concentration of 50 mg/L is well above this. Azamethiphos is not considered to be effective against chalimus larvae (reviewed by Roth et al. 1993). However, O'Halloran and Hogans reported a slight effect on chalimus stages (O'Halloran & Hogans 1996). Kaur et al. showed that the enzyme is not expressed at lower levels in chalimus stages compared to preadults and adults (Kaur et al. 2015b). Whether the reduced effect on chalimus larvae is caused by physical factors, such as hiding between the scales, or by recovery by parasites still being attached to the host, is yet unknown. Some uncertainty is therefore connected to this feature of azamethiphos. In a preliminary laboratory study performed in 2013, no effect on chalimus were observed (Table 3).

Table 3: Fish infested with chalimus larvae treated with a therapeutic dose of azamethiphos, parasites were counted after 7 and 29 days. Aza=azamethiphos, p=preadults, c=chalimus, a=adults, f=females, m=males.

Tank	Exp. to	Fish no.	No. of lice on day 0	No. of lice on day 8	No. of lice on day 30
6	Aza	1	8 (6p 2c)	6p+a	8 (6f 2m)
6	Aza	2	16 (15p 1c)	20p+a	10 (8f 2m)
6	Aza	3	18 (15p 3c)	22p+a	6 (5f 1m)
6	Aza	4	14 (11p 3c)	6p+a	11 (7f 4m)
6	Aza	5	7 (4p 3c)	17p+a	2 (0f 2m)
6	Aza	6	11 (8p 3c)	14p+a	8 (7f 1m)
10	Control	7	10 (8p 2c)	23 (22p 1 c)	18 (11f 7m)
10	Control	8	10 (6p 4c)	9p	5 (4f 1m)
10	Control	9	14 (12p 2c)	12p	9 (4f 5m)
10	Control	10	18 (13p 5c)	8p	4 (2f 2m)
10	Control	11	9 (7p 2c)	7p	8 (6f 2m)
10	Control	12	23 (19p 4c)	18p	7 (2f 5m)

In an epidemiological study by Jansen et al. (2016), resistance towards azamethiphos was found to be widespread along the Norwegian coast except for the northern parts. Although quite efficient in the 24-h assay (Table 4), propoxur is not likely to be introduced as an anti-sea lice compound, due to tentative cross-resistance with azamethiphos, and because of a general reluctance to use acetylcholinesterase inhibitors (Tierney et al. 2014). Azamethiphos is probably the first in line to be rested when novel products become available.

Table 4: Concentrations immobilizing 50 (EC_{50}) or 90 (EC_{90}) % of preadult *L. salmonis* exposed to inhibitors of acetylcholinesterase. The 90 % confidence intervals are provided in brackets.

Substance	Exposure time (h)	EC_{50} µg/L	EC_{90} µg/L
Propoxur	0.5	N.d.	N.d.
	24	<10	<10
Azamethiphos	0.5	N.d.	N.d.
	24	0.11 (0.05-0.22)	0.22 (0.09-0.54)

N.d.: Not determined

5.5.2 GABA- and glutamate-gated chloride channels; nervous system

The GABA-receptor is well conserved in many arthropods, as GABA is the most frequent occurring inhibitory neurotransmitter in the invertebrate nervous systems (Ozoe et al. 2013).

5.5.2.1 Phenylpyrazoles

Phenylpyrazoles, including pyriprole and the related compound fipronil, are non-competitive antagonists of this receptor (and the glutamate-gated chloride channel receptor), leading to disruption of inhibitory signals, hyperexcitability, and subsequently disrupting the action potential in the nervous system. These compounds are widely used as pesticides in agriculture, but only used on companion animals in veterinary medicine.

Given the effect of pyriprole on especially preadults (Table 5), a further elaboration of these compounds could be of interest. Fipronil, a chemical closely related to pyriprole, is a chiral compound, formulated as racemate (50/50 of + and – enantiomers) (Tierney et al. 2014), and the composition of these may influence the compound's efficacy. Fipronil also has a rather low LC_{50} value (median lethal concentration) towards rainbow trout when exposed in the water: 246 µg/L for 96 hours (Tingle et al. 2003). Konwick et al. reported a low toxicity (Konwick et al. 2006) when fipronil was administered orally, however the Environment Pollution Agency of USA characterized the compound as highly toxic to wildlife (reviewed by Gibbons et al. 2015). The presence of dissolved organic matter has been shown to increase the toxicity of fipronil to aquatic copepods, probably through slowing the light-dependent formation of less toxic metabolites (Bejarano et al. 2005). Although the compounds used in our experiments were protected from light before use, a slight decomposition during the exposure period cannot be ruled out. The anti-parasitic effect by compounds acting on the GABA-receptor is rapidly induced when exposure takes place in seawater, although the side effects on aquatic organisms are severe (Konwick et al. 2006). Another possible method of exposing parasites to phenylpyrazoles, although not yet commercially executed on any species, is oral application. The efficacy observed in the bioassay experiments (paper IV) promises convenient anti-parasitic effect on already low concentrations of the compound. Pyriprole does however not seem to inhibit naupliar development to a notable degree. Two novel classes of compounds working on the GABA receptor, 3-benzamido-N-phenylbenzamides and isoxazolines (Ozoe 2013, Zhao & Casida 2014), may also be of interest for further studies.

5.5.2.2 Macrocyclic lactones

Emamectin benzoate, one of the compounds already in use, is an avermectin, a group of compounds within the macrocyclic lactones. It is thought to affect glutamate-gated chloride channels and to a lesser extent also GABA-gated chloride channels, but recent evidence suggests that this compound also interacts with the nAChR, subunit α3 (Carmichael et al. 2013).

Table 5: Concentrations immobilizing 50 (EC_{50}) or 90 (EC_{90}) % of preadult *L. salmonis* exposed to substances within the group of compounds acting on GABA- and glutamate-gated chloride channels. The 90 % confidence intervals are provided in brackets when applicable.

Substance	**Exposure time (h)**	**EC_{50} µg/L**	**EC_{90} µg/L**
Emamectin benzoate	0.5	N.d.	N.d.
	24	38 (16-90)	77 (19-315)
Pyriprole	0.5	108 (N.d.)	118 (N.d.)
	24	0.9 (0.5-1.6)	2.2 (0.9-5.0)

N.d.: Not determined

5.5.3 Nicotinic acetylcholine receptors; nervous system

The nicotinic acetylcholine receptor was initially a particularly interesting target for combatting salmon lice. The acetylcholinesterase inhibitors (organophosphates and carbamates) indirectly exert their effect on this receptor system by elevating the acetylcholine levels in synapses. Because of its presence in all arthropod pest species, several compounds acting directly on the nAChR were chosen for the screening.

5.5.3.1 Cartap

Cartap is one such, blocking the nAChR. Cartap yielded the second lowest EC_{50} value of 51 mg/L in 30-min assay and 5.2 µg/L in 24-h assay (Table 7). Noticeably, it had little effect on nauplii (Table 13). Since no 24-h assay was conducted with nauplii, conclusions cannot be made regarding cartap's ability to affect moulting after long-term exposure. Nevertheless, being effective at a low concentration against preadults both in short-term and long-term exposures, this compound has interesting abilities. Cartap is a nereistoxin analogue, extracted from the marine annelid *Lubriconereis heteropoda* (Konishi 1968), and hydrolyses rapidly to nereistoxin in alkaline conditions (Asahi & Yoshida 1977). We have not analysed our seawater dilutions of cartap for purity or hydrolysed products. The experimental mortality may be caused by one or both compounds, and could be worthy further consideration. Unfortunately, cartap is highly toxic to salmonids

(http://www.pesticideinfo.org/List_AquireAcuteSum.jsp?Rec_Id=PC38463&Taxa_Group=Fish, accessed on Feb 23rd 2016, Lakote et al. 1980) with LC_{50} of 80 µg/L in 48-h assay, but degrades rapidly in seawater. However, this compound could make a strong candidate for a novel product, possibly as an in-feed formulation, when the toxicity towards adult Atlantic salmon in feed formulations has been assessed.

5.5.3.2 Neonicotinoids

The neonicotinoids, competitive modulators of the nAChR, is a group of compounds widely used as pesticides in today's agriculture (Krupke et al. 2012). Acetamiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram, thiacloprid, and thiamethoxam belong to this group of pesticides. Imidacloprid is the most utilized insecticide worldwide (Dively et al. 2008). Neonicotinoids are widely used on crops, and have been linked to severe adverse effects on non-target organisms, along with phenylpyrazoles and macrocyclic lactones (Lumaret et al. 2012). Neonicotinoids in general represent low acute toxicity to fish (Tierney et al. 2014, Gibbons et al. 2015). However, behavioural effects were observed following a 96 hours imidacloprid exposure to juvenile rainbow trout (CCMA 2007) with lowest observed effects concentration (LOEC) of 64 mg/L. As this group was highly interesting, two compounds, nitenpyram and imidacloprid, were included in the initial set-up. Imidacloprid yielded a significantly lower EC_{50} value than nitenpyram for both the 30-min and the 24-h assay, but neither were effective against egg strings and nauplii. The nAChR is in important part of the nervous system in arthropods. It was therefore surprising that the neonicotinoids were ineffective against early stages of the salmon louse.

In order to investigate possible differences in the efficacy between neonicotinoids, the seven compounds were included in a further bioassay experiment series with preadult parasites (Table 6). Each compound's EC_{50} value was calculated after 24 hours exposure. The lowest EC_{50} values were obtained by imidacloprid and thiachloprid. Our studies revealed varying EC_{50} values and indicated considerable specificity between compounds to the nAChR in *L. salmonis*. The solubility in freshwater varies between the compounds. Dinotefuran is for example highly soluble (39.83 g/L), and imidacloprid less soluble (0.51 g/L). These values are not necessarily comparable with the respective solubility in seawater, which may have influenced the results. The lipid solubility also varies greatly, with dinotefuran being the least soluble compound (log K_{ow}: -0.64) and acetamiprid, clothianidin, and imidacloprid showing higher lipid solubility (log K_{ow} 0.8, 0.7, and 0.57 respectively). Other physical factors, such as UV stability and hydrophility, may also have biased the outcome to some degree. The overall efficacy towards the parasites will depend on both the kinetic and dynamic properties of the individual compounds.

Table 6: Concentrations immobilizing 50 (EC_{50}) and 90 (EC_{90}) % of preadult *L. salmonis* parasites exposed to neonicotinoids for 24 hours. The 90 % confidence intervals are provided in brackets when applicable.

Substance	**EC_{50} (µg/L)**	**EC_{90} (µg/L)**
Imidacloprid	97.6 (74.2-148.6)	334.2 (152.3-733.75)
Thiacloprid	101.6 (25.1-411.2)	256.4 (23.8-2762.3)
Acetamiprid	325.8 (77.5-1368.8)	942.9 (143.2-6208.8)
Dinotefuran	447.2	528.5
Clothianidin	803.2	3050.7
Nitenpyram	1790.6 (411.2-7799.3)	70859.2 (3017.2-1664113.7)
Thiamethoxam	>1000	N.d.

N.d.: Not determined

5.5.3.3 Spinosyns

Spinosyns are a group of macrocyclic lactones considered to interact with nAChR (Perry et al. 2007, Silva et al. 2016), the exact mode of action is however under debate, as reviewed by Lumaret (2012). Orr et al. (2009) suggested an effect on voltage-gated calcium channels in the house fly (*Musca domestica*), as no effect on either imidacloprid or abamectin binding sites of nAChR could be proved. Spinosyns, of which several natural and synthetized isoforms exist, are derived from the actinomycete *Saccharopolyspora spinosa*. Spinetoram is one of the compounds effective on several species of diptera (Sparks et al. 2008), and was chosen as our model substance from this group along with spinosad. These compounds were by far the most difficult to dissolve in seawater, and the EC_{50} values may therefore be slightly inaccurate, explaining their moderate action on *L. salmonis*. Spinetoram was to some extent effective against preadults, but not against embryos within the egg strings or against nauplii. Taken into account the high medicine concentration, no conclusion regarding the presence of susceptible targets for spinosyns in salmon lice should be made from these experiments. Spinosyns are the least effective compounds working on the nAChR, and candidates should be recruited from groups other than this.

Table (7): Concentrations immobilizing 50 (EC_{50}) or 90 (EC_{90}) % of preadult *L. salmonis* exposed to substances acting on the nicotinic acetylcholine receptor. The 90 % confidence intervals are provided in brackets when applicable.

Substance	**Exposure time**	**EC_{50} (µg/L)**	**EC_{90} (µg/L)**
Cartap	0.5	4900 (700-33900)	38200 (11500-127000)
	24	5.2 (1.2-22.3)	152.6 (57.3-406.0)
Imidacloprid	0.5	8400 (3200-22300)	46.3 (7.4-289.6)
	24	97.6 (74.1-148.6)	334.2 (152.3-733.7)
Spinetoram*	0.5	51000 (13000-199600)	1845000 (77300-44049500)
	24	1919 (1200-3070)	4525 (2279-8982)

*: Not well dissolved

5.5.3.4 Molecular binding on the nAChR

The nicotinic acetylcholine receptor consists of different subunits in homomer- or heteromeric arrangements (Beck et al. 2015). The subunit construction varies between species, with very little known about this receptor in the salmon louse. The neonicotinoid binding site to the nAChR is not yet 100 % confirmed, but the α7-subunit D-loop is thought to be of importance in insects, at least for neonicotinoids possessing a nitroguanidine residue (Matsuda et al. 2000). In another study, Toshima et al. (2009) suggested that the binding site lies between the α4 and the β2 subunits expressed in *Xenopus* eggs. However, this binding site differs from the binding site of cartap, whereas nereistoxin is shown to bind to both sites in the honeybee (*Apis mellifera*) (Lee et al. 2003). The differences in EC_{50} values in the current study could reflect different binding sites and/or affinities to the subunits present in *L. salmonis*. Generally, the nAChR is an extremely difficult receptor complex to work with, and further research regarding this biochemical pathway may reveal interesting information.

5.5.4 Juvenile hormone analogues

Fenoxycarb and pyriproxyfen are grouped as juvenile hormone analogues in the IRAC-system. Recent research suggest that in *Drosophila*, the juvenile hormones interact with the ecdysone synthesis, a hormone responsible for moulting, influencing the growth rate (Mirth et al. 2014). By acting as a carbamate, fenoxycarb has an additional characteristic of affecting both acetylcholinesterase and the nicotinic acetylcholine receptors (Smulders et al. 2003). Both fenoxycarb and pyriproxyfen yielded quite low EC_{50} values with preadults in the 24-h assay (Table 8), and fenoxycarb immobilized more than 80 % of the nauplii on day 6 in the survival assay. Regarding combatting sea lice, these substances may be ineffective against parasites being resistant towards azamethiphos, since carbamates and organophosphates are both regarded inhibitors of the same enzyme. But the diverse nature of these

juvenile hormone analogues may suggest that this is not necessarily the case. Fenoxycarb is thought to impact aquatic wildlife to a significant degree, and may therefore not be suitable for application against sea lice in open cages (Gripe et al. 2003). Pyriproxyfen is rapidly degrading in seawater, and is susceptible to photodegradation (as reviewed by Sullivan & Goh 2008). Water solubility is low, but the compound is found to be persistent in sediments. Pyriproxyfen, or related substances should be regarded a candidate for novel sea lice products, either alone or in combination with another substance. It has successfully been implemented against the dengue vector *Aedes aegypti*, in combination with spinosad (Darriet & Corbel 2006).

Table 8: Concentrations immobilizing 50 (EC_{50}) or 90 (EC_{90}) % of preadult *L. salmonis* exposed to substances within the group the group of juvenile hormone analogues. The 90 % confidence intervals are provided in brackets when applicable.

Substance	**Exposure time (h)**	**EC_{50} (µg/L)**	**EC_{90} (µg/L)**
Fenoxcarb	0.5	N.d.	N.d.
	24	408 (256.0-651.1)	1209.1 (563.8-2593.1)
Pyriproxyfen	0.5	N.d.	N.d.
	24	676.6 (393.5-1163.3)	2862.0 (557.4-9595.8)

N.d.: Not determined

5.5.5 Chordotonal organ; perception

Pymetrozine is a modulator of the chordotonal organ according to the IRAC scheme. This group was until recently characterized as “feeding blockers”. Ausborn et al. (2005) described a mode of action affecting chordotonal mechanoreceptors, and Kaufmann et al. (2004) connected the serotonergic system to its mode of action. In our studies, pymetrozine inhibited larval development to an extent of 65 %. Studies with this compound and chalimus stages may be of interest, especially if toxicity studies with aquatic organisms reveal favourable results. The toxicity towards rainbow trout is low, with 96-h LC_{50} values of >128 ppm (Boeri et al. 1994). In a study by Sechser et al. (2002), its selectivity towards the target species was excellent, allowing predators of the target to survive. The effect on salmon lice may thus be further investigated.

5.5.6 Moulting processes

Two compounds acting on the moulting process, diflubenzuron and teflubenzuron, are currently on the market for use against salmon lice. One additional compound with a similar mode of action but longer efficiency, lufenuron, is close to being released commercially. They act on chitin synthesis, inhibiting the ability to re-construct the exoskeleton, which is a key process in the parasite's life cycle. The downside of such compounds are their side effects on non-target species cohabitating the water column with salmonids (Soltani et al. 2009, Samuelsen et al. 2014).

Diflubenzuron was included in the set-up as a positive control in the moulting assay, however it was also surprisingly effective at 5 mg/L against preadults in the 24-h assay (Table 9). The acknowledged action of benzoylureas is disruption of the chitin synthesis (van Eck 1979, Hajjar & Casida 1979). Diflubenzuron is also reported to work as an acetylcholinesterase inhibitor in *Prochilodus lineatus*, a freshwater fish (Maduenho & Martinez 2008). This action may explain the immobilization of preadults in the 24-h assay. Another compound affecting hatching through irreversible binding to the heterodimer consisting of the ecdysone and ultraspiracle receptor (Dhadialla et al. 1998, Retnakaran et al. 2003) is tebufenozide. The compound quite efficiently prevented hatching to copepodid larvae (paper III). Song et al. considered tebufenozide (and imidacloprid) to be more suitable for application in aquatic environment compared to aldicarb and dimethoate, because of their high degree of specificity and low toxicity towards nontarget crustaceans (Song et al. 1997). According to Wing & Aller (1990), RH-5849, a compound closely related to tebufenozide, is reasonable friendly to the environment. The ecdysone receptor may also represent an exciting target for vaccine development (Sandlund et al. 2015) and should be highly interesting for further research.

Table 9: Medicine concentrations immobilizing 50 (EC_{50}) or 90 (EC_{90}) % of preadult *L. salmonis* exposed to a substance within the group of benzoyl ureas. The 90 % confidence intervals are provided in brackets when applicable.

Substance	**Exposure period (h)**	**EC_{50} (µg/L)**	**EC_{90} (µg/L)**
Diflubenzuron	0.5	N.d.	N.d.
	24	5000	Could not be calculated

N.d.: Not determined

5.5.7 Acetyl coenzyme A carboxylase; fat metabolism

A tetronic acid belonging to the group of cyclic ketoenols, spiromesifen is a compound working on the lipid synthesis of several pest species (Nauen et al. 2005) through inhibition of a fat metabolism enzyme. The substance was efficacious against preadults at a concentration of 5 mg/L for 24 hours (Table 10). Kissoum & Soltani (2016) showed that spiromesifen elevated the energy demands in *D. melanogaster*, together with having a tissue damaging effect on pupal bodies. The compound is slightly toxic to rainbow trout in seawater exposure over a period of 96 hours, according to the California Department of Pesticide Regulation (2005). The environmental impact is considered to be moderate, with 99 % dissipation in plant material after 14-17 days (Sharma et al. 2007). In a study with 23 pesticides, spiromesifen was one of only four with no acute toxicity towards bumblebees *Bombus terrestris* (Besard et al. 2010). The possiblitiy of cross resistance is not likely because of the compounds distinctive mode of action (Marčić et al. 2011). Spiromesifen, or the related spirodiclofen, spirotetramat, or cycloxydim, all have good potential for further studies with salmon lice.

Table 10: Concentrations immobilizing 50 (EC_{50}) or 90 (EC_{90}) % of preadult *L. salmonis* parasites exposed to substances acting on the acetyl coenzyme A carboxylase. The 90 % confidence intervals are provided in brackets when applicable.

Substance	**Exposure period (h)**	**EC_{50} (µg/L)**	**EC_{90} (µg/L)**
Spiromesifen	0.5	N.d.	N.d.
	24	2039 (322-12880)	65577 (1972-2179696)

N.d.: Not determined

5.5.8 Calcium storage and channels, muscle contractions and cell haemostasis

Praziquantel is not a pesticide used in agriculture and therefore not mentioned in the IRAC insecticide program, but was included in our studies because its presumed mode of action made it an interesting compound to investigate. It induced a temporary immobilizing effect on preadult salmon lice with an exposure period of 30 minutes at the initial concentration of 50 mg/L. No mortality was observed either in this experiment or in the long-term exposure, but the ephemeral immobilization may be worth further investigation. This compound is effective against cestodes, and has a long history in both companion animal practice, production animal practice, and aquaculture. Its mode of action is still not completely verified, but involvement of calcium channels (reviewed by Greenberg 2005), calcium release from the endoplasmic reticulum after interaction with the caffeine binding site (reviewed by Martin et al. 1997), interaction with phospholipid membranes (Harder et al. 1988), and oxidative stress (Aragon et al. 2009) are among the suggested solutions. *In-vivo* experiments with praziquantel should

be undertaken to verify if any effect could be exerted through oral treatment at doses non-toxic to fish.

