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. Nauplii I and II, the infective copepodid,
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 (Hästein & Bergsjo 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 m³ 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

<table>
<thead>
<tr>
<th>IRAC no.</th>
<th>Mode of action</th>
<th>Substance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>Acetylcholinesterase inhibitor</td>
<td>Propoxur</td>
</tr>
<tr>
<td>1B</td>
<td>Acetylcholinesterase inhibitor</td>
<td>Azamethiphos</td>
</tr>
<tr>
<td>2B</td>
<td>GABA-gated chloride channel blocker</td>
<td>Pyriproxyfen</td>
</tr>
<tr>
<td>3</td>
<td>Sodium channel modulator</td>
<td>Cypermethrin</td>
</tr>
<tr>
<td>4A</td>
<td>Nicotinic acetylcholine receptor</td>
<td>Imidacloprid</td>
</tr>
<tr>
<td>4A</td>
<td>Nicotinic acetylcholine receptor</td>
<td>Nitenpyram</td>
</tr>
<tr>
<td>5</td>
<td>Nicotinic acetylcholine receptor</td>
<td>Spinetoram</td>
</tr>
<tr>
<td>5</td>
<td>Nicotinic acetylcholine receptor</td>
<td>Spinosad</td>
</tr>
<tr>
<td>6</td>
<td>Glutamate-gated chloride receptor</td>
<td>Emamectin benzoate</td>
</tr>
<tr>
<td>7B</td>
<td>Juvenile hormone mimic</td>
<td>Fenoxycarb</td>
</tr>
<tr>
<td>7C</td>
<td>Juvenile hormone mimic</td>
<td>Pyriproxyfen</td>
</tr>
<tr>
<td>9B</td>
<td>Modulator of chitin synthesis, type 0</td>
<td>Spinetoram</td>
</tr>
<tr>
<td>9C</td>
<td>Modulator of chitin synthesis, type 0</td>
<td>Pyriproxyfen</td>
</tr>
<tr>
<td>13</td>
<td>Uncoupler of oxidative phosphorylation</td>
<td>Sulfuramid</td>
</tr>
<tr>
<td>14</td>
<td>Nicotinic acetylcholine receptor</td>
<td>Cartap</td>
</tr>
<tr>
<td>15</td>
<td>Inhibitors of chitin synthesis, type 0</td>
<td>Diflubenzuron</td>
</tr>
<tr>
<td>17</td>
<td>Moulting disruptor</td>
<td>Cyromazine</td>
</tr>
<tr>
<td>18</td>
<td>Ecdysone receptor agonist</td>
<td>Tebufenozide</td>
</tr>
<tr>
<td>19</td>
<td>Octopamine receptor agonist</td>
<td>Amitraz</td>
</tr>
<tr>
<td>22</td>
<td>Voltage-dependent sodium channel blocker</td>
<td>Metalfumizone</td>
</tr>
<tr>
<td>23</td>
<td>Inhibitor of acetyl CoA carboxylase</td>
<td>Spiromesifen</td>
</tr>
<tr>
<td>28</td>
<td>Ryanodine receptor modulator</td>
<td>Chiorantraniilprole</td>
</tr>
<tr>
<td>N.a.</td>
<td>Calcium channel modulator</td>
<td>Phraziquantel</td>
</tr>
<tr>
<td>N.a.</td>
<td>β-Tubulin inhibitor</td>
<td>Thiazendazole</td>
</tr>
<tr>
<td>N.a.</td>
<td>Unknown</td>
<td>Azadirachtin</td>
</tr>
<tr>
<td>N.a.</td>
<td>Unknown</td>
<td>Pyridalyl</td>
</tr>
</tbody>
</table>

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 being 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 EC₅₀ 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 EC50 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 EC50 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 EC50 values, presented in Tables 3 and 4.

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-aminobutyric 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 EC50 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 EC50 values, followed by imidacloprid. The juvenile hormone mimics fenoxycarb and pyriproxyfen also resulted in EC50 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. EC50 values (in mg L⁻¹) calculated with probit analysis in JMP (90% confidence intervals in brackets where available)

<table>
<thead>
<tr>
<th>Substance</th>
<th>EC₅₀</th>
<th>EC₉₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyriprole</td>
<td>0.108 mg L⁻¹</td>
<td>0.118 mg L⁻¹</td>
</tr>
<tr>
<td>Imidacloprid</td>
<td>8.4 (3.2–22.3) mg L⁻¹</td>
<td>46.3 (7.4–289.6) mg L⁻¹</td>
</tr>
<tr>
<td>Cartap</td>
<td>4.9 (0.7–33.9) mg L⁻¹</td>
<td>38.2 (11.5–127.0) mg L⁻¹</td>
</tr>
<tr>
<td>Spinetoram</td>
<td>51.0 (13.0–199.6) mg L⁻¹</td>
<td>1845.0 (77.3–4049.5) mg L⁻¹</td>
</tr>
</tbody>
</table>

*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. Tarpons 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, whereas the 24-h assay was the start concentration in our study in order to mimic a bath treatment, whereas the 24-h assay was an attempt to simulate an oral feeding regime.

When not fulfilling these criteria, the parasites 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.

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 EC₅₀ values (in µg L⁻¹, calculated with probit analysis in JMP), with 90% confidence intervals in brackets (where available)

<table>
<thead>
<tr>
<th>Substance</th>
<th>EC₅₀</th>
<th>EC₉₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyriprole</td>
<td>0.9 (0.5–1.6) µg L⁻¹</td>
<td>2.2 (0.9–5.0) µg L⁻¹</td>
</tr>
<tr>
<td>Propoxur</td>
<td>&lt;10 µg L⁻¹</td>
<td>152.6 (57.3–406.0) µg L⁻¹</td>
</tr>
<tr>
<td>Cartap</td>
<td>5.2 (1.2–22.3) µg L⁻¹</td>
<td>334.2 (152.3–733.7) µg L⁻¹</td>
</tr>
<tr>
<td>Imidacloprid</td>
<td>97.6 (74.1–148.6) µg L⁻¹</td>
<td>1209.1 (563.8–2593.1) µg L⁻¹</td>
</tr>
<tr>
<td>Fenoxycarb</td>
<td>408 (256.0–651.1) µg L⁻¹</td>
<td>1209.1 (563.8–2593.1) µg L⁻¹</td>
</tr>
<tr>
<td>Pyriproxyfen</td>
<td>676.6 (393.5–1163.3) µg L⁻¹</td>
<td>2962.0 (557.4–9595.8) µg L⁻¹</td>
</tr>
<tr>
<td>Nitenpyram</td>
<td>1700 (227–12 281) µg L⁻¹</td>
<td>209 423 (4283–10 239 694) µg L⁻¹</td>
</tr>
<tr>
<td>Spinetoram</td>
<td>1919 (1200–3070) µg L⁻¹</td>
<td>4525 (2279–4962) µg L⁻¹</td>
</tr>
<tr>
<td>Spriomesifen</td>
<td>2039 (322–12 880) µg L⁻¹</td>
<td>65 577 (1972–179 698) µg L⁻¹</td>
</tr>
<tr>
<td>Diflubenzuron</td>
<td>5000 µg L⁻¹</td>
<td>Could not be calculated</td>
</tr>
</tbody>
</table>
These abilities were either registered either at 24 h after exposure, or after 24 hours’ residence in the diluted solution. Ephemeroid 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 dinofuran, 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.
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 (Whithorn 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 a 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 bensulide, thiocyclam and thiosultap-sodium. In this experiment, the EC50 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 \(\mu\)g 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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**References**


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