VESO Oslo

Selection of resistance of sea lice (*Lepeophtheirus salmonis*) to organophosphate and pyrethroid by combined treatment methods

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Summary

The objective of this study was to observe and investigate resistance development of a laboratory designed sea lice (*Lepeophtheirus salmonis*) population with a defined frequency of resistance markers to azamethiphos (Salmosan) and deltamethrin (AlphaMax) resistance. Altantic salmon postsmolts (255) were infestated with a mixed sea lice population. The frequency of resistance markers of the test population was found to be: Deltamethrin resistance: 29.2 % sensitive (S) and 70.8 % resistant (R), azamethiphos resistance: 37.5% homozygote sensitive (SS), 50% heterozygotes (RS), and 12.5% homozygote resistant (RR). The fish were divided in five groups and each were exposed to one of the regimes when the sa lice had reached the mobile pre-adult stage: AlphaMax: 2 µg/L deltamethrin (2 ppb) for 30 minutes, Salmosan: 100 µg/L azamethiphos for 60 minutes, Ecolice: 10 µg/L azamethiphos for 15 min before mixing in 2 µg/L deltamethrin (2 ppb) for 30 minutes, Combination: 2 µg/L deltamethrin (2 ppb) + 100 µg/L azamethiphos for 60 minutes and one untreated control group. All groups were treated with 1300 mg/L hydrogenperoxid (H_2O_2) for 20 minutes one week after the selective treatments to investigate if the first selective treatments had changed sensitivity to H_2O_2. Dead and moribund sea lice were sampled at timepoints 10, 20, 30, 60, 240, 1440 minutes and then each day for 6 days after start of selective treatments and then prior to and after H_2O_2 treatment. Randomly selected lice were analysed for genetic markers at each time between 10 – 1440 minutes and prior to and after H_2O_2 treatment. Total number of lice was registered after termination of the study by adding number of dead and moribund lice at each sampling point, dead and moribund lice after H_2O_2 treatment and lice surviving lice after H_2O_2 treatment. Each analysed louse was categorized in two categories for deltamethrin sensitivity, as either R – resistant or S – sensitive and for azamethiphos sensitivity in three categories for OP sensitivity on the basis of type of each allele; SS – homozygote sensitive, RS – heterozygote (one resistant- and one sensitive allele) and RR homozygote resistant.

Treatment efficacy was in decreasing order: Azamethiphos (80.4 %) > Combined (76.2 %) > Ecolice (68.4 %) > deltamethrin (32.3 %). The numbers of genotypes for pyrethroid and azamethiphos sensitivity of surviving sea lice of each regime was estimated. Only resistant genotypes were found in the analysed fraction of the survived lice after deltamethrin treatment. No sensitive (SS) genotypes were found and only a small fraction of heterozygotes (RS) (20 %) in the analysed fraction of the survived lice after azamethiphos treatment.

There was a higher frequency of resistant genotype to azamethiphos (RR) and lower frequency of RS and SS in lice surviving H_2O_2 compared to dead/ moribund lice.

A higher total mortality rate could result in longer intervals between treatments. Fewer treatments will result in less selective pressure for resistance. At the same time, treatments that leave only a small proportion of the sensitive alleles in the population can be a driving force towards a higher resistance level. These forces act in opposite directions, and the balance point has not been determined in the current study. Rotation between treatments with different classes of active ingredients will slow resistance to each chemical class and should always for part of an integrated pest management strategy.

Sammendrag

Hensikten med studien var å observere og undersøke utviklingen av resistens av en populasjon lakselus (*Lepeophtheirus salmonis*) som ble satt sammen til en populasjon med definert frekvens av genetiske markører for resistens mot azametifos (Salmosan) og deltametrin (AlphaMax). Atlantisk laks postsmolt (255) ble infestert med en blandet populasjon med lakselus. Frekvensen av resistens markører ble estimert til å være: Deltametrin resistens: 29.2 % sensitive (S) og 70.8 % resistente (R), azametifos resistens: 37.5 % homozygot sensitive (SS), 50 % heterozygote (RS) og 12.5 % homozygot resistente (RR). Fisken ble delt i fem grupper og hver av disse ble eksponert for et av følgende regimer når lakselusa var utviklet til preadult: AlphaMax: 2 µg/L deltametrin (2 ppb) i 30 minutter, Salmosan: 100 µg/L azametifos i 60 minutter, Ecolice: 10 µg/L azametifos i 15 min før innblanding av 2 µg/L deltametrin (2 ppb) i 30 minutter, Kombinasjon: 2 µg/L deltametrin (2 ppb) + 100 µg/L azametifos i 60 minutter. Det var også en ubehandlet kontrollgruppe. Alle gruppene ble behandlet med 1300 mg/L hydrogenperoksid (H_2O_2) i 20 minutter en uke etter de selektive behandlingene for å undersøke om de første behandlingene hadde endret følsomheten for H_2O_2. Døde og moribunde lakselus ble samlet inn ved tidspunkt:10, 20, 30, 60, 240, 1440 minutter, og deretter hver dag i 6 dager etter start av behandlingene, samt før og etter behandling med H_2O_2. Tilfeldig utvalgte lus ble analysert for genetiske markører fra hvert prøveuttak mellom 10 – 1440 minutter, og før og etter H_2O_2 behandling. Totalt antall lus ble beregnet etter avslutning ved å legge sammen døde og overlevende. Hver analysert lus ble kategorisert i to kategorier for følsomhet for deltametrin, enten R – resistent eller S – sensitiv, og i tre kategorier for følsomhet for azametifos på basis av type alleler; SS – homozygote sensitiv, RS – heterozygote (et resistent og et sensitivt allel) og RR – homozygote resistent.