5.5.9 Unknown mechanisms

Hydrogen peroxide is a disinfectant introduced in Norway and Scotland as an anti-sea lice compound in 1993 (Grave & Horsberg 2000, Lees et al. 2009). Initially, this product was used when the salmon lice abundance was much higher than the current situation, and populations resistant towards the currently available compounds were emerging in several locations. H_2O_2 was applied as an emergency treatment at the time. When resistance to the available medicinal compounds re-appeared over than a decade later, the compound was re-introduced. A marketing authorization as pharmaceutical compound was approved in Norway in 2012. The H_2O_2 consumption has escalated over the past few years, and the compound has been increasingly used over the last years (http://www.fhi.no/artikler/?id=117980, accessed on April 12th 2016, calculations with formula published by Grave et al. 2004). However, resistance problems have recently emerged with this compound as well (paper II). Also, a number of cases of massive fish mortality following H_2O_2 treatments have been reported lately, underlining the compound's low therapeutic index. Originally categorized as a disinfectant, the compound's mode of action is not clearly defined. An effect on the haemolymph has been suggested (Johnson et al. 1993). This would however not explain the detrimental effect on egg string hatching and subsequent nauplius and copepodid development. Furthermore, influence on the regulation of genes coding for cytochrome P450 causing oxidative stress is a plausible explanation as suggested for *A. aegypti* by Poupardin et al. (2010). Since salmon lice are immobilized after only a few minutes after exposure to H_2O_2, this mode of action seems unlikely. A possible effect on hatching executed by H_2O_2 was subject to an investigation early in the PhD period, in co-operation with industrial partners.

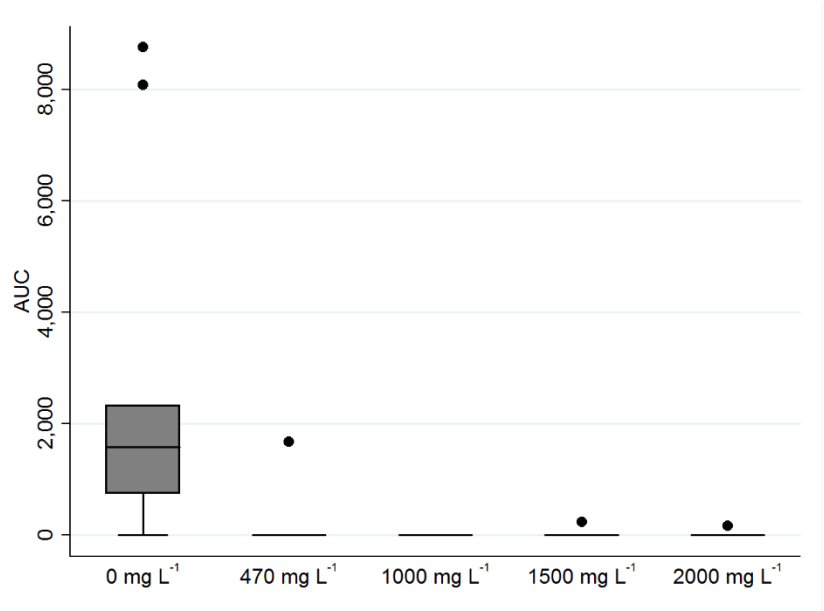

Figure 6: Hatching of egg strings after exposure to increasing concentrations of hydrogen peroxide. Boxplot of AUC values of larvae in control group and the four treatment categories. The results demonstrate that all categories had AUC values close to zero, except the group exposed to 470 mg/L, which produced a low number of larvae that did not develop to the infective stage.

Our study gave strong indications that the current practice of discharging residual water after H_2O_2 treatment water three kilometres away from the treatment site is unnecessary (Figure 6). Johnson et al. (1993) observed less activity in chalimus stages 24-h after exposure at concentrations of 3.0 and 4.0 g/l (20 min exposure). The same authors also observed a slightly lower survival rate in exposed chalimus larvae. H_2O_2 is administered as bath treatment. Under field conditions, an inhibiting effect on moulting is less likely on the parasitic stages of *L. salmonis* due to the short exposure period. For the same reason, effects on nauplii that are present in the cage during bath treatments may be observed, nevertheless no long-lasting effect can be expected.

5.5.10 The egg string hatching assay

Only one compound group, which is also commercially available, affected egg string hatching at therapeutic concentrations, namely hydrogen peroxide (paper I, Pert et al. 2015, Toovey & Lyndon 2000). Cypermethrin has previously been shown to inhibit hatching (Toovey & Lyndon 2000). Deltamethrin may also induce a similar effect due to its structural equality with cypermethrin. The current studies have illustrated that emamectin benzoate also reduces hatching and development to copepodids, however at concentrations far above the therapeutic level (Table 11). Cypermethrin and emamectin benzoate were included in the set-up as model substances, and their LC_{50} value towards egg strings and nauplii was therefore not further investigated. In general, egg strings are resistant to

chemical interference, at least in short-term exposures such as this. Bravo et al. (2015) however showed that 24 hours exposure of the current medicines were fatal to the egg strings of *C. rogercresseyi*. If any compound was found to have an effect on egg strings, it could be considered as a combined product with effect on other instars as well. The method of efficacy screening on hatching is functioning nicely, and may easily be applied for screening with for example semiochemicals or medicinal compounds over 24 hours.

According to the rationale behind paper I, an investigation of the hatching capability of egg strings after exposure to medicinal compounds was highlighted as beneficial. In the initial process, pilot experiments were performed to optimize this method. They revealed that a gentle movement of the water was essential to obtain consistent results. The development status of the egg strings was also taken into consideration. Mature egg strings are more vulnerable to hydrogen peroxide (paper I) and a similar effect to some of the other compounds could be expected. The maturation level of egg strings is not directly corresponding with the pigmentation observed by the eye, as the colour develops exponentially at the end of the process. Approximately 80 % of the egg strings were quite pale in the experiments described in paper III. Therefore, no division of egg strings according to pigmentation was undertaken.

Medicinal screening trials need reliable control groups. There are relatively many parameters of uncertainty for egg string hatching, such as fertilization status, number of eggs, age, water temperature (Tully 1989) length, quality, oxygen level (Piasecki & MacKinnon 1996), and maturity at hatching start. We chose to cut every egg string into two halves, in order to minimize the risk of false results. Some egg strings were removed from the mother with a pair of scissors, and some were released naturally. One half would then potentially be open in both ends in some of the egg strings, possibly contributing to an increased uncertainty. They were however randomly distributed into groups (controls or exposed) to avoid a systematic bias.

Table 11: Hatching study: Development of nauplii and copepodids of *L. salmonis* after egg strings were exposed to 23 medicinal compounds, presented as median of individual area under curves (AUC) for 12 parallels for each substance including 10/90 percentiles for each value. Exposed groups are compared to control groups in the Wilcoxon ranked-sum test.

IRAC no.	Substance	AUC exposed -median	10/90 percentile	AUC control – median	10/90 percentile	Wilcoxon P-value
	Control	114	0/3741.6	522	0/1191	P=0.77
1	Propoxur	690	0/8076	2640	0/9708	P=0.34
1	Azamethiphos	2458	0/9078	3883	0/9668	P=0.24
2	Pyriprole	2130	0/12966	1308	0/18360	P=0.54
3	Cypermethrin	378	0/2040	5388	0/10350	P=0.07
4	Imidacloprid	5094	1779.6/9579.6	4266	241,2/7272	P=0.40
4	Nitenpyram	1668	28.8/9678	4530	28.8/10554	P=0.14
5	Spinetoram	2970	960/9138	4740	86.4/11988	P=0.51
6	Emamectin benzoate	0	0/0	2280	0/10776	P=0.00
7b	Fenoxycarb	3420	28.8/13668	2940	90/7848	P=0.49
7c	Pyriproxyfen	5280	414/13668	4380	0/10464	P=0.82
9	Pymetrozine	6222	1824/8880	4884	3012/8653.2	P=0.77
13	Sulfluramid	4398	1567.2/7472.4	4224	0/8607.6	P=0.53
14	Cartap	4914	1484.4/10992	6618	1144.8/9040.8	P=0.69
15	Diflubenzuron	5352	213.6/10222.8	5712	1098/9696.6	P=0.91
17	Cyromazine	5898	1273.2/10260	3480	1510.8/8619.6	P=0.13
18	Tebufenozide	1158	0/5710.8	678	0/6667.2	P=0.62
19	Amitraz	4452	235.2/8965.2	4068	931.2/7161.6	P=0.77
22	Metaflumizone	600	0/6540	0	0/13884	P=0.67
23	Spiromesifen	276	0/5454	852	0/4633.2	P=0.75
28	Chlorantraniliprole	294	0/1359	2886	0/7642.8	P=0.24
29	Flonicamid	5430	367.2/10434	4464	228/9268.8	P=0.91
N.a.	Praziquantel	1860	284.4/4281.6	2052	0/7537.2	P=0.77
N.a.	Thiabendazole	1152	0/6177.6	2436	25.2/6009.6	P=0.30

As hydrogen peroxide inhibited hatching to a high degree, we expected other compounds to exert a similar effect. This inhibiting effect on hatching is probably connected to the nature of the compound, as it originally is classified as a disinfectant. It is reasonable to link the etching properties to declining fitness of the embryos. Another chemical substance with a similar effect, formalin, has occasionally been used off-label against sea lice in the past months. One could assume that formaldehyde also reduces hatching, given its disinfecting nature. As a general conclusion from the egg string experiments, egg strings are not very vulnerable to short-term medicine exposure. The embryos seem to be well protected within their egg sacs. In a field perspective, intervention with compounds only targeting egg strings would only have a transient effect, unless the compound also affects the next set of egg strings as well. Egg strings of all present sea lice will be replaced within a few days (Johnson & Albright 1991), and the effect on consecutive egg strings was not investigated. In retrospect, a 24-h

exposure of egg strings could have been beneficial in our experiments. A continuous effect by oral uptake through the salmon lice female into the egg string might interrupt the hatching ability and further development to copepodids. This argument may be considered in future studies.

5.5.11 The survival assay with nauplii

Chalimus larvae attached to fish may be registered by trained eyes, but in hectic operations such as lice counts, handling and re-organizing of fish, the early stages up to chalimus II are mostly overlooked. Most of the precautionary measures, except from plankton shielding, are directed towards preadults and adults. Therefore, protocols for evaluating the effect of medicinal compounds on other instars than the latter three are important for both medicine management and medicine development. The survival assay in paper III was developed for two purposes: Evaluating the mortality on the naupliar instar itself, and effects on the moulting process. The nauplius instar is physically different from the attached instars, and an interesting aspect was the comparison of physical features between planktonic stages and motile stages. Little is known about the expression of target molecules for medicinal compounds in the formerly mentioned instar, and the experiments provided some indications.

Table 12: Nauplii - survival analysis after 30 minutes exposure to medicinal compounds with an observation period of up to 14 days.

Substance	**Accumulated mortality in the control at day 7 (%)**	**Accumulated mortality in exposed parasites at day 7 (%)**	**Medicine induced mortality (%)**	**Wilcoxon ChiSq**	**Sample size (control/exposed)**
Propoxur	40	82	70	68.17	230/205
Azametiphos	49	100	100	127.15	180/181
Pyriprole	61	88	69	102.27	280/155
Cypermethrin*	55	100	100	183.13	170/110
Imidacloprid	50	60	20	22.07	410/232
Nitenpyram	21	28	9	2.76	290/195
Spinetoram	30	34	6	22.70	240/310
Emamectin benzoate	30	100	100	316.0	205/112
Fenoxycarb	20	89	86	1.68	142/170
Pyriproxyfen	32	60	41	49.74	270/160
Pymetrozine	20	72	65	115.54	200/240
Sulfluramid	20	43	29	24.34	175/140
Cartap	78	56	0	36.28	160/160
Diflubenzuron	0	100	100	281.42	140/190
Cyromazine	33	59	39	34.83	295/310
Tebufenozide	34	89	83	43.06	145/170
Amitraz	28	37	13	9.31	245/180
Metaflumizone	16	49	39	48.06	200/300
Spiromesifen	38	54	26	10.68	301/185
Chlorantraniliprole	74	46	0	175.0	320/340
Praziquantel	24	36	16	58.19	260/310
Thiabendazole	32	26	0	3.05	320/350

*: At day 6

The Wilcoxon value is the calculated Chi-square for the whole experimental period, with 1 degree of freedom. It compares the median survival time for the exposed and the control group. For this parameter, the value of 3.84 correlates with P=0.05 in standard statistical analysis.

The moulting process, ecdysis, is described by Anstensrud (1990) for the species *L. pectoralis*. Bron et al. (2000) has described the process in chalimus larvae of *L. salmonis*, concluding with processes similar to other crustaceans. When replacing the exoskeleton, the exuvium splits in two halves on the anterior side, and the salmon louse leaves the shed exoskeleton (Anstensrud 1990). Moulting is a crucial part of the development, and therefore a well-suited target for medicinal interference. The nauplius instar was selected for this assay. This instar facilitates the use of a high number of parasites compared to an assay with preadults, and eliminates further use of fish. The nauplius assays were relevant for investigation of the instar's baseline susceptibility to a range of medicinal compounds, and for evaluating the moulting ability after exposure (Table 12). The evaluation procedure was not straightforward. Since the start concentration was relatively high, an all or nothing effect exerted by the selected medicinal compounds was expected. A separation between control and exposed groups would consequently be easy to perform in the hatching cylinder. Using this approach, the number of actively swimming larvae was estimated at 48-72 hours intervals; the dead larvae were lying on the bottom of the cylinder. Another option tested was to explore larval viability through photographs of a selection of individuals at different time points after exposure. Pictures taken at 10-min intervals were overlaid and the number of larvae that had moved during this period were evaluated. This method differentiated well between the dead and moribund larvae, but not between moribund and fully fit parasites. Since only the latter are expected to be able to develop further to the infective copepodid, this method was abandoned. 24 hours assays with nauplii were not performed. Such assays could be a useful tool in further studies, but require additional equipment. Long-term assays have been applied with other crustaceans (Sasikumar et al. 1995). The constant aeration provided by the water movement would be important in this case. There is a possibility that some of the ineffective compounds could exert an effect on the moulting process when nauplii are exposed for a longer period than 30 minutes. In Figure 7, the medicine induced naupliar mortality is displayed. Generally, this assay differed from the hatching assay in that many compounds were effective. Out of 23 compounds, 9 induced more than 60 % mortality. It is worth mentioning that the concentration was very high.

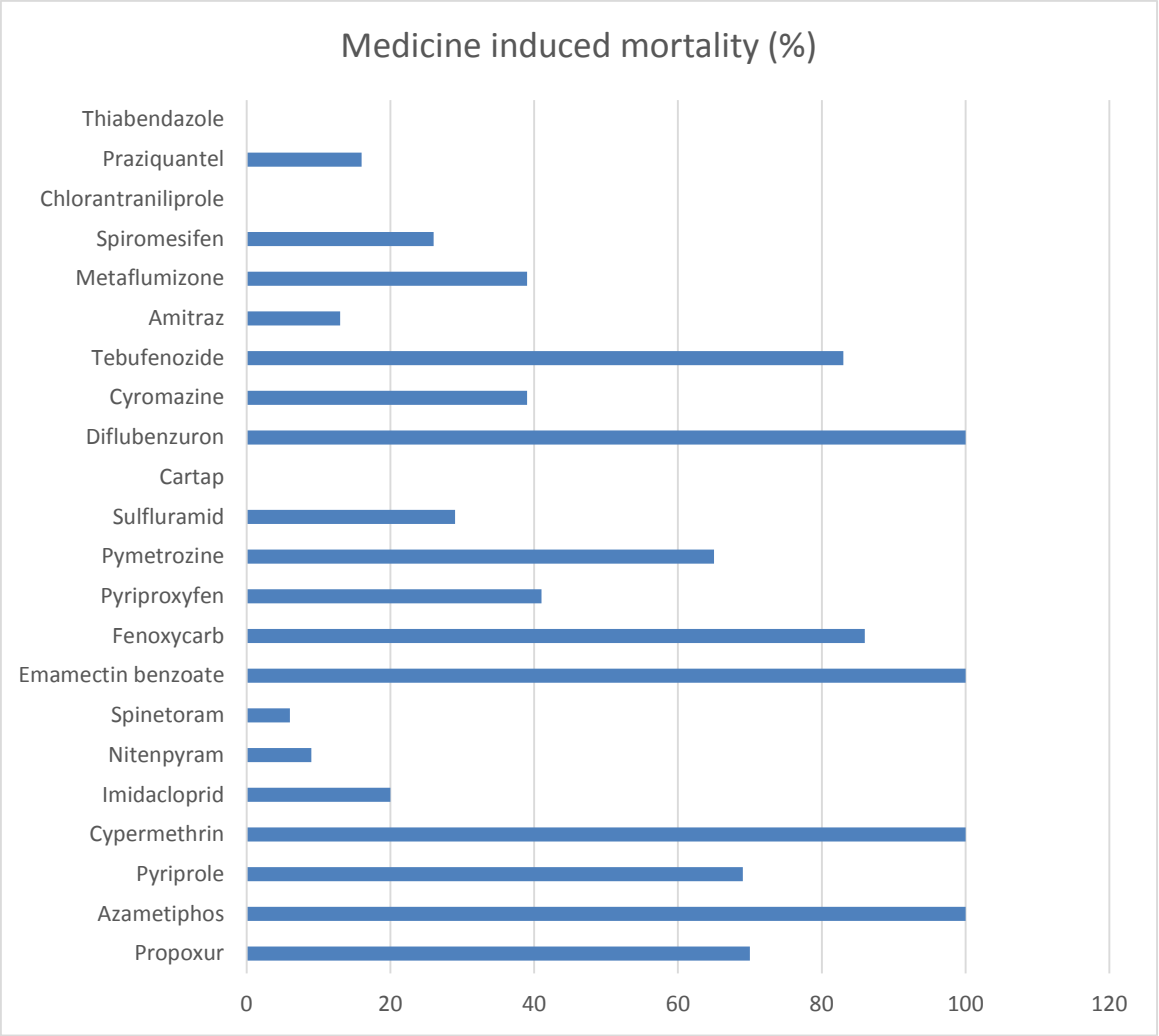

Figure 7: Survival assay: The net medicine induced mortality of naupliar larvae after exposure to 50 mg/L for 30 minutes (corrected with the Schneider-Orelli formula). Registration on day 7 after exposure (cypermethrin on day 6).

5.5.12 Repellent effects by the medicinal compounds

Repellent effects of some of the tested compounds may be expected. In a pilot experiment performed in our lab, imidacloprid and clothianidin (5 mg/kg fish as a single dose) was given orally via gavage. This concentration removed >80 % of the parasites within 7 days after application, with imidacloprid being slightly more efficient (>92 % efficacy). Some parasites had detached from the fish and were found attached to the wall of the tank, fully alive. This pointed to a repellent effect in addition to the toxic effect. No repellent effect was however recorded when fish were re-infected with copepodids 12 days after the medicine was given, as the infection level was equal between fish receiving the treatment and the control fish. The fish were not affected by the experimental dosage.

5.6 Integrated pest management

In agriculture, where resistant agents have existed for years, integrated pest management (IPM) is implemented all over the world to overcome these problems. IPM is a broad-based approach that integrates a multitude of methods to control pests. IPM is defined by the UN Food and Agriculture Organization as “the careful consideration of all available pest control techniques and subsequent integration of appropriate measures that discourage the development of pest populations and keep pesticides and other interventions to levels that are economically justified and reduce or minimize risks to human health and the environment”. IPM is utilized in fish farming as well, i.e. via fallowing, zonal coordination of anti-parasitical treatments and sea-transfer of fish, and use of physical barriers. Although called aquaculture, the veterinary aspect of fish farming principles are built on the heritage from husbandry more than agriculture. The current tendency, however, seems to be incorporation of the agricultural principles into the aquaculture sector. Sheep farmers intend to utilize two or more pastures, in order to prevent infection development by the next occurrence on the same area. This corresponds with zonal policy in aquaculture.

Since the sea lice also exist on non-controlled wild individuals, an eradication of the parasite is clearly impossible. This is similar to agricultural scenarios, where crop pest species have their reservoir in and outside the field. As long as the fish farms remain open to the environment, efforts to maintain sensitive parasites in and around the farms are needed. The definite advantage of fish farms is their ability to control every input, of water, oxygen and feed. Novel technology may realize this potential sooner rather than later, in order to avoid pathological agents and furthermore to decrease the production run-off.

Vaccination programs are reliable and considerably well-functioning, both for farmed animals and fish. However, the agents controlled by vaccines are mostly bacteria, whereas virus vaccines are connected with unpredictable effects. Aquaculture is anyhow an example of successful comprehensive vaccination performed with vast numbers of animals within a limited volume or area. A sea-lice vaccine would surely be a great asset for salmon farming, and the current experiments may provide useful information for this purpose.

Medicine administration in enclosed or semi-closed units is not well documented, and needs to be evaluated for novel medicinal compounds. Another important aspect is the presence of cleaner fish, which are definitely subject to medicine exposure in bath treatments, and possibly subject to oral treatment regimens, as they tend to eat salmon feed. The toxicity of potential medicinal products towards cleaner fish should be investigated thoroughly, taken the importance of this biological pest regulation into consideration. The presence of other fish pathogens also impact anti-salmon lice

measures, as they cause reduced fitness which make the livestock more vulnerable to stress related mechanic or medicinal treatment.

6 ETHICAL ISSUES

Keeping fish for cultivation of sea lice was a crucial part of our experiments. The fish were handled in compliance with animal welfare regulation, including proper anaesthesia, adequate water supply, daily feeding and inspection etc. The studies were approved by the local Animal Welfare Committee. Fish showing signs of suffering were euthanized. Working with substances that might be harmful to the environment has also been part of our discussions, and a large part of the discussion is related to this issue. Since the amounts of chemicals used in the bioassays were very small, this was not a problem in these studies, but is an important issue if any are to be used at a larger scale.

7 CONCLUSION

- Reduced sensitivity towards hydrogen peroxide, a compound hitherto considered as highly effective, was observed.
- Several medicinal compounds with effect on one or more instar of *L. salmonis* were identified in the screenings.
- A protocol for registering the moulting ability from nauplius I to nauplius II was developed, and a method for evaluating development to copepodids after the egg strings were exposed to medicinal compounds was also described.
- Variations in efficacy between neonicotinoids, substances acting on the nicotinic acetylcholine receptor, were investigated.
- Initial investigations of toxicity towards fish and environment was performed with some of the most interesting compounds.

Insecticides work on a range of physiological processes. However, the majority of insecticides target the nervous system. In salmon lice, this organ system is already exploited by the molecules emamectin benzoate, azamethiphos, cypermethrin and deltamethrin. Compounds acting on the nervous system often induce rapid mortality, and bioassays are usually easily interpreted. In these screening series, the mapping of physiological pathways present in salmon lice became an important issue. In addition to further compounds acting on the nervous system, substances interfering with pathways other than this may be highly interesting for upcoming studies.

8 PERSPECTIVES FOR THE FUTURE

Sea lice will remain a problem in salmon farming in the future, and the current thesis will hopefully contribute to development of novel medicinal products. The potential of such inventions in connection with current non-medicinal measures is to keep sea lice numbers at an acceptable level, in order to secure economic growth accompanied by sustainable biological production of salmonids. Our experiments show that compounds acting on the GABA-gated chloride channel and the nicotinic acetylcholine receptor were highly effective in low concentrations, and possess the capacity of removing sea lice effectively under field conditions. The main obstacle for these agents to be introduced commercially is the environmental aspect, as the controversy surrounding these compounds may happen to be the biggest obstacle for a novel anti-sea lice product. The rapid excretion of neonicotinoids however make them suitable candidates for a product with oral administration. The acetyl coenzyme A carboxylase inhibitor compounds represent another interesting group of insecticides, offering low toxicity to the environment, and only moderate toxicity towards salmonids. These aspects may be extremely valuable in the current situation, where sudden mortalities after de-lousing, or dispersal of toxic substances to the environment are fatal for an industry already struggling to live up to the standards set by the population in highly industrialized countries. Last but not least, the nereistoxin equivalents are also highly interesting due to cartap's ability of immobilizing *L. salmonis* at very low concentrations. Other factors, such as toxicity towards cleaner fish, are important to take into consideration when developing new anti-sea lice products for Atlantic salmon. So also the chemical's persistence in aquatic environments. Fish farming has substantially changed since the last introduction of a novel medicinal compound around the millennium change, so the administration route has to be evaluated thoroughly. In the end, development of anti-sea lice compounds constitute a political question, where the environmental footprint needs to be measured against the impact on coastal societies and economic costs. The future of the fish farming industry does not depend on a novel compound, but a breakthrough would at least offer good premises for further growth.

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ENCLOSED PAPERS I-IV

PAPER 1

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Impact of hydrogen peroxide on hatching ability of egg strings from salmon lice (*Lepeophtheirus salmonis*) in a field treatment and in a laboratory study with ascending concentrations

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Impact of hydrogen peroxide on hatching ability of egg strings from salmon lice (*Lepeophtheirus salmonis*) in a field treatment and in a laboratory study with ascending concentrations

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ABSTRACT

Well boat treatments with hydrogen peroxide are used to control sea lice infections on farmed salmonids in many salmon producing countries. A study was performed to investigate the hatching ability of salmon lice egg strings following exposure to hydrogen peroxide in both a field treatment and a laboratory experiment. Egg strings were collected prior to and following exposure to the test compound and subsequently hatched in a laboratory. In the laboratory experiment, the inhibiting concentration of hydrogen peroxide on egg string hatching was investigated by exposures to various concentrations. No hatching was observed after the field treatment with 1750 mg L^{-1} for 31–32 min at 6.4 °C. Even low concentrations proved to affect the hatching when egg strings were exposed for 36 ± 3 min at 8.0 °C. No infective larvae were observed after exposure to concentrations above 470 mg L^{-1} in the laboratory study. Thus, the current practice of discharging residual well boat hydrogen peroxide treatment water 3 km from the treatment site seems unnecessary and reduces on-site treatment capacity.

1. Introduction

In 2010, 1.5 million tonnes of Atlantic salmon (*Salmo salar*) were produced worldwide with the main producing countries being Norway, Chile, UK, Ireland and Canada (http://www.salmonfarming.org/industry-development/, International Salmon Farmers Association). A Norwegian salmon farm can house up to 2 million fish (10,000 tonnes) during the sea water production phase of around 18–20 months. However, the standing biomass of livestock varies considerably within this range, and is regulated to some extent in all salmon farming countries.

Estimates made by Costello (2009) suggest a worldwide cost of sea lice control at around € 305 million in 2006. This figure includes both surveillance and treatments, making sea lice the most costly parasite in salmon production. Due to preventive measures and strict regulations on the maximum allowed number of parasites per fish (https://www.lovdata.no/for/sf/fi/ti-20121205-1140-0.html), pathological effects are seldom seen in farmed fish in Norway. Infection with salmon lice is, however, potentially fatal for wild salmonid smolts residing in or migrating through waters where the level of infective larvae is high (Birkeland, 1996; Costello, 2009; Torrissen et al., 2013; Tully and Whelan, 1993).

The life cycle of the salmon louse (*Lepeophtheirus salmonis* (Krøyer)) consists of initial pelagic stages followed by parasitical stages. After hatching from the egg string, the pelagic nauplius I larva is free floating in the water masses where it molts first to nauplius II and finally to the infective copepodid stage.

After successfully attaching to a host, the copepodids develop to non-motile chalimus stages, before molting to preadult and final adult stages. Mating takes place only when adults of both sexes are situated on the same fish, and a spermatophore can be transferred from a male to a female (Hull et al., 1998; Ritchie et al., 1996). From one single mating, the female develops a number of successive egg strings, up to 11 pairs have been observed in laboratory experiments. Egg strings are found as singles or pairs, the latter being the most frequent, where one egg string can carry up to 700 embryos (Heuch et al., 2000).

Embryos are successively organized into the egg string while this is folded within the mother (Schram, 2000). The proximal part of the egg string does not contain embryos. When the egg string is completed, it is unfolded externally and remains attached to the mother by a hook (Heuch et al., 2000). The embryos in the egg strings undergo a maturing process while in this position, pigment develops, giving the egg strings a darker color. The egg string membrane ruptures and larvae are released, either while still attached to the mother or while released from the mother (Schram, 2000). Little is published regarding the ultrastructure, molecular composition and permeability of the egg string membrane.

Several medicinal products are used to maintain low levels of salmon lice in fish farms, with hydrogen peroxide (H_2O_2) being one. This compound is mainly used for the immediate removal of pre-adult and adult lice. Treatments with this substance have primarily been performed in well boats, but have also been carried out as regular bath treatment in tarpaulin enclosed cages. Previous studies have demonstrated

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reduced hatching and larval development from exposed salmon lice egg strings (McAndrew et al., 1998; Toovey and Lyndon, 2000).

It is not known whether adult female salmon lice previously exposed to hydrogen peroxide are able to produce viable progeny from later egg strings. Furthermore, uncertainty concerning the hatching ability of egg strings from females exposed to H_2O_2 has resulted in regulations being imposed regarding the dumping of residual water following well boat treatments. These regulations require the dumping of residual water at least 3 km downstream from the fish farm, resulting in a substantial loss of well boat capacity.

The study objective was to investigate potential inhibition of egg string hatching and larval development after exposure to hydrogen peroxide. In addition, the identification of the inhibiting concentration of the compound on hatching was sought. In order to detect a possible link between the level of maturation and hatching success, egg strings were separated according to their pigment status at the time of exposure.

2. Materials and methods

2.1. Field treatment with hydrogen peroxide

A field treatment with hydrogen peroxide, Interox Paramove 50 (IP50), Solvay Chemicals, was performed in April 2012 at a commercial fish farm located in Mid Norway. Average fish weight was 1.8 kg, the water temperature was 6.4 °C and salinity was 35.0‰. The fish stock had not previously been exposed to any delousing agent. In the well boat, three separate wells were used for the anti-parasitical treatment. Once the fish had been transferred to the wells, H_2O_2 was distributed to the water through multiple water jets until the correct concentration was achieved. Each well had a separate titration station, called Applikon 2040, produced by Methron. Every 3 min the concentration level was titrated with sulfuric acid and cerium sulfate, with results presented as mg L^{-1}. This frequency allowed a continuous supply of the delousing agent when the concentration dropped below the desired level. After the exposure period, the residual water was flushed out and replaced with fresh sea water which was pumped into the wells. The treatment conditions are listed in Table 1.

The treatment efficacy was registered by the prescribing veterinarian by counting the number of parasites on individual fish just before and 6 days after treatment. On 67 fish, no adult female salmon lice were found after the treatment. Other motile stages were reduced from 0.75 per fish ($n = 65$) before treatment to 0.10 after treatment. In these terms, the treatment was judged successful. The data are presented in Table 2.

Control egg strings were sampled prior to treatment. Fish were collected from the cage with a landing net and salmon lice were removed from the fish with forceps. The egg strings were transferred directly to a plastic bottle containing 1500 mL of sea water from the site. After treatment, as residual water had been flushed out, salmon lice and egg strings were collected with a plankton mesh. The egg strings were transferred to the NIVA Marine Research Station in the Oslofjord area for the laboratory part of the experiment.

Exposed mature and non-mature egg strings were divided into three groups according to their degree of pigmentation: low pigmentation, medium pigmentation, high pigmentation. All egg strings were cut into 10 mm parts with a scalpel. The hatching equipment was a 1-liter box supplied with running seawater from the bottom. Both the inlet and the outlet water were filtered using a plankton mesh of 50 μm. Each hatching unit contained three egg strings which were 10 mm in length. The water temperature was 8.0 ± 0.4 °C and the salinity was within 33.8 ± 0.1‰ throughout the experiment.

Table 1
Parameters for initiation and maintenance of hydrogen peroxide concentrations in the well boat treatment at 6.4 °C.

Well no.	Total treatment time	Infusion time of H_2O_2	Concentration of H_2O_2 after infusion
1	31 min	6 min	1750 mg L^{-1}
2	31 min	9 min	1750 mg L^{-1}
3	32 min	6 min	1750 mg L^{-1}

Table 2
Treatment efficacy after well-boat treatment with 1750 mg L^{-1} hydrogen peroxide for 31–32 min. Efficacy was registered 6 days after treatment. Numbers are given as total number of parasites, and per fish (mean).

Sea lice category	Before treatment	After treatment
Number of fish counted	65	67
Non-motile lice	9 (mean: 0.14)	2 (mean 0.03)
Motile lice except female adults	49 (mean 0.75)	7 (mean 0.10)
Female adults	11 (mean 0.17)	0 (mean 0.00)

Hatching was observed every two to three days for the 24 days of the study period. The number of live larvae was counted on days 6, 10 and 18 after initiation of the trial. On days 6 and 10, the number of vivid larvae per unit was counted thoroughly by trained personnel. Dead larvae were not counted during the trials. On day 18, the exact number of parasites was registered as each larva was removed one by one from the hatching unit using a plastic pipette.

In both trials, nauplii were scored dead if they showed no sign of movement when lying on the bottom of the hatching unit. Live larvae were recorded as copepodids when they survived for more than 50 degree days after hatching. The results were expressed as number of larvae per 5 mm egg string, and as a function of time.

2.2. Laboratory experiment with hydrogen peroxide

Adult female salmon lice with egg strings were collected at a fish processing plant in December 2012. The fish originated from a fish farm in Mid Norway and had previously been exposed to several anti-parasitic compounds, but not hydrogen peroxide. Salmon lice were removed from the fish with forceps and put into a plastic bottle containing sea water from the site. The biological material was sent to The Norwegian School of Veterinary Science, Oslo, by air freight and arrived the following day. The water temperature was 7.0 °C upon arrival. Egg strings were divided into three groups similar to the field treatment egg strings and cut into 5 mm pieces with a scalpel. The sea water used for exposure to hydrogen peroxide was taken from a depth of 60 m at the NIVA Marine Research Station, Solbergstrand, Norway on the day before the arrival of the material.

Two hydrogen peroxide formulations were tested in parallel: Interox Paramove 50 (IP50), Solvay Chemicals, batch PM50-255 and Hydrogenii peroxidum 30% (HP30), Apotekproduksjon AS, Oslo, batch 11F049/2. Four egg strings from each group were exposed to approximately 470, 1000, 1500 and 2000 mg L^{-1} of hydrogen peroxide, 24 h after sampling. The H_2O_2 concentrations were calculated as weight per volume of sea water. Two egg strings from each pigment level were exposed to each of the test substances.

Egg strings were exposed to their respective concentrations in 70 mL containers which were placed on a shaker plate (Biosan Orbital Shaker) for 36 ± 3 min. After exposure, the egg strings were washed in three successive sea water baths. The egg strings were then returned to the rinsed sea water filled container and incubated at 12.0 °C for 16–18 h. Finally, the egg strings were randomly distributed to the hatching equipment described by Hamre et al., 2009. The input water temperature was 7.8 ± 1.1 °C and the salinity was 34.4 ± 0.2‰ throughout the trial. The hatching was observed every two to three days until the end of the experiment on day 28. The groups were blinded for the observer. Since distinguishing between nauplii and copepodids was challenging, parasites of both categories were registered and referred to as "larvae" in both experiments and in this paper. To be

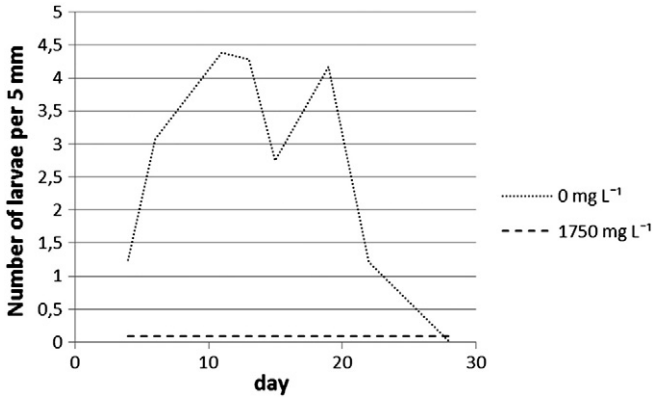

Fig. 1. Field trial. Curves representing mean number of salmon lice larvae (nauplii and copepodids) per 5 mm egg string during the 28 day study period.

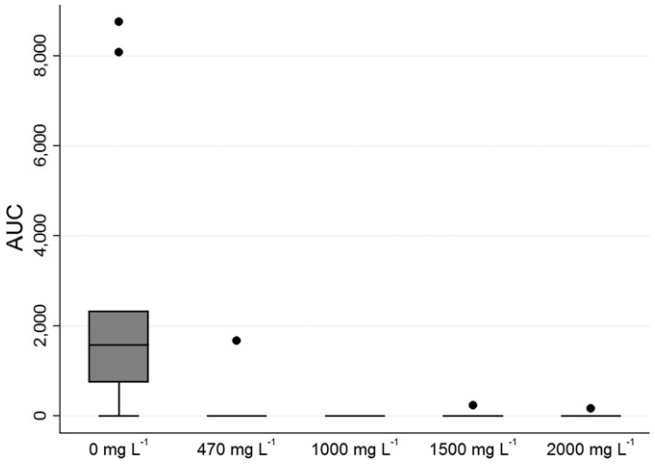

Fig. 3. Boxplot of AUC values of larvae in control group and the four treatment categories. The results are demonstrating that all categories had AUC values close to zero, except the group exposed to 470 mg L^{-1}, which produced a low number of larvae that did not develop to the infective stage.

classified as copepodid larvae, a group of parasites in a hatching unit had to remain alive for more than 50 degree days.

2.3. Data management and statistical methods

Data was compiled in a spreadsheet (Microsoft Excel) and controlled for errors by checking minimum and maximum values. The data was then re-read for entry errors. The statistical unit was the 5 mm egg string. Results are expressed as the number of larvae per 5 mm egg string and as a function of time (Figs. 1 and 2 from Excel). Data was transferred to Stata (StataCorp LP) for further graphical presentation and statistical analysis (Fig. 3).

The AUC (area under curve) was calculated using the trapezoidal method for every unit to evaluate the development of larvae over time. Results from the laboratory trial were graphically presented in a boxplot, Fig. 3. A non-parametric test (Wilcoxon signed-rank test) was used to decide whether AUC variables of treatment groups differed from the control group, in both the field trial and the laboratory trial. The effect of pigmentation level was assessed with one-way ANOVA test performed with JMP software (SAS).

3. Results

3.1. Field experiment

The treatment efficacy is presented in Table 2. Adult females were reduced from 0.17 to 0.00 individuals per fish, other motile stages were reduced from 0.75 to 0.10 per fish. Hatching and development data is presented in Table 3. The number of viable larvae increased with the level of pigmentation in the control group. Out of 18 control egg strings, 13 hatched almost completely. Exposed egg strings did not produce viable progeny. However, in one hatching unit containing highly pigmented egg strings at start, two of these egg strings were to a certain extent swollen and 10 dead naupliar larvae were observed on day 8. These larvae were not observed floating or swimming at any given time point. Hatching was initiated, but no viable larvae were observed in the container. A graphical presentation of the results from the field experiment is found in Fig. 1.

The hatching represented as AUCs were compared with the Wilcoxon-test, giving a highly significant result between treated and non-treated egg strings ($P < 0.005$).

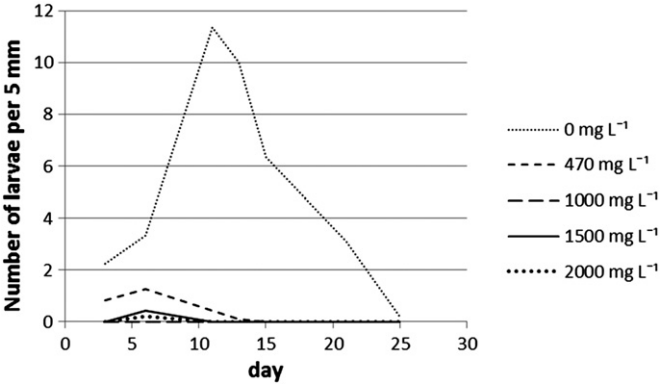

Fig. 2. Laboratory trial. Curves representing mean number of salmon lice larvae (nauplii and copepodids) per 5 mm egg string over a period of 24 days, corresponding to initial exposure concentration.

3.2. Laboratory experiment

The same trend was demonstrated in the laboratory experiment, where seven out of nine control group egg strings hatched, and produced a variable number of naupliar larvae. Out of 46 exposed egg strings a total number of 12 hatched, giving a total of 32 viable naupliar larvae, of which 25 originated from two egg strings exposed to a concentration of 470 mg L^{-1} of hydrogen peroxide. Five of these larvae were alive for up to 48 degree days, the rest lived for a shorter period. None of the larvae reached the copepodid stage. Of the 12 egg strings that hatched to a certain degree, nine produced small amounts of larvae that were not registered as viable. From the remaining 34 egg strings no larvae were observed. The control group larvae lived for up to 21 days, the majority staying alive for more than 10 days, indicating a development to copepodids for the majority of the planktonic larvae. The results are presented in Table 4 and Fig. 2.

Table 3
Hatching and development of larvae from egg strings exposed to a field treatment with IP50, 1750 mg L^{-1} compared to control egg strings. The hatching success and the number of larvae counted on days 6, 10 and 18 are presented as number of hatched egg strings/viable larvae.

	Pigmentation level at start	0 days	6 days	10 days	18 days
0 mg H_2O_2 L^{-1}	Low (N = 6)	0/0	0/0	3/12	3/2
	Medium (N = 6)	0/0	2/16	5/59	5/12
	High (N = 6)	0/0	4/95	5/97	5/135
1750 mg H_2O_2 L^{-1}	Low (N = 9)	0/0	0/0	0/0	0/0
	Medium (N = 9)	0/0	0/0	0/0	0/0
	High (N = 9)	0/0	0/0	2/0	2/0

Table 4
Hatching and development of larvae in the laboratory experiment. Control groups were exposed to sea water, the other groups were exposed to 470, 1000, 1500 or 2000 mg L^{-1} hydrogen peroxide, IP50 and HP30. The hatching success and the number of larvae counted on days 6, 10 and 15 are presented as number of hatched egg strings/viable larvae.

	Pigmentation level at start	0 days	6 days	10 days	15 days
0 mg H_2O_2 L^{-1}	Low (N = 3)	0/0	0/0	1/10	2/9
	Medium (N = 3)	0/0	0/0	3/42	3/13
	High (N = 3)	0/0	1/30	2/50	2/40
470 mg H_2O_2 L^{-1}	Low (N = 4)	0/0	1/15	2/5	2/0
	Medium (N = 4)	0/0	0/0	0/0	0/0
	High (N = 4)	0/0	0/0	2/0	2/0
1000 mg H_2O_2 L^{-1}	Low (N = 4)	0/0	0/0	0/0	0/0
	Medium (N = 4)	0/0	0/0	0/0	0/0
	High (N = 4)	0/0	0/0	2/0	2/0
1500 mg H_2O_2 L^{-1}	Low (N = 4)	0/0	1/5	0/0	0/0
	Medium (N = 4)	0/0	0/0	1/0	1/0
	High (N = 4)	0/0	1/2	2/0	2/0
2000 mg H_2O_2 L^{-1}	Low (N = 3)	0/0	1/2	1/0	1/0
	Medium (N = 4)	0/0	0/0	0/0	0/0
	High (N = 3)	0/0	0/0	2/0	2/0

From these data, control group AUC and exposed group AUC from both trials were summarized in a table (Table 5). The effect of hydrogen peroxide on the hatching of egg strings with different degrees of pigmentation is shown in this table. The effect on medium versus low-pigmented egg strings was tested by ANOVA. No significant effect of pigmentation could be demonstrated ($p = 0.35$). The results from egg strings with low and medium pigmentation were therefore combined in further statistical analyses.

A boxplot was created for the laboratory trial (Fig. 3). The Wilcoxon-test was performed to compare the AUC values of larvae from control and exposed egg strings, rendering a P-value <0.005, which describes a highly significant difference.

4. Discussion

In the field trial, 50% of the low pigmented control egg strings failed to hatch. Prior to sampling, the mean number of adult female lice per fish was = 0.17 and the mean number of other motile stages was 0.75 per fish. Considering that non-fertilized females also produce egg strings, the low hatching rate may be explained by the absence of mating with male and subsequent failure to produce fertilized eggs (Krkosek et al., 2013; Stormoen et al., 2013). Toovey and Lyndon (2000) observed that hatching rate increased with late egg string separation from the mother. Physical stimuli such as change of temperature may also influence hatching ability. Other reasons for lack of hatching from medium or highly pigmented egg strings may be the result of misclassification, or handling at crucial time points during the hatching process. The results indicate that laboratory hatching of egg strings is unpredictable and requires relatively large groups of egg strings in order to render reliable results.

Table 5
Mean area under curve (AUC) for larval development from salmon louse egg strings exposed to various concentrations of hydrogen peroxide. The egg strings were sorted by level of pigmentation prior to exposure.

Field experiment

	0 mg L^{-1}	1750 mg L^{-1}
Pigment: low	128	0
Pigment: medium	918	0
Pigment: high	3628	0

Laboratory experiment

	0 mg L^{-1}	470 mg L^{-1}	1000 mg L^{-1}	1500 mg L^{-1}	2000 mg L^{-1}
Pigment: low	784	417	0	0	56
Pigment: medium	1744	0	0	60	0
Pigment: high	5616	0	0	0	0

In the laboratory trial, every tested concentration of hydrogen peroxide affected the hatching ability of egg strings, independent of the developmental stage of the embryos. Furthermore, even the lowest concentration of 470 mg L^{-1} hydrogen peroxide inhibited development of copepodids from exposed egg strings.

Lethal effect of hydrogen peroxide on salmon lice is thought to be caused by the presence of oxygen bubbles in the hemolymph (Bruno and Raynard, 1994; Thomassen, 1993). A direct effect on membranes is also probable, possibly disrupting the integrity of the egg sac and the embryos. Egg strings with low level of pigment hatched more successfully than highly pigmented egg strings when exposed to a low concentration of hydrogen peroxide. This indicates a reduced effect of the compound on immature compared to mature egg strings.

In this study, two separate experiments sharing several similarities were performed. Similar, but not identical equipment was used for the hatching studies in the studies. Egg string length was adjusted between the field experiment and the laboratory experiment. Furthermore, the field trial was performed in May, and the laboratory trial was performed in December. Heuch et al. (2000) described a seasonal difference in the number of embryos per egg string. Season and equipment may have contributed to the variable hatching rate of the control group egg strings.

Converting field conditions to the laboratory is not always conceivable. Similarly, laboratory results from controlled conditions cannot always be transferred to field situations since there will be many uncontrollable variables in the field. Hence, the interface of the two experiments provides core information about the subject.

The purpose of grouping the egg strings according to pigmentation level is supposed to reflect the state of maturation. In the current study, the pigmentation was classified by comparing the egg strings with the color of a template. However, individual differences may be expressed as nuances which are potentially hard to detect and a certain biological diversity will influence both color and development time. Egg strings express pigment during a short period in the latter part of the development. In fish farms, egg strings containing a small amount of pigment will in most cases outnumber highly pigmented egg strings at any time. In the current study, the challenge lay in obtaining enough highly pigmented egg strings. Separation into two instead of three groups might have been a better option.

In previous experiments (Johnson et al., 1993; Toovey and Lyndon, 2000), 30 minute bath exposures were used, where egg strings were situated in still water during the exposure. In field treatments, a certain period of up-dosing is required in order to reach the treatment concentration. The substance also remains in the treatment volume as a declining concentration during re-fill of water. Furthermore, the recommended therapeutic concentration varies with water temperature. In this trial, an approximation to field conditions was desired, resulting in exposure exceeding 30 min.

The study results were in accordance with what others have found. Johnson et al. (1993) discovered that salmon lice egg strings exposed to 1500 mg L^{-1} in a laboratory were less likely to produce viable progeny than control group egg strings. Toovey and Lyndon (2000) observed similar results, although some hatching and development to the copepodid stage were registered. However, no stirring of the exposed medium was carried out in these studies. This seems to be a crucial part of a laboratory set-up, since it mimics field treatment conditions. When no stirring was performed inconsistent results were observed (data not shown). However, with stirring, the results became constant.

In the studies performed by Johnson et al. (1993) and Toovey and Lyndon (2000), the effect of low, presumably sub-lethal concentrations for the parasite were not studied. Thus, the current observation that

even exposure to 470 mg L^{-1} affects the hatching of egg strings and development of larvae is novel.

Control measures against salmon lice in fish farms are more successful when aimed at being effective against all instars. As shown in this experiment, treatment with hydrogen peroxide is fatal to egg strings. The effect against non-motile stages is thought to be insufficient (Johnson et al., 1993), possibly due to chalimus larvae tending to "hide" between the skin shells. This may result in reduced mortality of this instar in field treatments. Questions have been raised as to whether or not the effect on motile stages is lethal, as eventual survivors would be able to re-attach to fish. The occurrence of this scenario, though, is considered to be diminutive, as adults, and females in particular, are poor swimmers. This experiment indicates that the well boat procedure of dumping residual water at a pre-designated location about 3 km away from the site is not necessary for the protection of fish stock from copepodids originating from egg strings on female parasites immobilized by hydrogen peroxide treatment.

Even though egg strings attached to the mother were rendered incapable of hatching and producing viable progenies after exposure to hydrogen peroxide, it would also be of interest to study whether or not the next pairs of egg strings produced by an adult female would be sterile in upcoming studies.

Resistance against hydrogen peroxide has been reported in salmon lice (Treasurer et al., 2000). It is not known whether resistant salmon lice are able to produce resistant embryos, resulting in resistant egg strings.

5. Conclusion

This study showed that the impact made by even low concentrations of hydrogen peroxide on egg strings is comprehensive. The egg strings are vulnerable, and represent an ideal target for salmon lice prevention when combined with substances killing living parasites. Some of the trial concentrations were sub-therapeutic, as the concentrations in field treatments usually span from 1500 to 1750 mg L^{-1}. This should serve a certain long-term effect at salmon lice populations in fish farms, as the immediate reproductive output will be minimized after the procedure.

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PAPER 2

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First report of reduced sensitivity towards hydrogen peroxide found in the salmon louse *Lepeophtheirus salmonis* in Norway

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Short communication

First report of reduced sensitivity towards hydrogen peroxide found in the salmon louse *Lepeophtheirus salmonis* in Norway

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ABSTRACT

Reduced sensitivity towards chemotherapeutants in the salmon louse *Lepeophtheirus salmonis* (Krøyer) is an increasing problem for the fish farming industry. Most fish farmers are dependent on chemical treatments in order to maintain salmon lice numbers below permitted levels. However parasites showing reduced sensitivity contribute to complicating this task. Hydrogen peroxide (H_2O_2) is used as a delousing agent in bath treatments and until recently treatment failures due to reduced H_2O_2-sensitivity have not been documented in Norway. The aim of the current study was to develop a bioassay protocol suitable for testing H_2O_2-sensitivity in *L. salmonis*. If failed treatments were found to be caused by parasite insensitivity to H_2O_2 the possibility of this reduced sensitivity being hereditary was looked into. The results show that bioassays permit differentiation between strains of salmon lice with regards to H_2O_2-sensitivity, coinciding with treatment efficacies. Up to ten times variance in sensitivity between two strains was recorded. The progeny of the least sensitive salmon lice also showed reduced sensitivity to H_2O_2 in a bioassay, which indicates that reduced sensitivity towards H_2O_2 is hereditary. The current study presents the first case report of reduced sensitivity towards H_2O_2 in salmon lice in Norway. This change in sensitivity imposes a threat to the Norwegian fish farming industry and should be monitored closely.

1. Introduction

The salmon (or sea) louse *Lepeophtheirus salmonis* (Krøyer) infests both wild and farmed salmonids in the Northern Hemisphere (Costello, 2006; Pike and Wadsworth, 2000; Torrissen et al., 2013). Control of the salmon lice in fish farms is important in order to protect the farmed fish from parasite-related stress and injuries as well as minimizing the infestation pressure on wild salmonids. Most fish farms are dependent on chemical treatments in order to keep the parasite numbers below national maximum permitted levels, but few chemical treatment agents are available (Burka et al., 1997; Roth, 2000; Westcott et al., 2004). This has subsequently led to the development of reduced sensitivity in *L. salmonis* towards most of the available chemical treatments (Jones et al., 1992; Lees et al., 2008a; Roth et al., 1996; Sevatdal and Horsberg, 2003; Treasurer et al., 2000). The geographical dispersion of the reduced sensitivity towards each treatment agent varies (Grøntvedt et al., 2014; Jones et al., 2013; Lees et al., 2008b). Chile, the major salmonid producing country in the Southern Hemisphere is having problems with the sea louse *Caligus rogercresseyi* (Johnson et al., 2004). In Chile, chemical treatments are essential in order to control sea lice levels; however this task has become increasingly difficult as the parasites have developed reduced sensitivity towards both pyrethroids and the avermectin emamectin benzoate (Bravo et al., 2008, 2013; Helgesen et al., 2014).

In Norway, hydrogen peroxide (H_2O_2) was used to a certain extent as a delousing agent between the years 1993 and 1997 (Grave et al., 2004). This use was terminated due to the introduction of more efficient chemicals with larger safety margins. The emerging occurrence of reduced sensitivity towards other compounds however led to the re-introduction of H_2O_2 for salmon lice treatment in 2009 (Norwegian Institute of Public Health, 2014). In order to delay the development of reduced sensitivity, an increased range of chemical treatment agents is desirable. This permits rotation between compounds with various modes of action subsequently postponing the development of reduced sensitivity (Brooks, 2009; Denholm et al., 2002). Unfortunately, the extensive use of H_2O_2 as a delousing agent in various parts of Norway (Grøntvedt et al., 2014) has increased the risk of developing reduced sensitivity towards

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this compound. In Scotland, reduced sensitivity towards H_2O_2 in salmon lice was reported in the year 2000, following extensive use of this treatment agent over several years (Treasurer et al., 2000). Due to the toxicity and low safety margin of H_2O_2 in fish, only minor increases in treatment time and concentration can be introduced (Bruno and Raynard, 1994; Thomassen, 1993). In order to avoid unsuccessful treatments due to reduced parasitic sensitivity, a method for sensitivity testing in salmon lice is therefore required.

Incidences of reduced treatment efficacy following H_2O_2-treatments in Norway have raised the issue of possible reduced sensitivity. Treatment failure may be due to inadequate delousing procedures or reduced sensitivity in the parasites (Denholm et al., 2002). Biological assays (bioassays) are employed to test for reduced sensitivity in arthropods when the resistance mechanisms are unknown (Robertson et al., 2007). Bioassays have been developed for sensitivity testing in *L. salmonis* towards pyrethroids, emamectin benzoate and the organophosphate azamethiphos (Helgesen and Horsberg, 2013; Sevatdal and Horsberg, 2003; Westcott et al., 2008; Whyte et al., 2013). Treasurer et al. (2000) tested for H_2O_2-sensitivity using small scale treatments, however this method requires the use of fish and due to animal welfare and practical reasons should be avoided if possible.

Possible mechanisms for reduced H_2O_2-sensitivity in salmon lice include increased antioxidant enzymes activity such as catalase, glutathione peroxidase or glutathione-S-transferase, all of which have been found in mammalian cells in conjunction with H_2O_2-resistance (Fiander and Schneider, 2000; Spitz et al., 1992; Baud et al., 2004). Increased catalase activity has also been seen in H_2O_2-resistant bacteria and fungi (Amin and Olson, 1968; Elkins et al., 1999; Nakamura et al., 2012; Uhlich, 2009). Other possible enzymes involved in reduced H_2O_2 sensitivity are superoxide dismutase, superoxide reductase, glutathione reductase and thioredoxin, as they are proven to possess activity against reactive oxygen species (Nordberg and Arner, 2001).

The primary objective of the present study was to develop bioassay protocols suitable for H_2O_2-bioassays. Whether reduced treatment effects from H_2O_2-treatments were caused by reduced salmon lice sensitivity was also investigated. This required using bioassays on both field collected parasites and their laboratory reared progeny.

2. Materials and methods

2.1. Salmon lice

Salmon lice originating from seven different farms in Norway were employed in the current study (Table 1). All salmon lice with the exception of Ls A originated from the northern part of Mid-Norway. Ls V came from a farm reporting of reduced treatment efficacy in H_2O_2-treatment since one year back. The treatment performed three months prior to parasite collection for field bioassays had 74.1% efficacy in mobile stages (salmon lice from 80 fish collected from 4 fish cages were counted on the day of treatment and on the following day to calculate efficacy). The treatment efficacy was 51.4% in mobile stages in the treatment performed one month before the Ls V F0 bioassay was performed (salmon lice from 30 fish from 3 cages were counted on two consecutive days, with treatment on day 1, to evaluate treatment efficacy). Ls Ky, Ls D and Ls Aa all came from farms with a history of H_2O_2-treatments against salmon lice, but without having experienced reduced treatment efficacies. Ls S and Ls Kl originated from the same area, but from farms which had not treated with H_2O_2 themselves. Ls A was a laboratory strain originally collected from the northern part of North-Norway in 2011. Bioassays and small scale treatments had shown this strain to be sensitive to pyrethroids, azamethiphos and emamectin benzoate (Helgesen and Horsberg, 2013). Ls A had never been exposed to H_2O_2, neither in field nor in the laboratory.

The salmon lice designated for field bioassays were collected from anesthetized fish at the sea farms and transported in cooled sea water to the laboratory. These bioassays, six in total, were performed by Aqua Kompetanse AS at their laboratory in Flatanger, Norway. The other five bioassays were performed at The Norwegian University of Life Sciences in Oslo, Norway. Table 1 provides the details. The salmon lice for the five latter assays were reared on fish at the NIVA Marine Research Station in Drøbak, Norway. Ls A was kept in a continuous culture on Atlantic salmon (*Salmo salar*), while Ls V was sent to the laboratory as egg strings. After hatching and development into copepodites, 20 sea trout weighing about 150 g each were infested with the parasites. Approximately 50 copepodites per fish were employed for the infestation, which was conducted in 30 l of aerated sea water for 45 min. Pre-adult parasites and adult males were used for the bioassays depending on which instars were available at the time. All bioassays were initiated within 8 h after parasite collection.

2.2. Bioassays

Two types of bioassays were performed: 30-min and 24-h bioassays. The 30-min bioassays were performed according to the protocol for pyrethroid bioassays described in Sevatdal and Horsberg (2003) with some modifications. The parasites were exposed to between six and twelve different concentrations of H_2O_2 using Interox Paramove 50 (H_2O_2 50%, w/w, Solvay Chemicals, Belgium) in Oslo and Eka HP T49 S (H_2O_2 49.7%, w/w, Azko Nobel, Sweden) in Flatanger, diluted in sea water. Nominal concentrations ranging from 0 to 5000 mg L^{-1} were utilized and varying concentrations within this range were chosen according to the expected sensitivity level of the respective salmon lice strain. The salmon lice were distributed in sea water filled polystyrene bioassay boxes, with approximately 10 parasites (6–13) in each box. The different concentrations of H_2O_2 were prepared by adding the appropriate amount of H_2O_2 to cooled sea water (10–12 °C) in one litre polypropylene containers. In each of the concentrations applied in each bioassay, two of the bioassay boxes were submerged. For the field bioassays with Ls Ky, Ls D, Ls Aa and Ls Kl only one box of parasites was used for the control group. The boxes were kept in the solution for 30 min and the results were immediately recorded. Parasites attached to the wall of the box or swimming in a straight line were considered alive. All others were regarded as immobilized or dead. For the bioassays on Ls A and Ls V F1 the boxes containing the parasites were relocated to fresh sea water after the initial evaluation, without removing any parasites, and kept at 12 °C under constant aeration for the following 24 h. The results were then re-evaluated.

The 24-h bioassays were performed according to the protocol for pyrethroids, azamethiphos and emamectin benzoate bioassays described in Helgesen and Horsberg (2013), with some modifications. Between eight and seventeen parasites from Ls A were exposed to six or seven different nominal concentrations of H_2O_2, ranging between 0 and 120 mg L^{-1}, in one litre glass bottles. The bottles were kept at 12 °C for 24 h and supplied with constant aeration. Ls V was exposed to 11 different nominal H_2O_2-concentrations between 0 and 1800 mg L^{-1}. Between 30 and 61 parasites were used for each concentration. After the exposure period the results were recorded by turning the bottles upside down three times and then moving them in circles with a diameter of 20 cm 10 times. When the water had settled it was poured out into a beaker. All parasites remaining in the bottle or able to attach to the beaker wall or swim in a straight line were considered alive. All other parasites were regarded as immobilized or dead.

Table 1
Bioassays. All salmon lice strains were collected in the northern part of Mid-Norway with the exception of Ls A which came from the northern part of North-Norway. Ls V F0 and Ls V F1 were the same strain, but bioassays were performed both with parasites from the field (F0) and with laboratory-reared parasites from field collected egg strings (F1). Two types of bioassays were performed, with both 30 min and 24 h parasite exposure to hydrogen peroxide. Six to eleven different concentrations were employed for each bioassay and the dose-response curve were modelled in JMP (SAS Institute Inc., Cary, NC, USA). The concentrations in mg L^{-1} immobilizing 50% of the parasites (EC_{50}) with 95% confidence intervals (CI) are given.

Strain	Origin	Date	Place	Assay	EC_{50}	Lower 95% CI	Upper 95% CI
Ls A	Laboratory	10.10.2013	Oslo	30-min	216	153	305
Ls V F1	Laboratory	13.11.2013	Oslo	30-min	1767	1494	2090
Ls V F0	Field	11.10.2013	Flatanger	30-min	2127	1253	3610
Ls S	Field	03.02.2014	Flatanger	30-min	539	386	754
Ls Ky	Field	03.02.2014	Flatanger	30-min	693	483	993
Ls D	Field	31.01.2014	Flatanger	30-min	563	435	730
Ls Aa	Field	30.01.2014	Flatanger	30-min	541	403	727
Ls Kl	Field	30.01.2014	Flatanger	30-min	538	373	776
Ls A	Laboratory	12.02.2014	Oslo	24-h	45.9	29.8	70.9
Ls A	Laboratory	23.06.2014	Oslo	24-h	64.7	43.4	96.4
Ls V F1	Laboratory	13.11.2013	Oslo	24-h	138	112	171

The bioassay results were modelled using probit-analysis in JMP 10.0.0 (SAS Institute Inc., Cary, NC, USA) to find EC_{50}-values, which is the concentration immobilizing 50% of the parasites, with 95% confidence intervals (CI).

3. Results

Ls A had the lowest EC_{50}-values for both the 30-min and the 24-h bioassays with 216 and 45.9 to 64.7 mg L^{-1} respectively. Ls V had the highest EC_{50}-values for both types of bioassays, with EC_{50}-values of 1767 and 2127 mg L^{-1} in the 30-min bioassays and 138 mg L^{-1} in the 24-h bioassay.

The five other strains tested in the 30-min bioassay showed intermediate EC_{50}-values, ranging from 538 to 693 mg L^{-1}. The dose-response curves for Ls A and Ls V F1 with 90% CI are presented in Fig. 1 (30 min exposure) and Fig. 2 (24 h exposure). All results after modelling are displayed in Table 1.

No control group mortality was seen in any of the bioassays with 30 min exposure and immediate evaluation. In the 24-h bioassay, 0 and 8.3% mortality was observed in the Ls A control groups, while 7.5% of the parasites in the Ls V-bioassay-control group were dead after 24 h.

With respect to the results for the 30-min Ls A and Ls V F1 bioassays, fewer parasites were regarded as dead or immobilized at the re-evaluation 24 h after exposure than in the first evaluation. With respect to Ls A the total percentage immobilized parasites decreased from 68 to 51% when the results from all concentrations were included. For Ls V F1 the same group decreased from 48 to 21%. The attempts to model the re-evaluation results gave illogical dose-response curves.

4. Discussion

Assuming that H_2O_2-sensitivity is similar in both attached and free-swimming parasites; one would expect reduced treatment efficacy from treatment of fish infested with Ls V. The treatment regime, outlined in the summary of product characteristics for Paramove (49.5% H_2O_2, Solvay chemicals), is exposure to 1500 mg L^{-1} H_2O_2 for 20 min (Norwegian Medicines Agency, 2014). In the Ls V bioassays, less than 50% of the parasites were immobilized when exposed to this concentration for 30 min. The results from the bioassays may not however be directly interpreted into treatment results. The bioassays were performed on parasites detached from the fish and sensitivity to chemicals may differ between parasites attached to and detached from the fish (Sevatdal and Horsberg, 2003). Furthermore during treatment, a constant H_2O_2-level is maintained through-out the exposure time. In contrast the bioassays were performed by adding the substance to sea water only at the beginning of exposure. In order to develop bioassays into an accurate prediction tool for treatment efficacy, the correlation between bioassay results and treatment results need to be elucidated.

The intermediate bioassay results found in five of the tested strains could be a sign of reduced sensitivity. This hypothesis is supported by the fact that all of the lower 95% CI values in these strains were above the upper 95% CI for the fully sensitive strain, Ls A. The intermediate results could also represent the range of EC_{50}-results found in sensitive parasites. This latter theory is supported by the results obtained by Bruno and Raynard (1994). Evaluating their bioassay results using probit analysis in JMP, an EC_{50}-value of 890 mg L^{-1} could be calculated for pre-adult parasites and 503 mg L^{-1} for adult salmon lice. In an experiment where salmon lice infested salmon were treated with different concentrations of H_2O_2, an EC_{50}-value of 800 mg L^{-1} was obtained (Thomassen, 1993). In both trials results were evaluated immediately after exposure. However the shorter exposure period in these trials (20 min) would have increased the EC_{50}-values. This in comparison to a protocol with 30 min exposure time. Furthermore both experiments were performed at lower temperatures (10 °C in Bruno and Raynard's experiment and 6–9 °C in Thomassen's experiment) than in the current study, which might also have affected the results. The toxic effect of H_2O_2 on salmon lice increases at higher temperatures (Treasurer and Grant, 1997). EC_{50}-values would therefore be expected to be higher at lower temperatures. Thomassen's experiment was performed on parasites attached to fish. Given that salmon lice are more susceptible to pyrethroid bath treatments when detached from fish, (Sevatdal and Horsberg, 2003) and if this is also the case for H_2O_2, then the same parasites would have shown a lower EC_{50}-value if Thomassen's experiment had been performed on parasites which had been removed from the fish. Whether the differences in EC_{50}-values between different life stages of salmon lice, as seen by Bruno and Raynard (1994), were present in the current study, was not evaluated. If differences were present this might have biased the results as the parasitic instars were not allocated evenly within or between the experiments. Future research should look into the possible differences of H_2O_2-sensitivity between instars of *L. salmonis* as this might influence both sensitivity assessments and treatment effects.

A large proportion of the parasites immediately characterized as immobilized, were 24 h later re-evaluated as alive. Recovering parasites after H_2O_2-exposure were also observed by Hodneland et al. (1993), Johnson et al. (1993) and Treasurer and Grant (1997). This implies that salmon lice have mechanisms to fight the effect of H_2O_2. These mechanisms seem to be induced by the treatment, as sensitive parasites are immediately knocked down and

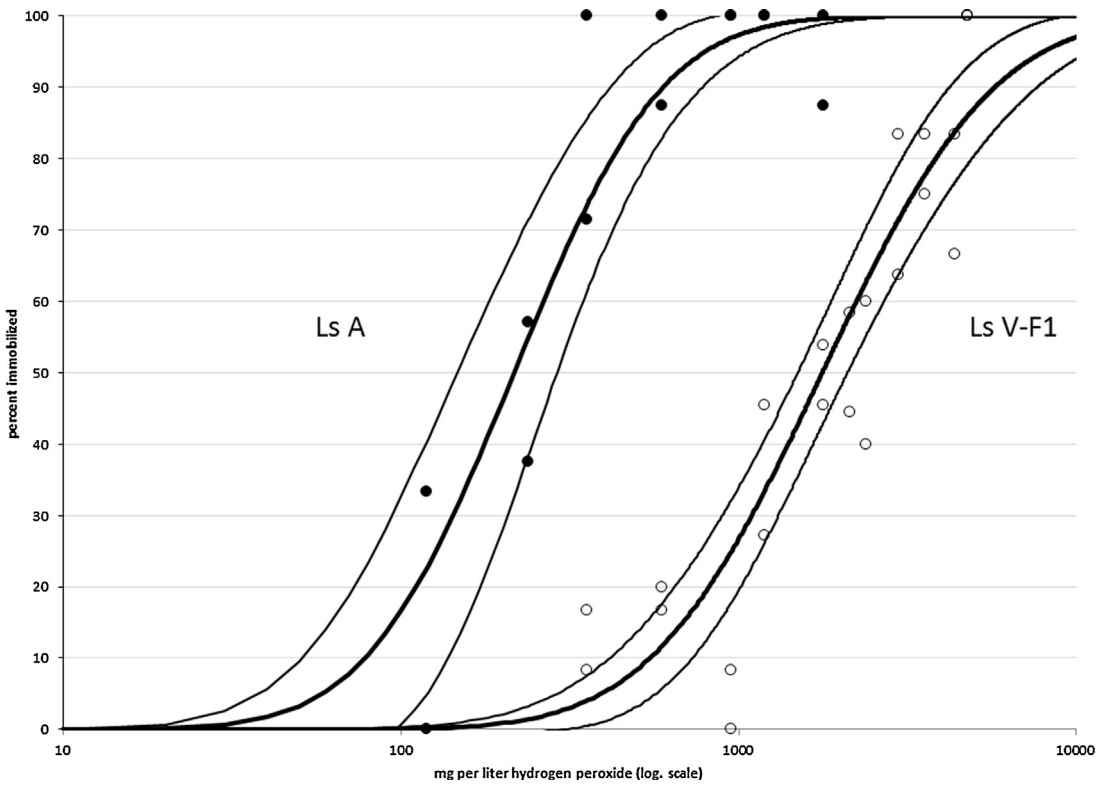

Fig. 1. 30-min bioassay. The dose-response curves (90% CI) and the observed immobilization rates for two of the salmon lice strains tested for sensitivity towards hydrogen peroxide in a 30-min bioassay. The observed rates for the sensitive strain (Ls A) are indicated by filled circles, while the corresponding values for the strain with reduced sensitivity (Ls V) are indicated by open circles.

later recover. Recovery, following treatment-induced immobilization, was observed to a greater extent in parasites from the least sensitive strain. This was in addition to the fact that a higher concentration of H_2O_2 was necessary for their immobilization. The cause of this might be dual; both an increase in the already existing inducible mechanism and an additional mechanism that is present prior to exposure. The exact mechanisms behind reduced sensitivity towards H_2O_2 in salmon lice are not yet clarified, and research into this field is therefore required. The knowledge on mechanisms for reduced sensitivity is important in order to develop mechanism-specific tools for sensitivity testing and -surveillance, as well as avoiding pursuing existing mechanisms when developing new chemicals treatment agents (Denholm et al., 2002).

Due to the revitalization of the parasites the results from the 30 min-exposure bioassays could not be evaluated in the same manner as the Sevatdal and Horsberg (2003) pyrethroid bioassays. Sevatdal and Horsberg allowed a waiting period of 24 h in fresh sea water following the exposure period. When this protocol was applied to the H_2O_2-bioassays, it resulted in illogical dose-response curves. The immediate evaluation of the bioassays however gave plausible dose-response curves in addition to low control group mortalities. This was therefore chosen for the H_2O_2-bioassay protocol. The chosen protocol was considered a good proxy for lice survival under farm treatment conditions due to similar exposure periods. The parasites that recovered after first being immobilized were not considered important with regards to field treatments, as they were not seen to re-infest fish in the study by Treasurer and Grant. This coincides with the Norwegian field treatment experience. However, a laboratory study showed that both adult males and pre-adult parasites have the ability to re-infest salmon independent of the parasites' previous exposure to H_2O_2 (McAndrew et al., 1998).

The 24-h bioassay also differentiated between parasite strains with differences in sensitivity. The EC_{50}-value for the least sensitive strain was two to three times higher than for the most sensitive strain. The lower difference in EC_{50}-value, compared to the 30-min bioassays, might be caused by the mechanisms for reduced sensitivity and/or H_2O_2-degradation. Since exposure time does not exceed 30 min in normal H_2O_2 field treatments, it may be concluded that the parasite has not undergone selection in order to develop mechanisms to withstand long term H_2O_2-exposure. Developing a 24-h bioassay is an attempt to standardize all salmon lice bioassays to the set up by Helgesen and Horsberg (2013), thereby making field bioassays easier to perform. To accomplish this task a degradation curve of H_2O_2 under the circumstances given in the bioassay protocol should be employed and the correlation between bioassays and treatment results should be established.

The development of reduced sensitivity towards H_2O_2 in *L. salmonis* was expected, as reduced sensitivity towards pyrethroids, azamethiphos and emamectin benzoate has previously been described by bioassays performed in Norway (Grøntvedt et al., 2014; Helgesen and Horsberg, 2013; Sevatdal et al., 2005; Sevatdal and Horsberg, 2003). As a result of the development of reduced sensitivity towards other available chemical treatments, the use of H_2O_2 for anti-salmon lice treatments has increased since the product was re-introduced to the Norwegian market in 2009. In 2013, 8262 metric tonnes of H_2O_2 was applied in treatment while the figure was 2538 metric tonnes the previous year (Norwegian Institute of Public Health, 2014). The outbreak of amoebic gill disease (AGD) may also have contributed to the increased use of H_2O_2 in 2013. Treatments against AGD will nonetheless simultaneously combat infested salmon lice and therefore impose selection pressure on both parasitic species. Grøntvedt et al. (2014) showed that the most intense H_2O_2 treatment regime against salmon lice was found in the northern part of Mid-Norway and in the southern part of

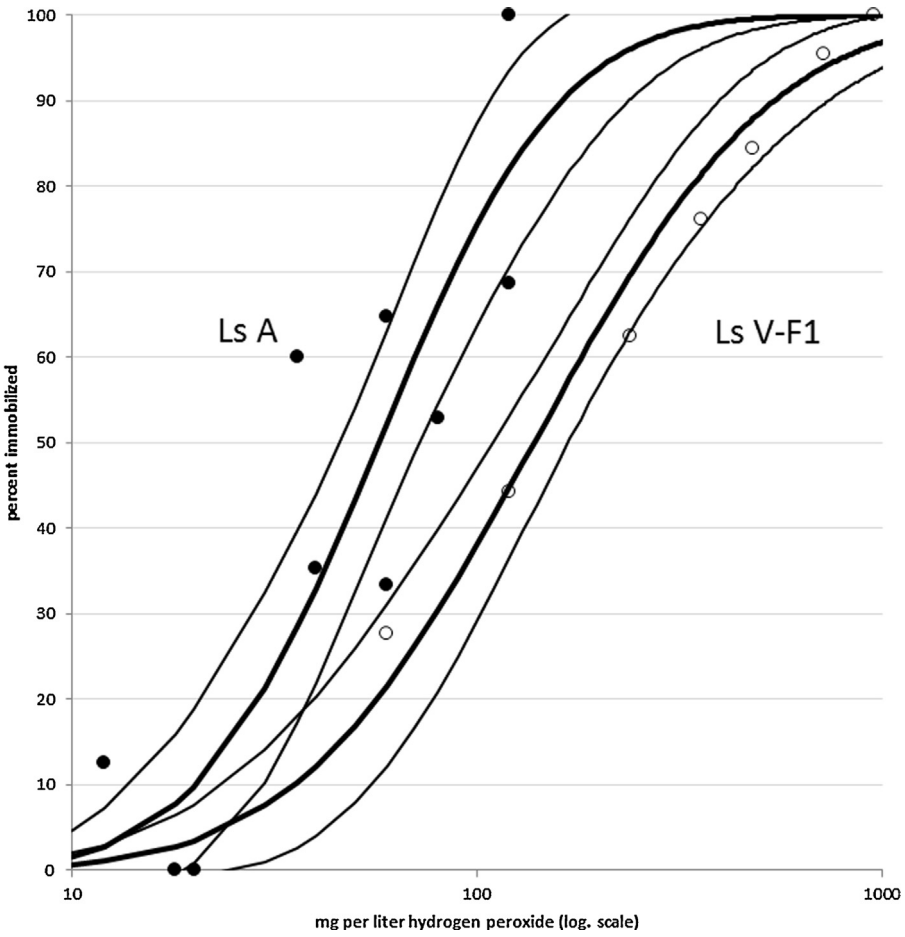

Fig. 2. 24-h bioassay. The dose-response curves (90% CI) and the observed immobilization rates for the two strains of salmon lice tested for sensitivity towards hydrogen peroxide in a 24-h bioassay. The observed rates for the sensitive strain (Ls A) are indicated by filled circles, while the corresponding values for the strain with reduced sensitivity (Ls V) are indicated by open circles. The dose-response curve for Ls A is modelled with results from two different bioassays.

North-Norway. The development of reduced sensitivity towards H_2O_2 in these particular areas was therefore not unexpected as repeated treatments provide fast lane evolution towards reduced sensitivity. In the current study reduced H_2O_2-sensitivity was found in one strain of salmon lice in combination with reduced treatment efficacy. To decide if reduced sensitivity is a settled trait in the salmon lice population more sensitivity tests should be conducted on farms reporting of reduced treatment efficacy.

Reduced H_2O_2-sensitivity in *L. salmonis*, due to extensive use of the chemical as a delousing agent, was first reported from Scotland in 1999, seven years after H_2O_2-treatment was introduced to the Scottish market (Treasurer et al., 2000). Using experimental bin treatments, 15% effect of treatment was recorded for the gravid females and 25% effect for the other mobile stages. Fish, infested with salmon lice previously unexposed to H_2O_2, were treated and used as a control with an effect of 87% or more for all groups. The study by Treasurer et al. was performed on the F0-generation of salmon lice, while the current study also included the F1-generation, which had not previously been exposed to H_2O_2. This structure provided stronger evidence for the heritability of reduced H_2O_2-sensitivity.

The bioassay results presented in the current paper show differences in the sensitivity level towards H_2O_2 between various strains of *L. salmonis*. These differences coincide with the differences in treatment efficacies. 30-min bioassays revealed up to tenfold variations in EC_{50}-values between the different strains of salmon lice. Reduced sensitivity towards H_2O_2, evident in both reduced treatment efficacy and increased bioassay EC_{50}-values, is most likely an inherited trait. This is suggested by the fact that the F1-generation showed a relatively high EC_{50}-value, despite having not been exposed to H_2O_2-treatment. The current study presents the first case report of reduced H_2O_2-sensitivity in salmon lice in Norway. This reduced sensitivity imposes a threat to the Norwegian fish farming industry and should be monitored closely.

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PAPER 3

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A screening of medicinal compounds for their effect on egg strings and nauplii of the salmon louse *Lepeophtheirus salmonis* (Krøyer)

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A screening of medicinal compounds for their effect on egg strings and nauplii of the salmon louse *Lepeophtheirus salmonis* (Krøyer)

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Abstract

Egg strings and nauplii of the salmon louse *Lepeophtheirus salmonis* were exposed to a variety of medicinal compounds at 50 mg L^{-1} for 30 min in two experiment series. This medicine concentration was selected as a starting point for a screening series. Hatching of egg strings and development to copepodid larvae were monitored in one experiment, and the survival and development of nauplii were monitored in the other. Two compounds, emamectin benzoate and cypermethrin, inhibited hatching effectively. Several compounds affected nauplii, either directly or through inhibiting development to the infective stage. A total of 50 mg L^{-1} of azamethiphos, cypermethrin, emamectin benzoate and propoxur was lethal to >70% of the larvae. Diflubenzuron, fenoxycarb, pymetrozine, pyriprole and tebufenozide diminished the ability of nauplii developing to copepodids.

Keywords: egg strings, medicines, nauplii, salmon lice, screening.

Introduction

Sea lice are ectoparasitic copepods that represent a fundamental hindrance for fish farmers in all salmonid producing areas. In the fish farming industry, these parasites mainly constitute of *Lepeophtheirus salmonis* in the Northern hemisphere and *Caligus rogercresseyi* in the Southern hemisphere. Their presence may cause reduced fish welfare (Dawson *et al.* 1999), host immunosuppression (reviewed by Fast 2014), considerable economic expenditure (Costello 2009), in some cases detrimental impacts to wild fish populations (Krkošek *et al.* 2007; Skilbrei *et al.* 2013; Thorstad *et al.* 2015) and administrational inconvenience such as lice counting and surveillance programs. In general, sea lice are a major obstacle facing the sustainability of salmonid farming. This paper will focus mainly on the salmon louse *L. salmonis*.

The salmon lice life cycle consists of eight stages: initially, two planktonic nauplii stages are followed by the infective copepodid. After moulting to chalimus I and II, these stages being attached to the fish by a protein filament, the parasites develop to motile preadult (I + II) and adults (Hamre *et al.* 2013). Adult males and females reproduce sexually; where copulation is followed by the female releasing several sets of egg strings in succession. After maturation, the egg sacs unravel and disperse the planktonic nauplii into the water. Each egg string may contain up to 700 individual eggs (Heuch, Nordhagen & Schram 2000), but this number varies greatly in relation to time of year, water temperature and the consecutive sequence of the egg string; the earlier being more vital than the later. The reproductive potential is thus vast, and favoured by high host density.

The control of sea lice infestations has relied heavily on treatments with ectoparasiticide treatments. Anti-sea lice medicinal agents are

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traditionally applied as either in-feed formulations or bath treatments. Oral medicines are distributed via intestinal uptake, systemically distributed to skin and mucus of the fish, thereby affecting sea lice ingesting these components. The treatment regimens usually run for up to 7 days, with medicated feed being given daily (Stone *et al.* 1999; Horsberg 2012; Samuelsen *et al.* 2015). Bath treatment, or topical application, is administered to the fish as a short-term exposure, ranging from 20–60 min, the concentration dependent on water temperature, fish size, health condition and parasite sensitivity. Bath treatments occur either directly in tarpaulin-enclosed net pens, or in well boats or other transferable units. They are potentially harmful to the fish as the safety margins for some treatment substances are relatively low, the fish stock is crowded during the procedure through tightening of the net pen leading to decreased oxygen levels (Grant 2002). However, treatment efficacy has usually been quite predictable as long as the parasites were generally sensitive to the treatment agents.

The five compound groups pertinent to sea lice removal are as follows: avermectins, benzoyl ureas, disinfectants, pyrethroids and organophosphates. The last chemical introduced for control of salmon lice in Norway was emamectin benzoate in 1999. Regarding *L. salmonis*, there have been reports of reduced sensitivity against all therapeutic agents except the benzoyl urea compounds (Aaen *et al.* 2015).

The rather widespread occurrence of salmon lice resistant to existing remedies has triggered a renewed interest in finding alternative salmon lice treatments. One approach is to screen a number of model substances with different modes of action for their efficacy against different instars of the parasite. The Insect and Resistance Action committee (IRAC 2015) has classified insecticides according to their modes of action, a list that is an appropriate starting point for selection of model compounds to test.

Hatching and moulting are two biological processes constituting possible new targets for chemicals. However, little knowledge concerning egg string structure exists. The embryos are stacked and wrapped in an egg sack, each embryo slightly turned relative to the next. The egg sac unravels, releasing nauplii capable of swimming. In addition, the gravid females tend to drop their eggs strings under stressful conditions (Heuch *et al.* 2000). Thus, following chemical treatments that are not harmful to the embryos, egg strings are still capable of hatching even after the adult females have been neutralized. Egg strings should therefore also be considered desired targets for chemotherapeutants. The moulting process is already a target of medicinal intervention through the benzoyl urea compounds. In other pest species, several metabolic pathways involved with moulting are targets of medicinal compounds; this feature could represent a potential asset for novel anti-sea lice medicines as well. Copepodids have previously been used for screening assays or regular bioassays, but little is known about the effect of medicinal compounds on nauplii.

The aim of this experiment series was to develop robust methods for testing the effect of various chemicals on the hatching of egg strings and development of nauplii to copepodids and, furthermore, to utilize these methods with a selection of medicinal compounds.

Materials and methods

Compounds possessing known insecticidal activity included in this study were primarily selected using the IRAC classification scheme, and grouped according to mode of action (MoA). The compounds and their category are provided in Table 1. All compounds were purchased from either Sigma-Aldrich, Nerliens Meszansky or VWR, with one compound supplied from Elanco, St. Aubin, Switzerland, and one supplied from Bayer, Norway. The cypermethrin formulation used was the commercial product Betamax® (Elanco).

Hatching assay

The seawater used in the experiment was at ambient temperature and salinity, taken from 60 m depth, filtered through a sand filter and subsequently a plankton mesh (150 μm). The egg strings all originated from a fully sensitive population (Helgesen & Horsberg 2013a). Atlantic salmon of 150–800 g infested with salmon lice from this strain were kept in 100-L seawater tanks. After anaesthetizing the fish in 100 mg L^{-1} of metacaine, the egg strings were detached from the lice using scissors. Each egg string was halved using a scalpel, with one half exposed to a medicinal compound, and the other half used as a

Table 1 Medicinal substances included in this experiment series, grouped by mode of action

IRAC no.	Mode of action	Substance	CAS	Provider
1A	Acetylcholinesterase inhibitor	Propoxur	114-26-1	Sigma-Aldrich
1B	Acetylcholinesterase inhibitor	Azamethiphos	35575-96-3	Sigma-Aldrich
2	GABA-gated chloride channel blocker	Pyriprole	394730-71-3	Elanco
3	Sodium channel modulator	Cypermethrin	52315-07-8	Elanco
4	Nicotinic acetylcholine receptor competitive modulator	Imidacloprid	138261-41-3	Sigma-Aldrich
4	Nicotinic acetylcholine receptor competitive modulator	Nitenpyram	150824-47-8	Sigma-Aldrich
5	Nicotinic acetylcholine receptor allosteric modulator	Spinetoram	187166-40-1	Nerliens Meszansky
6	Glutamate-gated chloride channel allosteric modulator	Emamectin benzoate	155569-91-8	Sigma-Aldrich
7B	Juvenile hormone mimics	Fenoxycarb	72490-01-8	Sigma-Aldrich
7C	Juvenile hormone mimics	Pyriproxyfen	95737-68-1	Sigma-Aldrich
9B	Modulator of chordotonal organs	Pymetrozine	123312-89-0	VWR
9C	Modulator of chordotonal organs	Flonicamid	158062-67-0	Sigma-Aldrich
13	Oxidative phosphorylation uncoupler	Sulfluramid	4151-50-2	Nerliens Meszansky
14	Nicotinic acetylcholine receptor blocker	Cartap	15263-52-2	VWR
15	Inhibitors of chitin biosynthesis, type 0	Diflubenzuron	35367-38-5	Sigma-Aldrich
17	Moulting disruptor	Cyromazine	66215-27-8	Sigma-Aldrich
18	Ecdysone receptor agonist	Tebufenozide	112410-23-8	Sigma-Aldrich
19	Octopamine receptor antagonist	Amitraz	33089-61-1	Sigma-Aldrich
22	Voltage dependent sodium channel blocker	Metaflumizone	139968-49-3	Sigma-Aldrich
23	Inhibitor of acetyl CoA carboxylase	Spiromesifen	283594-90-1	Sigma-Aldrich
28	Ryanodine receptor modulator	Chlorantraniliprole	50008-45-7	Sigma-Aldrich
N.a.	Calcium channel modulator	Praziquantel	55628-74-1	Bayer
N.a.	β-Tubulin inhibitor	Thiabendazole	148-79-8	Sigma-Aldrich

control. A total of 12 egg strings were designated to each compound, exposed in three replicate groups each comprising 4 egg strings. No classification according to egg string maturity was performed as they were of the approximate same age when collected. The most commonly used exposure period for bath treatments is 30 min, thus this exposure period. Egg strings were exposed to 50 mg L^{-1} of the test substance, a concentration selected after pilot studies and considered high enough to identify interesting compounds. The compounds were formulated by weighing 5 mg of each substance into glass test tubes and dissolving them in 50 μL room-tempered dimethyl sulphoxide (DMSO, Sigma-Aldrich) and 50 μL heated (40 °C) blank emulsion (4.5 g polysorbatum 80, 2 g sorbitan monostearate, 100 mL propylene glycol and 150 mL purified fresh water, mixed and heated to 50 °C [Muan *et al.* 1985; Aaen & Horsberg 2016]). Every test tube was vortexed until the test substance was sufficiently dispersed. To each test tube, 5 mL filtered sea water was added, before being vortexed for 60 s. This volume was then added to a glass bottle containing 95 mL of 4 °C seawater, and shaken gently. Finally, the total volume of 100 mL was divided in three plastic containers, each receiving 33 mL. The control groups were exposed to DMSO and blank emulsion only. Each box was placed on a BioSan shaker plate for 30 min, allowing the medicinal compound to be properly dispersed in the water throughout the exposure. After exposure, the egg string pieces were washed three times in sea water, before being incubated separately for 20–24 days in small continuous flow incubators (Hamre, Glover & Nilsen 2009). The number of nauplii and copepodids were recorded every 48–72 h. The water temperature ranged from 7.4 °C to 12.0 °C and the salinity from 31.4‰ to 34.5‰ during the experiment.

Hatching and development of larvae from each egg string was registered over time, as number of larvae and hours from exposure. The observation period endured 20–24 days, where all egg strings were observed for several days after hatching completion to ensure that the hatching was terminated. After plotting these figures, curves were generated, and finally, the area under the curve (AUC) was calculated for each egg string, yielding a value of reproductive output over time. Every trial compound was tested on 12 egg strings, giving 12 separate AUC values for each compound.

Survival assay

The egg strings were collected as described for the hatching assay and cut in two halves, each egg

string contributing to one control group and one treatment group. At day 0 of the experiment, each half was placed individually in separate small continuous flow incubators. When a sufficient number of nauplii (>20) were present in both parallels, the nauplii were exposed to a medicinal compound. Egg string residuals were removed prior to exposure. Development of nauplii was recorded every 48–72 h.

The medicinal compounds were prepared from a stock solution of 10 g L^{-1} in DMSO. They were diluted with DMSO to a final volume of 125 μL, mixed with an equal volume of blank emulsion before being mixed with 200 mL filtered sea water. The substances were prepared <48 h prior to the experiments.

The nauplii were exposed to 50 mg L^{-1} of the test substance on their first or second day post-hatch. Each well including nauplii was swiftly moved from the incubator rack to a 50-mL glass (VWR 213-1121) containing the test substance dissolved in sea water. The glass with content was then gently shaken for 30 min on a BioSan shaker plate. Finally, each well was transferred back to the incubator rack and continuous water supply was restored (1 L min^{-1}). Post-exposure, the larval development was monitored for 8–14 days. For each test compound, four parallels were applied.

Pilot studies demonstrated that the mortality of nauplii after twice undergoing this procedure was minimal. Both the hatching assay and the survival assay series were conducted at NIVA Marine Research Station in the Oslofjord area.

Statistical analysis

Hatching assay: counting of vivid larvae (floating in the water column), was performed at several time points, and plotted in a diagram. From these figures, the AUC was calculated for each egg string. An example of AUC is given in Fig. 1. Every test compound was tested on 12 egg strings, giving 12 separate AUC values for each compound. The Wilcoxon ranked-sum test was performed to compare the outcome of the AUC calculations between controls and exposed groups. A significance level of $\alpha \leq 0.10$ was chosen for the analyses, to avoid exclusion of potentially interesting compounds with a larger variation between parallels.

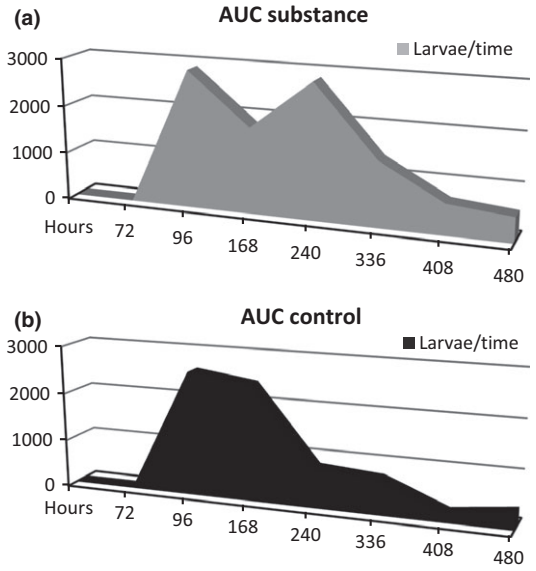

Figure 1 Example of AUC calculation of the egg string output after exposing one egg string half to a medicinal substance (a) and the other half to a control (b) (blank emulsion and dissolvent). The number of hatched larvae was registered every 48–72 h after exposure for up to 20 days.

Survival assay: counting of nauplii and copepodids was performed at several time points. A Kaplan–Meier survival analysis was performed in JMP (SAS Institute Inc.) to compare the viability of the larvae after exposure to the medicinal compounds. The direct effect of the compound was adjusted using the Schneider-Orelli formula (Schneider-Orelli 1947).

Results

Hatching assay

For one substance, emamectin benzoate, the experiment was aborted after 16 days because of the deleterious effect of the compound on hatching activity, as there were no larvae to be counted in the exposed groups. The AUC value analysis showed that emamectin benzoate, cypermethrin, metaflumizone and chlorantraniliprole reduced the hatching with more than 50%. Only cypermethrin and emamectin benzoate induced a significant reduction, with Wilcoxon $P < 0.1$. The other compounds had little or no effect on the developing embryos within the egg string. Details on all tests are provided in Table 2 and Fig. 1.

Survival assay

A total of 132 egg strings were included in the moulting assay. In 68% of the cases, the two corresponding egg string parts had hatched within 48 h and released a similar amount of nauplii. A cut-off value of 50% was set, excluding egg strings yielding an asymmetric number below this limit. In 12% of the cases, only one of the egg strings yielded nauplii at the time of planned exposure, and they were therefore omitted from the experiment. At the next registration, 48–72 h thereafter, nauplii were present in both parallels. However, in 14 of 132 egg strings (11%), only one part of the egg string resulted in nauplii. Finally, 8% of the egg strings were incapable of producing either a sufficient amount of nauplii or even any nauplii at all.

The survival rates on day 7 after exposure are presented in Table 3. When corrected for the mortality in the control groups, azamethiphos, cypermethrin, emamectin benzoate and diflubenzuron resulted in 100% mortality within 7 days. Propoxur, fenoxycarb, pymetrozine, cartap and tebufenozide yielded 50% mortality or more, while the remaining compounds resulted in less than 50% mortality.

The medicinal compounds could be grouped either as inducing direct mortality (70% or more at day 2), inhibiting larval development (significant difference in the median survival time) or having no effect (no significant difference in the median survival time). Table 4 presents the test compounds grouped after their efficiency range.

The period of time until occurrence of larval inactivity was visualized using a Kaplan–Meier survival analysis in JMP. One example for each group according to Table 4 is presented in Fig. 2.

Discussion

To avoid great abundances of sea lice in salmon farms, a continuously growing set of control measures are available. Over the last 10 years, a general trend of replacing medicines with other control measures has been prevailing, mainly because of reduced sensitivity to one or more of the available compounds, but also due to possible adverse environmental effects, where uncertainty regarding accumulation of anti-sea lice compounds is

Table 2 Hatching of *L. salmonis* egg strings and development of nauplii and copepodids over time after exposure to 23 medicinal compounds, presented as median of individual area under curves (AUC) for the 12 parallels including 10th/90th percentiles for each value. Exposed groups are compared to control groups in the Wilcoxon ranked-sum test

IRAC no.	Substance	AUC exposed median	10/90 percentile	AUC control median	10/90 percentile	Wilcoxon *P* value
	Control	114	0/3741.6	522	0/1191	0.77
1	Propoxur	690	0/8076	2640	0/9708	0.34
1	Azamethiphos	2458	0/9078	3883	0/9668	0.24
2	Pyriprole	2130	0/12 966	1308	0/18 360	0.54
3	Cypermethrin	378	0/2040	5388	0/10 350	0.07
4	Imidacloprid	5094	1779.6/9579.6	4266	241.2/7272	0.40
4	Nitenpyram	1668	28.8/9678	4530	28.8/10 554	0.14
5	Spinetoram	2970	960/9138	4740	86.4/11 988	0.51
6	Emamectin benzoate	0	0/0	2280	0/10 776	0.00
7b	Fenoxycarb	3420	28.8/13 668	2940	90/7848	0.49
7c	Pyriproxyfen	5280	414/13 668	4380	0/10 464	0.82
9b	Pymetrozine	6222	1824/8880	4884	3012/8653.2	0.77
9c	Flonicamid	5430	367.2/10 434	4464	228/9268.8	0.91
13	Sulfluramid	4398	1567.2/7472.4	4224	0/8607.6	0.53
14	Cartap	4914	1484.4/10 992	6618	1144.8/9040.8	0.69
15	Diflubenzuron	5352	213.6/10 222.8	5712	1098/9696.6	0.91
17	Cyromazine	5898	1273.2/10 260	3480	1510.8/8619.6	0.13
18	Tebufenozide	1158	0/5710.8	678	0/6667.2	0.62
19	Amitraz	4452	235.2/8965.2	4068	931.2/7161.6	0.77
22	Metaflumizone	600	0/6540	0	0/13 884	0.67
23	Spiromesifen	276	0/5454	852	0/4633.2	0.75
28	Chlorantraniliprole	294	0/1359	2886	0/7642.8	0.24
N.a.	Praziquantel	1860	284.4/4281.6	2052	0/7537.2	0.77
N.a.	Thiabendazole	1152	0/6177.6	2436	25.2/6009.6	0.30

addressed (Burridge & Van Geest 2014). As a result, non-medicinal precautions have grown in numbers. Cleaner fish, permanent tarpaulins of material impermeable to infective parasite larvae, lasers, fresh- or hot water baths, prolonged freshwater phase, anti-attachment diets and enclosed cages are among the preventive actions performed by farmers. In addition, governmental instructions regarding sea lice abundance and preventive measures exist in all salmonid producing countries. Thus, the current measures for controlling salmon lice are diverse. Even though the non-medicinal approaches are showing promising results, effective chemical treatments will most likely still be necessary. A reasonable way to proceed with medicine use against sea lice is to direct the treatment towards specified instars and to delay resistance towards novel compounds through sensitivity testing and balanced use.

To achieve comparable results in these screening assays, all compounds were tested with a standard concentration of 50 mg L^{-1} and an exposure time of 30 min. In screening studies, the initial experimental concentration is usually very high, to capture also compounds with a moderate efficacy. The parameters used in these studies are not necessarily relevant for concentrations that egg strings and nauplii may be exposed to during a clinical treatment in the field.

Hatching assay

The water temperature and salinity varied somewhat throughout the experimental period and may have affected the hatching rate in the hatching experiment where the temperature varied from 12.1 °C in the initial phase, to 9.3 °C by closure. The salinity was relatively stable within the period, with the value ranging from 33.8‰ to 35.5‰.

Among the 23 substances tested for effect on egg string hatching, only 2 proved to be significantly effective in an overall view. Emamectin benzoate was in these terms the most efficacious at a concentration of 50 mg L^{-1} for 30 min, that is a much shorter exposure time and a much higher concentration than the recommended dose of 50 μg per kg fish for 7 days through oral treatment. The lowest concentration inhibiting egg string hatching was not further investigated. Emamectin benzoate has been demonstrated to affect hatching in *C. rogercresseyi* egg strings (Bravo *et al.* 2015), when these were exposed for a time period of 24 h. Emamectin benzoate has an effect on glutamate-gated chloride channels, resulting in paralysis, and speculated also to be active on nicotinergic acetylcholine receptors (Carmichael *et al.* 2013). The fact that emamectin benzoate, although at a high concentration, inactivated embryos and nauplii, could be interesting in a practical view. Its pharmacokinetic properties, that is ability to cross egg string membranes, from the water column or even from the mother, could be subject to further investigation.

Cypermethrin was the other compound inhibiting hatching. The recommended dose for this substance, when 80:20 cis:trans relationship, is 0.015 mg L^{-1} for 30 min, which is very much lower than the concentration used in the present experiment. Toovey & Lyndon (2000) found an effect of this compound on hatching and also on subsequent development to copepodids, which is in line with our observations. The main mode of action for pyrethroids like cypermethrin is interference with the voltage-gated sodium channels, resulting in disruption of signals from the nervous system to muscles. A possible explanation for the effect of cypermethrin might be incapability of the parasite to emerge from the egg or moult, as these processes require considerable muscle activity. Cypermethrin also proved to inhibit hatching of *C. rogercresseyi* egg strings after 24-h exposure (Bravo *et al.* 2015).

Propoxur and azamethiphos, both acting on the enzyme acetylcholinesterase, had lethal effects on some of the egg strings, although the effect was not statistically significant ($P = 0.34$ and 0.24, respectively). The maturity status of each egg string was not registered in the experiment, but we suspect the most mature egg strings to have lost their hatching ability after exposure to these compounds. This would be in accordance with Aaen, Aunsmo & Horsberg 2014; where the effect of hydrogen peroxide was more expressed on mature than on immature embryos (Aaen *et al.* 2014). The gene coding for neuronal acetylcholinesterase (ace1a) was by Kaur *et al.* (2015) demonstrated to be significantly expressed in the first larval instar, nauplius I. Thus, there seems to be a difference in the lethal effect on different instars that cannot be explained by the mere presence of the enzyme acetylcholinesterase, the target protein for organophosphates and carbamates, as Kaur *et al.* (2015) demonstrated expression of the

Table 3 The direct effect of medicinal compounds after exposing nauplii to a 50 mg L^{-1} bath treatment for 30 min and a subsequent observation period of 7 days (51.8–84.0 degree days). The mortality in the exposed group was corrected for the mortality in the corresponding control group using the Schneider-Orelli formula

Substance	Accumulated mortality in the control at day 7 (%)	Accumulated mortality in exposed parasites at day 7 (%)	Medicine-induced mortality (%)	Wilcoxon	Sample size (control/exposed)
Propoxur	40	82	70	68.17	230/205
Azamethiphos	49	100	100	127.15	180/181
Pyriprole	61	88	69	102.27	280/155
Cypermethrin[a]	55	100	100	183.13	170/110
Imidacloprid	50	60	20	22.07	410/232
Nitenpyram	21	28	9	2.76	290/195
Spinetoram	30	34	6	22.70	240/310
Emamectin benzoate	30	100	100	316.0	205/112
Fenoxycarb	20	89	86	1.68	142/170
Pyriproxyfen	32	60	41	49.74	270/160
Pymetrozine	20	72	65	115.54	200/240
Sulfluramid	20	43	29	24.34	175/140
Cartap	78	56	0	36.28	160/160
Diflubenzuron	0	100	100	281.42	140/190
Cyromazine	33	59	39	34.83	295/310
Tebufenozide	34	89	83	43.06	145/170
Amitraz	28	37	13	9.31	245/180
Metaflumizone	16	49	39	48.06	200/300
Spiromesifen	38	54	26	10.68	301/185
Chlorantraniliprole	74	46	0	175.0	320/340
Praziquantel	24	36	16	58.19	260/310
Thiabendazole	32	26	0	3.05	320/350

[a]Experiment ended at day 6.

Table 4 Grouping of tested medicinal compounds according to their effect *on L. salmonis* naupliar larvae

Inducing direct mortality	Inhibiting larval development	No significant effect
Azamethiphos	Diflubenzuron	Amitraz
Cypermethrin	Fenoxycarb	Cartap
Emamectin benzoate	Pymetrozine	Chlorantraniliprole
Propoxur	Pyriprole	Cyromazine
	Tebufenozide	Flonicamid
		Imidacloprid
		Metaflumizone
		Nitenpyram
		Praziquantel
		Pyriproxyfen
		Spinetoram
		Spiromesifen
		Sulfluramid
		Thiabendazole

gene coding for this enzyme to be present in all instars.

Some of the compounds seemed to have an enhancing effect on the hatching, but none of these observations were statistically significant results. The possibility of enhancing effects caused by substances such as juvenile hormone analogues cannot be excluded, although the results may also have been a result of natural variation of parted egg strings.

As very few of the substances enrolled in this experiment series had an effect on the hatching, it seems that *L. salmonis* egg strings are not very vulnerable when exposed to medicinal compounds over short periods, even when doses are far above concentrations therapeutic to fish. This finding suggests that egg strings quite competently endure several medicinal molecules, indicating a resistant nature of the outer sacs. The 24 h applied by Bravo *et al.* (2015) seems to be a better choice for evaluating effects on egg strings by medicinal compounds, although this would infer a simulation of other medicinal interference than bath treatments.

The counting of larvae in this experiment was performed by visual observation and could in some cases be a little inaccurate. A better way of registering the mortality could be to increase the number of larvae and evaluate the motility in smaller groups with a microscope. Instead, we opted to utilize a lower number of larvae, in order to cover a higher number of medicinal compounds. We were also seeking compounds with a

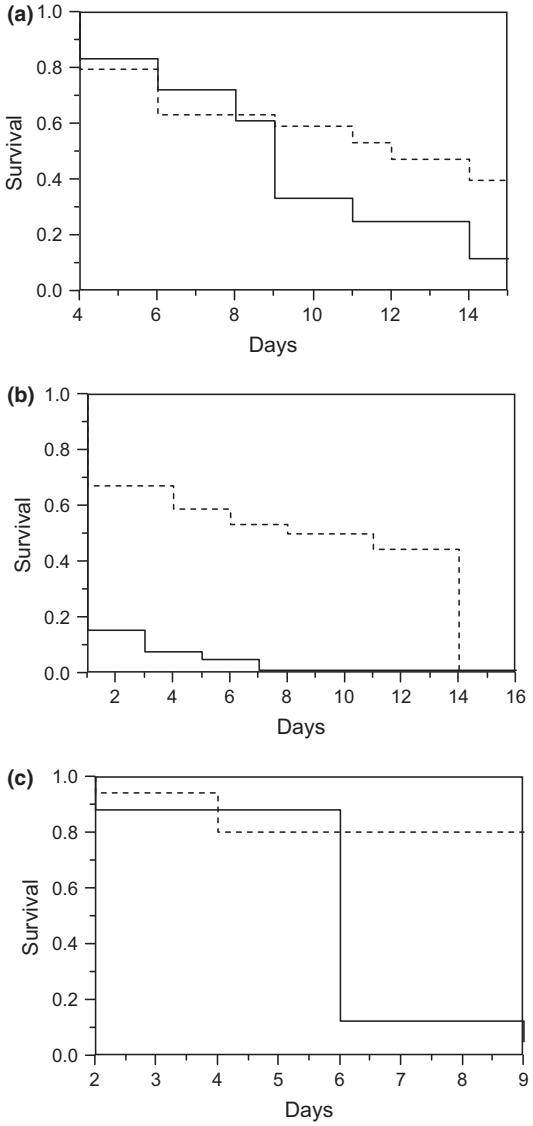

Figure 2 Survival analysis for nauplii exposed to medicinal compounds at day 0, given in % per day. Control group represented by the stipled line, exposed group by the solid line. (a) Amitraz (no significant effect); (b) Azamethiphos (direct mortality); (c) Fenoxycarb (inhibition of development).

100% effect on the larvae, a response that would be fully discovered by visual observations.

The naupliar stage of *L. salmonis* is planktonic and not parasitic. Therefore, this instar possesses other properties than the parasitic instars chalimus, preadults and adults. A thorough comparison with *L. salmonis* and other parasitic species with respect to the medicinal compounds mentioned in this manuscript is available in another text (Aaen & Horsberg 2016).

The observation frequency in this experiment was between 48 and 72 h. By reducing the intervals to 24 h, a more accurate response might be registered. However, our purpose was to minimize this error by increasing the concentration, in order to obtain valid results. Most likely, a total mortality or immobilization of hatched larvae would be observed irrespective of the registration frequency.

The cutting of egg strings in two halves is a source to inaccurate results. For example, the AUC median for metaflumizone control was 0 (Table 2); however, the 90th percentile of the control group was twice as high as that of the exposed group. In this case, it is likely that most of the embryos were present in the half that was exposed.

Survival assay

Nauplii are well suited for screening experiments for several reasons. They were easily reared and easily monitored and, as opposed to egg strings, quite susceptible to medicinal compounds. Their viability could be measured either as direct mortality, or as inability to moult into the infective instar; the copepodid.

Egg string residue were removed at the initiation of medicinal exposure, but it is still possible that some minor egg string residues were present in some of the wells, possibly hatching during exposure and resulting in a few nauplii undergoing a shorter exposure period than 30 min. In addition, small fragments of egg strings may have remained in the well, releasing additional nauplii after exposure. However, these factors were considered to be of minor importance.

The age of the female might also have influenced the results to some extent. According to Mustafa, Conboy & Burka (2000), the later egg strings of one female produce less viable and fewer copepodids than the initial sets of egg strings, although the very first pair are reported to contain particularly few embryos (Heuch *et al.* 2000). The females used in our experiments were producing egg strings for up to approximately 1128 degree days, and thus, the later egg strings may have contained fewer embryos than the initial originating from these respective parasites. This phenomenon may explain the relatively high variation in larvae from egg strings throughout both experiment series.

Although the test solutions were prepared within hours prior to experiment initiation, the stability of the compounds in water might still have influenced the results to some degree. Imidacloprid, for example, is reported to have a half-life of 146 min in water when exposed to light (Wamhoff & Schneider 1999). In the current experiment, one imidacloprid stock solution was prepared 5 days prior to the exposure, whereas in a parallel experiment, the stock solution was prepared immediately before exposure. However, no difference in the effect on moulting could be detected. The stability status of another compound, propoxur, was thoroughly examined by Sun & Lee (2003). They stated that hydrolysis is heavily dependent on light intensity, where sunlight would initiate faster decomposing than indoor light. Under alkaline conditions, in which category seawater belongs, the degradation appeared to happen faster than 8 h. At a pH of 8.5, propoxur would be completely hydrolysed after 24 h. These experiments were performed to detect chemotherapeutants effective against sea lice, and thus, the initial concentrations of the compounds were high. The risk of missing out on effective compounds was therefore minimized, as a minor degradation of the medicinal compounds could be tolerated. A laboratory investigation of the actual concentration of each compound would have been too time-consuming in this initial phase of the study.

Other physiochemical properties also vary between compounds, possibly interfering with the actual concentration in the water and the ability to penetrate the outer layers of the parasite. The material of the test equipment has previously been demonstrated to affect the medicine concentrations achieved during bioassays (Helgesen & Horsberg 2013b). In the current screenings, glass bottles were used whenever possible. As the exposure time was only 30 min and the concentrations applied rather high (50 mg L^{-1}), the effect of the test equipment was considered to be of less importance.

The compounds inducing direct mortality, azamethiphos, cypermethrin, emamectin benzoate and propoxur all act on the nervous system of the parasite. They are either already used as anti-sea lice products, or perform their actions on a biochemical pathway already exploited by pharmaceuticals (Table 1). Interestingly, azamethiphos induced some mortality on the nauplii in this study, as shown in Fig. 1. However, in an experiment performed in our laboratory, *L. salmonis* of the chalimus stage were exposed *in vivo* to the azamethiphos treatment concentration of 100 ppb for 30 min, as recommended by the manufacturer. When registered 29 days later, no significant difference regarding sea lice abundance could be detected between the exposed group and the control group. This is in accordance with the findings of Roth *et al.* (1996). Thus, there seems to be a difference in the lethal effect on different instars that cannot be explained by the mere presence of the enzyme, as Kaur *et al.* (2015) demonstrated expression of the gene coding for this enzyme to be present in all instars.

Four compounds inhibited moulting, thereby arresting the nauplii in this instar, and making them incapable of developing to copepodids. One of these, diflubenzuron, is already on the market for use against sea lice together with the similar compound teflubenzuron, working as a chitin synthesis inhibitor (Soltani, Lechekhab & Smagghe 2009). The synthesis of chitin is essential for the development of a functional exoskeleton in the larvae, and the effect of this compound is explained by this process. Diflubenzuron was used as a model substance in our experiment. Another compound inhibiting moulting similarly to diflubenzuron, and not currently used as an anti-sea lice compound was the ecdysone agonist tebufenozide, where a significant effect was recorded on day 7. The process of shedding exoskeleton, the ecdysis, is a complicated matter constituting of several phases and hormones (Retnakaran *et al.* 2001). Further investigation connected to this pathway and the interference of tebufenozide should be conducted in upcoming studies.

Furthermore, the juvenile hormone analogue, fenoxycarb, which possesses carbamate properties, also inhibited moulting. Pyriproxyfen, another juvenile hormone mimic, is a growth regulator as described by Darriet *et al.* (2010). This compound did however not yield any significant mortality. This observation represents a perfect example of the specificity of chemical compounds and arthropods, as fenoxycarb and pyriproxyfen exert a similar effect according to their IRAC grouping, however proving differently efficacious within the same species.

Pymetrozine is a neuroactive insecticide thought to affect the chordotonal organ. This compound induced mortality to nauplii in our experiments. The mode of action is described by Ausborn *et al.*

(2005) to be interfering with the chordotonal mechanoreceptor, an organ not considered to be present in *L. salmonis*. However, Kaufmann *et al.* (2004) suggested that pymetrozine acts on a novel mechanism connected to a signalling pathway of serotonin. The presence of a serotonergic system has been described in another crustacean, the American lobster, *Homarus americanus* (Harzsch 2003), but has to our knowledge not been studied in *L. salmonis*. The effect of pymetrozine could, however, be investigated further in the context of development inhibition in early instars of *L. salmonis*.

Finally, pyriprole, an antagonist of GABA-gated chloride channels, also inhibited moulting. The moulting inhibitory mechanism is not clear, but it has been demonstrated that a similar compound, fipronil, interferes with hormone regulation by causing a sixfold upregulation of the ecdysone receptor in another copepod, *Amphiascus tenuiremis* (Gaertner *et al.* 2012). Cary *et al.* (2004) showed that fipronil resulted in reproductive dysfunction in the same crustacean, *A. tenuiremis*. The inhibition was connected to gender, a feature that may have been the case in our experiment as well.

Cyromazine is a moulting disruptor utilized against several pest species. The mode of action is at present not fully discovered (Van de Wouw, Batterham & Daborn 2006), but the mortal effect on target organisms seems to be delayed after exposure. This compound was a possible candidate to affect the moulting process of sea lice, but this did not seem to be the case in our study. A reason for this could be inability of the compound to penetrate the sea louse cuticle, insufficient accumulation of the substance in the organism due to the short exposure period, or absence of its corresponding receptor in nauplii of *L. salmonis*.

One compound that to some extent, but not significantly ($P = 0.24$) affected egg string hatching and subsequent larval development, was the ryanodine receptor modulator chlorantraniliprole. The ryanodine receptor is a specific intracellular calcium channel occurring in muscle tissue and neurons. This compound yielded a weak effect on egg strings but not on nauplii in our assay, processing input to the investigation of the ryanodine receptor in sea lice, as related chemotherapeutants with higher efficacy towards these species may exist.

Conclusion

A few medicinal compounds affected the hatching ability of egg strings when these were exposed during 30 min baths at high concentrations. A further inhibiting effect on hatching through oral uptake by the gravid female feeding on fish mucus and skin could be investigated. Nauplii are quite vulnerable to direct, short-term exposure, and the survival assay may be useful for further screening of medicinal compounds or other substances with effect on physiological pathways. In this study, moulting inhibition of salmon lice was achieved with several medicinal groups, of which some can potentially be utilized as targets for future commercial products. This study revealed a high degree of specificity regarding the effect of several medicinal compounds towards sea lice, an attribute connected to the pharmacokinetic properties induced by presence in seawater, and to the presence of specific receptors and target molecules in *L. salmonis*.

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PAPER 4

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A screening of multiple classes of pharmaceutical compounds for effect on preadult salmon lice *Lepeophtheirus salmonis*

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A screening of multiple classes of pharmaceutical compounds for effect on preadult salmon lice *Lepeophtheirus salmonis*

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Abstract

The salmon louse, *Lepeophtheirus salmonis* Krøyer, is the major obstacle facing a sustainable future for farmers of salmonids in the North Atlantic Ocean. Medicinal compounds have been the most utilized tool to prevent salmon lice infestation; however, the active compounds have become less effective or considered environmentally unfriendly in the past years. Novel medicinal compounds are thus highly desired. In two experiment series, 26 medicinal compounds were screened for their efficacy against salmon lice, in a 30-min exposure and 24-h exposure, respectively. Pyriprole, imidacloprid, cartap and spinetoram were effective at 50 mg L^{-1} in the short-time exposure. In the 24-h exposure, pyriprole, propoxur, cartap, imidacloprid, fenoxycarb, pyriproxyfen, nitenpyram, spinetoram, spiromesifen and diflubenzuron induced a high level of immobilization at 5 mg L^{-1}. The EC_{50} values of the effective compounds were calculated in further titration studies for both exposure periods. Several physiological and biochemical pathways were discovered as possible targets for medicinal intervention against the salmon louse.

Keywords: aquatic toxicology, copepod, *Lepeophtheirus salmonis*, preadults.

Introduction

The presence of parasitic copepods, sea lice, in commercial salmonid farming is a major obstacle facing a sustainable industry, as their economic impact (Costello 2006) and effect on wild salmonids (Krkošek *et al.* 2007) are potentially gross. Local conditions, however, play a significant role in sea lice abundance, and the presence of the parasite is not necessarily fatal to wild fish (Torrissen *et al.* 2013; and reviewed by Thorstad *et al.* 2015). Over thousands of years, these parasites' biology has adapted to a relatively low host density, displayed by vast reproduction potential, minor host morbidity when parasites colonize in low numbers, energy efficient growth and selective infestation mechanisms. The increasing occurrence of salmonid farms has, however, improved the parasite's conditions in terms of efficacious dispersal to surrounding water masses, where wild fish may reside.

The main producers of salmonids are Canada, Chile, Scotland, Norway and the Faroe Islands, with some production also taking place in the USA, Ireland and Australia (Nowak *et al.* 2011). Atlantic salmon, *Salmo salar* L., is produced in all these countries and is the major farmed salmonid species. Rainbow trout, *Oncorhyncus mykiss* Walbaum, is produced in the second largest quantum. In the Northern Hemisphere, *Lepeophtheirus salmonis* Krøyer is the most prevalent, whereas in Chile, *Caligus rogercresseyi* is dominant. Other Caligus species, such as *Caligus elongatus*, are also present in some areas, although to a lesser degree than the two aforementioned species. In this manuscript, *L. salmonis* is mainly emphasized, although some parallels to *C. rogercresseyi* have been drawn when relevant.

Eight stages are present in the salmon louse life cycle. Nauplius I and II, the infective copepodid,

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two chalimus stages attached to the fish with a protein filament, two preadult and finally the adult stage (Hamre *et al.* 2013). The naupliar larvae live as planktonic organisms in the water column, as does the copepodid, until it finds a host. The other stages develop on the host, with the development from embryo to adult male expected to take around 50 days at 10 °C (Pike & Wadsworth 1999), a bit longer for females. Preadults and adults have traditionally been subject to the most intense combat, with most of the remedies being directed towards these instars in particular.

Antiparasitic medicines were introduced to salmon farming in the 1970s (Håstein & Bergsjø 1976), as were antibiotics against a variety of bacterial infections. However, as the use of antibiotics has been drastically reduced after effective vaccines were put in play; the use of antiparasitics has increased massively (http://www.fhi.no/artikler/?id=114175 [accessed on 2 November 2015]). The various medicinal compounds are administered at different dosages; therefore, utilized volumes are not an appropriate method to compare consumption of such products (Helgesen *et al.* 2014). The number of treatments per slaughtered fish has increased extensively. There are several causes for this trend. Lowered thresholds for compulsory therapeutic interference have led to frequent treatments, which in line has led to reduced sensitivity towards one or more chemical compounds, resulting in a vicious cycle evoking resistant parasites that likely disperse to neighbouring farms.

The last antiparasitic product to be introduced was emamectin benzoate in 1999 (Aaen *et al.* 2015a). Reduced sensitivity was detected as early as in 2006, in the species *C. rogercresseyi* in Chile and in *L. salmonis* in Ireland and Scotland (Horsberg 2012). This resistance mechanism has been spread widely among *L. salmonis* in the Northern Atlantic Ocean (Besnier *et al.* 2014), within a relatively short time-span of a maximum 11 years. The history of resistance and supplementary increasing sea lice numbers in all areas reflects the need for novel medicinal products effective against *L. salmonis* and *C. rogercresseyi*.

The aim of this study was to screen a wide range of substances for their *in vitro* effect on preadult *L. salmonis* using the IRAC classification scheme of insecticides as a basis for the selection of compounds (http://www.irac-online.org/modes-of-action/ [accessed on 17 September] (Aaen, Hamre & Horsberg 2016). Furthermore, the aim was to identify sensitive physiological and biochemical pathways in the salmon lice, with which medicinal compounds were able to interfere. For these screenings, possible adverse effects such as toxicity to the fish, bio-accumulation, side effects towards other arthropods or administrational challenges were not considered.

Materials and methods

Medicinal compounds were purchased from Sigma-Aldrich, Nerliens Meszansky and VWR. One compound was kindly supplied by Elanco, Switzerland, and one by Bayer, Norway. The compounds are listed in Table 1.

Water

The seawater used in the experiment was taken from 60 metres depth, filtered through a sand filter and subsequently a plankton mesh (150 μm).

Salmon lice

The parasites originated from the sensitive strains Ls A (Helgesen & Horsberg 2013) or Ls G (Hamre, Glover & Nilsen 2009). Maintenance and cultivation of salmon lice was conducted at the Marine Research Station Solbergstrand NIVA in the Oslofjord. Laboratory-reared Atlantic salmon of various size and weight were kept in tanks containing from 0.1 to 1 m^3 of seawater for cultivation of salmon lice. Salmon lice egg strings were harvested from adult females serving the sole purpose of breeding, as they would produce progeny of the same generation in successive rounds. The experiments were performed 3–5 weeks after copepodid infestation. Mainly preadult parasites were included, but some adult individuals were also present in some of the groups at different time points.

The salmon lice were harvested on the day of exposure. Fish were anaesthetized with 100 mg L^{-1} metacaine for 2–3 min. The parasites were gently removed from the fish with forceps and placed directly in glass containers filled with either 250 mL (30-min) or 1000 mL (24-h) of filtered seawater and kept on ice. Within 4 h following de-attachment from the fish, the exposure to medicinal compounds was commenced. The water temperature was between 7.0 °C and 9.0 °C. Two test series were performed, in order

Table 1 List of substances utilized in this study, grouped according to the IRAC mode of action classification

IRAC no.	Mode of action	Substance
1A	Acetylcholinesterase inhibitor	Propoxur
1B	Acetylcholinesterase inhibitor	Azamethiphos
2B	GABA-gated chloride channel blocker	Pyriprole
3	Sodium channel modulator	Cypermethrin
4A	Nicotinic acetylcholine receptor competitive modulator	Imidacloprid
4A	Nicotinic acetylcholine receptor competitive modulator	Nitenpyram
5	Nicotinic acetylcholine receptor allosteric modulator	Spinetoram
5	Nicotinic acetylcholine receptor allosteric modulator	Spinosad
6	Glutamate-gated chloride channel allosteric modulator	Emamectin benzoate
7B	Juvenile hormone mimic	Fenoxycarb
7C	Juvenile hormone mimic	Pyriproxyfen
9B	Modulator of chordotonal organs	Pymetrozine
9C	Modulator of chordotonal organs	Flonicamid
13	Uncoupler of oxidative phosphorylation	Sulfluramid
14	Nicotinic acetylcholine receptor channel blocker	Cartap
15	Inhibitors of chitin synthesis, type 0	Diflubenzuron
17	Moulting disruptor	Cyromazine
18	Ecdysone receptor agonist	Tebufenozide
19	Octopamine receptor agonist	Amitraz
22	Voltage-dependent sodium channel blocker	Metaflumizone
23	Inhibitor of acetyl CoA carboxylase	Spiromesifen
28	Ryanodine receptor modulator	Chlorantraniliprole
N.a.	Calcium channel modulator	Praziquantel
N.a.	β-Tubulin inhibitor	Thiabendazole
N.a.	Unknown	Azadirachtin
N.a.	Unknown	Pyridalyl

to investigate the effect of short- and long-term exposure to the medicinal compounds.

Test substance dilution: 30-min exposure

Every group of parasites was exposed to 50 mg L^{-1} of the test substance for 30 min. The correct concentration of the medicinal compound was obtained by weighing 12.5 mg of each substance in glass test tubes, dissolving it in 125 μL room-tempered dimethylsulphoxide (DMSO, Sigma-Aldrich) and 125 μL heated (40 °C) emulsion (Muan *et al.* 1985; Aaen *et al.* 2016). Every test tube was vortexed vigorously until the test substance was sufficiently dispersed. To each test tube, 5 mL seawater was added, before being vortexed for another 60 s. This volume was then added to a glass bottle (Duran, Item Number 215-1786, VWR) containing 245 mL of 8 °C seawater, herein distributing the medicinal compound to the total water volume by rotating the bottle. After exposure, the medicated water was poured out through a filter to collect moribund parasites; the bottle was rinsed with approximately 100 mL of fresh seawater, before it was refilled with 1000 mL of seawater. The bottles were placed in an incubator (10 °C) for 24 h before registration of mortality. The control groups were treated similarly, but only exposed to 125 μL DMSO and 125 μL emulsion being diluted in 250 mL of seawater. The bottles were supplied with constant aeration from air pumps throughout the 24-h incubation period. Groups of 6–25 parasites were used, and the group size was dependent on the available number of sea lice on that particular day. Effective compounds were subject to a dilution series with concentrations of 25, 10, 3, 1 and 0.1 mg L^{-1}.

Test substance dilution: 24-h exposure

Each group of parasites was exposed to 5 mg L^{-1} of the test substance for 24 h. The dilution procedure was the same as that for the 30-min assay, with 5 mg of each substance dissolved in 1000 mL of seawater. The control groups were exposed to a mixture of 125 μL DMSO and 125 μL emulsion diluted in 1000 mL of seawater. The groups consisted of 7–25 parasites. The bottles were finally incubated at 10 °C according to Helgesen & Horsberg (2013) for 24 h.

In both experiment series, the medicinal compounds immobilizing 75% or more of the parasites were subject to titration studies (assays with descending concentrations), in order to generate EC_{50} values, meaning the concentration immobilizing 50% of the parasites in the assay. The medicinal compounds underwent the same dilution procedures as in the initial experiments: a fivefold dilution system was selected, using concentrations of 1 mg L^{-1}, 200 μg L^{-1}, 40 μg L^{-1}, 10 μg L^{-1}, 2 μg L^{-1}, and 0.4 μg L^{-1}.

Registration

After the incubation period was over, each bottle was turned upside down three times and rotated carefully in a circle ten times (as described by

Helgesen & Horsberg 2013) to give the live parasites a chance to re-attach to the bottle wall. The water was then poured out through a funnel containing a filter, where immobilized parasites were collected. These parasites were given a second chance to prove their swimming ability in a Petri dish filled with fresh seawater. Immobile parasites were counted, classified according to sex and developmental stage and registered as immobilized. Parasites remaining attached to the bottle wall were classified as alive. Parasite sex and developmental stage was determined after filling the bottles with hot water, pouring the content through the same funnel and filter system collecting the parasites.

Statistical analysis

For compounds being effective in both assays, the EC_{50} value was calculated with data from titration assays using Probit analysis in JMP (SAS Institute Inc.).

Results

A summary of the results from the initial assays is provided in Table 2.

The EC_{50} value is considered to reveal the comparable toxicity of a substance, as it is mentioned in all toxicity studies with medicinal substances. The experiments yielded the following EC_{50} values, presented in Tables 3 and 4.

Table 2 The number of alive and immobilized parasites exposed to 50 mg L^{-1} of the test substance for 30 min or 5 mg L^{-1} for 24 h

IRAC no.	Substance	30-min exposure (live/immobilized)	24-h exposure (live/immobilized)
1	Propoxur	13/3	0/17
1	Azamethiphos	2/13	0/14
2	Pyriprole	0/9	0/25
3	Cypermethrin	0/11	0/17
4	Imidacloprid	0/7	0/17
4	Nitenpyram	11/4	2/12
5	Spinetoram	3/9	1/12
5	Spinosad	13/0	19/4
6	Emamectin benzoate	0/8	0/12
7b	Fenoxycarb	12/12	1/23
7c	Pyriproxyfen	9/0	0/20
9b	Pymetrozine	26/3	16/1
9c	Flonicamid	11/0	11/1
13	Sulfluramid	19/1	14/5
14	Cartap	3/20	0/16
15	Diflubenzuron	8/0	1/22
17	Cyromazine	13/0	23/0
18	Tebufenozide	10/1	16/3
19	Amitraz	17/2	19/0
22	Metaflumizone	8/1	17/0
23	Spiromesifen	10/0	0/15
28	Chlorantraniliprole	12/1 + 10/0	14/1
N.a.	Praziquantel	9/2	19/1
N.a.	Thiabendazole	8/0	11/1
N.a.	Pyridalyl	Not performed	11/1
N.a.	Azadirachtin	6/0	Not performed
	Controls	34/1, 27/1, 30/2, 16/2	7/1, 8/0, 18/1, 9/3, 9/0

30-min assay

Compounds from two different groups were effective when acting over a short period against preadult salmon lice. Pyriprole, a substance acting as an antagonist on the gamma-amino butyric acid (GABA)-gated chloride channels, quickly induced mortality and was therefore selected for further titrating studies. The nicotinic acetylcholine receptor (nAChR) was also subject to activity from several substances that interfere with this receptor in different ways. Both cartap hydrochloride, a blocker of this receptor, spinetoram, a modulator, and finally imidacloprid, a receptor activator, were effective in the initial study. These compounds were selected for further titration studies. Nitenpyram, a compound structurally similar to imidacloprid, was also included, as was spinosad, a compound that is structurally similar to spinetoram. Pyriprole was the most efficacious compound, with an EC_{50} value in the region of 100 μg L^{-1} in the 30-min assay and 1 μg L^{-1} in the 24-h assay.

24-h assay

This study revealed a list of compounds that knocked out *L. salmonis* when exposed to 5 mg L^{-1} concentrations for a period of 24 h: propoxur, fenoxycarb, cartap, spiromesifen, nitenpyram, pyriprole, spinetoram, imidacloprid and diflubenzuron were proved effective at this concentration. These substances were further investigated in titration studies using decreasing concentrations. Pyriprole and cartap yielded the lowest EC_{50} values, followed by imidacloprid. The juvenile hormone mimics fenoxycarb and pyriproxyfen also resulted in EC_{50} values below 1 mg L^{-1}.

Table 3 Cohorts of sea lice exposed to declining concentrations of antiparasitics, with the outcome being immobilized or alive. Exposure period of 30 min, followed by 20- to 24-h residence in clean seawater with constant aeration. EC_{50} values (in mg L^{-1}) calculated with probit analysis in JMP (90% confidence intervals in brackets where available)

Substance	EC_{50}	EC_{90}
Pyriprole	0.108 mg L^{-1}	0.118 mg L^{-1}
Imidacloprid	8.4 (3.2–22.3) mg L^{-1}	46.3 (7.4–289.6) mg L^{-1}
Cartap	4.9 (0.7–33.9) mg L^{-1}	38.2 (11.5–127.0) mg L^{-1}
Spinetoram[a]	51.0 (13.0–199.6) mg L^{-1}	1845.0 (77.3–44 049.5) mg L^{-1}

[a]Not well dissolved.

Discussion

Application of medicinal compounds to organisms living in seawater could occur using several methods. Bath treatment is an obvious solution that has been widely implemented in fish farming already. Three of the compound groups, pyrethroids, organophosphates and hydrogen peroxide, are applied as bath treatments in the cages. Tarpaulins surrounding the treatment volume ensure the maintenance of the wanted concentration, which once removed, initiate a rapid concentration drop. The advantage of bath treatment is a reasonably even dispersion of the compound due to aeration of the cage and fish movements. Fish and parasites are exposed irrespective of their appetite. The other current approach to medicate fish infested with sea lice is oral treatments using medicinal feed. Two substance classes, avermectins and chitin synthesis inhibitors, are the current remedies available with this application route. This method demands less mechanical and physical intervention; however, fish with low appetites may receive a subtherapeutic dose and following treatment may be in danger of facilitating the release of infective larvae from surviving sea lice to the surroundings. The 30-min assay was included in our study in order to mimic a bath treatment, whereas the 24-h assay was an attempt to simulate an oral feeding regime.

For the 30-min challenge, 50 mg L^{-1} was chosen as the start concentration. In comparison, the recommended dose used for the available medicinal substances for bath treatment, deltamethrin, cypermethrin and azamethiphos, is 0.002, 0.015 and 0.1 mg L^{-1}, respectively. For the 24-h assay, a tenfold dilution of this concentration was chosen: 5 mg L^{-1}. Substances resulting in a mortality of 75% or more underwent further trials, where the concentration killing 50% of the parasites (EC_{50}) was sought. Herein, a fivefold dilution series with these compounds was performed until the EC_{50} value was determined. The work-flow of these experiments is presented in Fig. 1. Initial concentrations at, respectively, 50 and 5 mg L^{-1} were considered appropriate for sea lice studies, as they separated non-effective and effective substances well, and were also reasonably convenient to work with in multiple experiment set-ups.

The outcome of bioassays is usually dead, moribund or alive. This has in many cases led to statistical challenges. In our study, the sea lice were classified as either immobilized or alive; the live criterion being the ability to attach to the bottle wall or being able to swim in a straight line. When not fulfilling these criteria, the parasites were classified as immobilized (Sevatdal &

Table 4 Cohorts of sea lice exposed to declining concentrations of antiparasitics, with the outcome being immobilized or alive. Exposure period of 24 h with constant aeration. Medicinal compounds presented with decreasing EC_{50} values (in μg L^{-1}, calculated with probit analysis in JMP), with 90% confidence intervals in brackets (where available)

Substance	EC_{50}	EC_{90}
Pyriprole	0.9 (0.5–1.6) μg L^{-1}	2.2 (0.9–5.0) μg L^{-1}
Propoxur	<10 μg L^{-1}	
Cartap	5.2 (1.2–22.3) μg L^{-1}	152.6 (57.3–406.0) μg L^{-1}
Imidacloprid	97.6 (74.1–148.6) μg L^{-1}	334.2 (152.3–733.7) μg L^{-1}
Fenoxycarb	408 (256.0–651.1) μg L^{-1}	1209.1 (563.8–2593.1) μg L^{-1}
Pyriproxyfen	676.6 (393.5–1163.3) μg L^{-1}	2862.0 (557.4–9595.8) μg L^{-1}
Nitenpyram	1700 (237–12 281) μg L^{-1}	209 423 (4283–10 239 694) μg L^{-1}
Spinetoram	1919 (1200–3070) μg L^{-1}	4525 (2279–8982) μg L^{-1}
Spiromesifen	2039 (322–12 880) μg L^{-1}	65 577 (1972–2 179 696) μg L^{-1}
Diflubenzuron	5000 μg L^{-1}	Could not be calculated

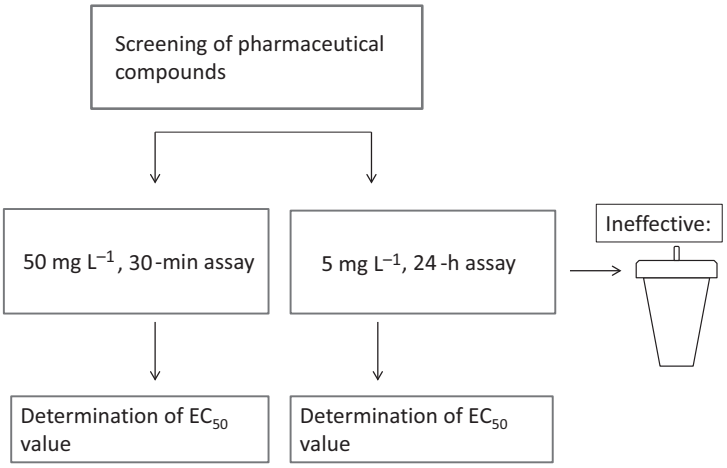

Figure 1 Flow diagram of the screening procedure for determining the parasite sensitivity towards pharmaceutical compounds.

Horsberg 2003). These abilities were either registered either at 24 h after exposure, or after 24 hours' residence in the diluted solution. Ephemeral immobility after short-time exposure was thus not included in the formal registration.

Agricultural pesticides are subject to degradation by many factors, for example, sunlight, hydrolysis, and in pest targets themselves. The worldwide usage of pesticides is formidable and subject to criticism. These factors should also be considered in the rapidly growing fish farming industry. This set of experiments have illustrated that dissolving medicinal substances in seawater, to which sea lice are strictly committed, is a challenging task. Several of the molecules only partially dissolved in seawater due to the interaction with ubiquitous salts and other oligo-elements. Other substances dissolved easily in organic solvents such as DMSO or ethanol. However, high solubility in such solvents does not necessarily mean that the compound is soluble in seawater. A mix of DMSO and an emulsion (Muan *et al.* 1985; Aaen *et al.* 2016) proved to be a suitable way of overcoming this problem, offering low toxicity to the parasite and sufficiently dissolving most of the compounds. In a pilot study, a 50/50 mixture of the emulsion and DMSO was the most gentle to preadult sea lice.

Medicinal compounds featuring modes of actions not already utilized against sea lice have been sought for years. The existing remedies act on the acetylcholinesterase enzyme (azamethiphos), the voltage-gated sodium channels (deltamethrin/cypermethrin), the glutamate-gated chloride channels (emamectin benzoate), the chitin synthesis (diflubenzuron/teflubenzuron) or as a general disinfectant (hydrogen peroxide).

In the current study, several additional physiological and biochemical pathways vulnerable to various medicinal compounds were identified. Compounds from group 1 of the IRAC classification, organophosphates and carbamates, have been in use since the early stages of salmon farming in the 1970s (Grave & Horsberg 2000). Azamethiphos is the only current anti-sea lice compound with acetylcholinesterase inhibitory properties. In a therapeutic context, organophosphates are only effective against preadults and adults and not chalimus instars, probably because their target, the acetylcholinesterase, is less expressed in the earlier stages of the parasite development. Surprisingly, the carbamate propoxur was not effective in our experiment, even at the high concentration of 50 mg L^{-1} in the 30-min assay. The compound was, however, very effective in the 24-h assay.

The nicotinic (neuronal) acetylcholine receptor (nAChR) proved to be a suitable target for medicines in these experiments. One group of compounds acting on this receptor are the neonicotinoids. Consisting of seven separate insecticides, imidacloprid, thiacloprid, thiamethoxam, acetamiprid, nitenpyram, clothianidin and dinotefuran, the neonicotinoids are used to combat pest organisms on a wide range of crops (codex alimentarius: http://www.codexalimentarius.net/pestres/data/pesticides/details.html;jsessionid=208EB91253CF44C876A52B43153128D8?d-16497-o=2&id=206&d-16497-s=3) and parasites on animals. In

Australia and New Zealand, products containing compounds from this group are available for use on sheep; otherwise, companion animals are the main consumers of these substances. A prominent feature of neonicotinoids is their specificity to invertebrate nAChR compared to vertebrate nAChR (Matsuda *et al.* 2001). Furthermore, this group of compounds is reported to induce toxic effects on crustaceans when distributed in extremely low concentrations (Morrissey *et al.* 2015).

Neonicotinoid compounds have become quite controversial recently, especially as they have been linked to the massive decline in bee hives (Whitehorn *et al.* 2012). Their relatively long persistence in aquatic environments complicates its use as an antiparasitic compound (reviewed by Morrissey *et al.* 2015; Goulson 2013). In our experiment, imidacloprid was highly effective against *L. salmonis*; however, the related compound nitenpyram did not yield an similar effect. This indicates that the nicotinic acetylcholine receptor displays properties affecting the affinity of the ligands, connecting a high degree of specificity to this receptor.

A blocker, or inhibitor of the nAChR, cartap, was found to be highly effective in both assays. This compound is an analogue of nereistoxin, a product of the marine annelid *Lumbriconereis heteropoda* (Ray 1991). Cartap is found within the group 14 of the IRAC scheme, joint with bensultap, thiocyclam and thiosultap-sodium. In this experiment, the EC_{50} value of cartap was calculated to 4.9 mg L^{-1} (confidence interval 0.7–33.9) in the 30-min assay, and 5.2 μg L^{-1} (confidence interval 1.2–22.3) in the 24-h assay, which were the second lowest values of all tested compounds. Cartap is described to have high affinity to a noncompetitive blocker site on this receptor in *Apis mellifera* (Lee, Tomizawa & Casida 2003). In contrast to neonicotinoids, and in fact nereistoxin itself, this compound is not thought to bind to the imidacloprid binding site, at least in this species (*A. mellifera*). Asahi & Yoshida (1977) claimed that cartap rapidly hydrolyses to nereistoxin under alkaline conditions. In addition, cartap has been found to be far more effective at pH 7.4 than at 6.1 (Lee *et al.* 2004).

In an experiment where rats were orally fed with cartap over 2 years (Hartley & Kidd 1987), no pathological lesions were observed. The half-life of cartap in water is short; 10 min at pH 7 and 25 °C. In addition, the half-life of nereistoxin was reported to be 2.4 years at the same pH (Menzie 1980). In our study, it is therefore not clear whether the effect of cartap on sea lice is due to cartap itself, or the metabolized form nereistoxin. In general, the group of nereistoxin analogues represents a great potential for sea lice combat. The LC_{50} of *Pomacea canaliculata*, a snail, is 2.0 mg L^{-1} in a 48-h assay. In comparison, the LD_{50} value for oral exposure in rats is 340 mg kg^{-1}. The availability of molecular methods and possibly other histological/interference studies offers a great asset for assessing the definite binding site, affinity levels and the correct molecule configuration of this molecule group.

Spinetoram, a macrocyclic lactone also executing its effect on the nAChR, was also effective in both assays. However, this compound was the most difficult of all compounds to dissolve in seawater, so the EC_{50} values are most likely inaccurate. The best results were obtained when dissolving it overnight in DMSO and the formulated emulsion, followed by lengthy vortexing. Spinosad, a related compound, was less effective than spinetoram. Emamectin benzoate, also a macrocyclic lactone, was as expected highly effective in both initial screenings. The EC_{50} value was not further investigated, as this compound is already in use as an in-feed treatment agent.

Medicinal compounds interfering with GABA-gated chloride channels are widely used against insects and other arthropods in both pest combat and against ectoparasites on animals. Our model substance from this group, pyriprole, also proved highly effective in both assays, down to a fairly low concentration. The substance was easily dissolved in seawater. Pyriprole belongs to the group of phenylpyrazoles, along with ethiprole and fipronil. Phenylpyrazoles are tested on aquatic copepods, and varying sensitivities towards different crustaceans are reported by Chandler *et al.* (2004). Cary *et al.* (2004) observed a huge difference in sensitivity related to gender in *Amphiascus tenuiremis*, a copepod living in brackish water, as males were four times more sensitive than adult females. An effect of such calibre was, however, not observed (for any compound) in our experiment, but may be present when the exposure period exceeds 24 h.

Phenylpyrazoles, together with neonicotinoids, constitute approximately one-third of the reported

agricultural insecticide consumption worldwide (Simon-Delso *et al.* 2015), but are also widely used for companion animals (Jennings *et al.* 2002). Cary *et al.* (2004) showed that fipronil induced reduced fertility in males of *A. tenuiremis*. The degradation products of fipronil possess properties harmful to terrestrial ecosystems (Konwick *et al.* 2006). Phenylpyrazoles thus may imply severe adverse effects on other organisms living in waters close to fish farms, possibly making them inappropriate for use against sea lice.

In summary, neonicotinoids, phenylpyrazoles and cartap hydrochloride proved to affect salmon lice preadults at the lower range of the μg L^{-1} in this experiment series, as shown in Tables 3 and 4. Other compounds were also effective, however, at substantially higher concentrations than the previously mentioned compound groups.

The IRAC group 7 includes the juvenile hormone mimics, of which two were tested here, fenoxycarb and pyriproxyfen. Fenoxycarb has the chemical structure of a carbamate, but also acts as a hormone analogue in pest organisms. This compound was to some extent effective in the 30-min assay, possibly due to its carbamate nature, affecting the acetylcholine esterase. In the 24-h assay however, it yielded 100% mortality at 5 mg L^{-1}, supported by the result of the sister compound pyriproxyfen, which also immobilized 100% of the parasites at the same concentration. The EC_{50} value of fenoxycarb was calculated to 408 μg L^{-1} (255–651) and for pyriproxyfen 676.6 μg L^{-1} (292.5–1163.3) after 24 h exposure. Carbamates are considered acetylcholinesterase inhibitors, similar to organophosphates. They may also work as nACh-receptor inhibitors, as described by Smulders *et al.* (2003). Fenoxycarb and pyriproxyfen, or related compounds such as methoprene or its equivalents, could represent a promising group of novel anti-sea lice medicines.

The acetyl coenzyme A carboxylase inhibitor spiromesifen was another effective compound on preadult sea lice in this experiment. After 24 h, 100% of the parasites were immobilized at the initial concentration of 5 mg L^{-1}. However, in the following titration studies, no immobilization was observed. This compound differs somewhat from the rest of the efficacious substances, as it acts on other physiological pathways than the components of the nervous system. Spiromesifen belongs to the chemical class of spirocyclic phenyl-substituted tetronic acids and is effective on both eggs and several instars of other arthropod species, such as the greenhouse whitefly, *Trialeurodes vaporariorum* (Bi & Toscano 2007). In another study, species such as *Daphnia magna*, rainbow trout and honeybee were vulnerable to concentrations way below the EC_{50} value of sea lice, in the lower μg L^{-1} area (California Department of Pesticide Regulation, 2005). The tobacco fly, *Bemisia tabaci*, was vulnerable to concentrations similar to the EC_{50} value of *L. salmonis* (Kontsedalov *et al.* 2008). These values are although not directly comparable, as the *B. tabaci* were exposed via spraying and the *L. salmonis* via bath application. This substance's distinctive mode of action makes it eventually interesting for sea lice combat, as it is unlikely to be related to any existing resistance mechanisms (Guthrie, Denholm & Nauen 2003). A similar compound called spirodiclofen could also be of interest (Dekeyser 2005).

Praziquantel, a traditional compound used against cestodes in both vertebrates and fish, was included in the experiment. Its mode of action is not clearly defined, but several theories have been suggested. In 2005, Greenberg reviewed the matter and concentrated on the calcium channels and calcium homeostasis as possible molecular targets (Greenberg 2005). Interactions between praziquantel and phospholipids in membranes have also been subject to investigation (Harder, Goossens & Andrews 1988). In the 30-min assay, a distinct temporary immobilization caused by praziquantel was observed. However, after 24 h, all parasites had fully recovered. In the 24-h assay, no effect of the compound was detected. Praziquantel has a high safety margin towards fish. Forwood, Harris & Deveney (2013) bathed silver perch in 10 mg L^{-1} for 48 h, with no signs of pathology registered, and with good efficacy towards a freshwater cestode, *Lepidotrema bidyana* Murray (Forwood *et al.* 2013). Both oral feeding and bath treatment with this compound were effective against the targeted parasite species. Although no lethal effects were observed in salmon lice subjected to this treatment agent, further investigation should be conducted with praziquantel or related compounds, as the initial immobilization of the parasite could be of great value.

The chitin synthesis inhibitors teflubenzuron and diflubenzuron are in-feed formulations, being effective against instars that are undergoing moulting. Diflubenzuron was included in this

experiment as a model substance as a bath treatment. The compound is known to disrupt the growth process, including in sea lice, by inhibiting the synthesis of chitin, an important structure of the exoskeleton (Roth, Richards & Sommerville 2006). No mortality was observed in the 30-min assay, but surprisingly, it was highly effective at a concentration of 5 mg L^{-1} after 24 h of exposure. Grosscourt (1976) suggested a stomach poison property of the compound (Grosscourt 1976) which may explain the relatively immediate impact of diflubenzuron.

In these tests, we are looking for positive results, namely immobilized sea lice. The power of the significance test is the same thing as the sensitivity of a screening test (Colcuhoun 2014). False positives are to a great degree ruled out through the use of control group, but cannot be neglected. However, the high concentration followed by decreasing concentration of the titrating study compensated for this. The high starting concentration should rule out false negatives (effective compounds failing to be so because of insufficient disintegration in the dissolvent).

The reproductive ability of parasites surviving either concentrations close to the EC_{50} value or the initial concentration of 5/50 mg L^{-1} was not further investigated.

Conclusions

The nicotinic acetylcholine receptor and the GABA-gated chloride channels were identified as sensitive targets for novel salmon lice medicines. The acetyl coenzyme A of salmon lice is another possible candidate for provocation, as well as juvenile hormone analogues.

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