Behandlingseffekt i minskende rekkefølge: Azametifos (80.4 %) > Kombinasjon (76.2 %) > Ecolice (68.4 %) > deltametrin (32.3 %). Antall genotyper for pyretroid- og azametifos følsomhet som overlevde fra hvert regime ble estimert. Kun resistente (R) genotyper ble funnet i fraksjonen som ble analysert etter behandling med deltametrin. Ingen sensitive (SS) genotyper ble funnet, og kun en liten fraksjon heterozygote (RS) (20 %) ble funnet in den analyserte fraksjonen av lus som hadde overlevd behandling med azametifos.

Det ble funnet en høyere frekvens av den resistente genotypen for azametifos (RR) og en lavere frekvens av heterozygote (RS) og sensitive (SS) i lus som hadde overlevd H_2O_2, sammenlignet med døde / moribunde lus.

Høy total dødelighet kan resultere lenger intervaller mellom behandlinger og færre behandlinger, noe som igjen medfører redusert selektiv press mot resistens. Behandlinger som lar kun få sensitive alleler være igjen i populasjonen, kan være en drivende kraft bak utvikling av et høyere nivå av resistens. Disse to kreftene virker i motsatt retning, og balansepunktet, har ikke blitt bestemt i denne studien. Rotering av behandlingsmidler med ulike klasser aktive substanser, vil forsinke utviklingen av resistens, og bør alltid være en del av en integrert bekjempelsesstrategi.

Title
Selection of resistance of sea lice (*Lepeophtheirus salmonis*) to organophosphate and pyrethroid by combined treatment methods.

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Introduction

Sea lice (*Lepeophtheirus salmonis*) are the most severe parasitic problem in Atlantic salmon aquaculture. Atlantic salmon with sea lice are treated with a range of substances in Norway. Bath treatments: Synthetic pyrethroids (deltamethrin – AlphaMax (Pharmaq)), cypermethrin – (Betamax (Novartis)) and organophosphate (OP - azamethiphos – Salmosan (Fish Vet Group)). In feed treatments: Avermectin (Emamectin benzoate – Slice (MSD)), chitin synthesis inhibitors (diflubenzuron – Releeze (Ewos) and Teflubenzuron – Ektobann (Skretting)). Hydrogen peroxide (H_2O_2) (Paramove, AquaPharma) is also used as a bath treatment. Atlantic salmon are treated at relatively low infestation levels. In Norway the regulation is average 0.5 adult female sea lice per fish at site level in the entire year (01 January – 31 December). This low treatment threshold is set to protect wild salmon and sea trout, and to keep a low reproduction rate to reduce re-infestation. Other methods are also used to control sea lice because of resistance, namely cleaner fish, fallowing, synchronised area treatments, mechanical removal of sea lice and a combination of treatment products.

Resistance in sea lice is documented for most medicinal products (Sevatdal et al. 2005a, Fallang et al. 2005, Tribble et al. 2007, Bravo et al. 2008). Resistance develops because of repeated treatments with the same substance or related substances (as with pyrethroids) over long periods, and because treatments are rarely 100 % effective. Due to a number of factors, there will always be surviving lice that can reduce the effective concentration of the substance in question. So far there is no reported resistance to chitin synthesis inhibitors.

Resistance to azamethiphos and pyrethroids

Azamethiphos (OP) and pyrethroids act on different target sites in the arthropod nervous system, i.e. the mechanisms behind resistance should be different. There is the possibility of resistance development which is caused by mechanisms based on enhanced multi-function oxidases (MFO) or esterase activity, both of which are identified as a possible resistance mechanism in sea lice (Sevatdal et al. 2005b). Both mechanisms have the potential to affect OP`s and pyrethroids. MFO-based systems selected by OP`s or pyrethroids could conceivably extend to other unrelated compounds including teflubenzuron and the avermectins, whose molecules are also vulnerable to oxidative attack.

Azamethiphos (and other OP`s) acts by inhibiting the enzyme acetylcholinesterase (AChE). One mechanism behind resistance to azamethiphos in sea lice has been identified. Resistant

sea lice have developed a type of AChE that is not totally inhibited by azamethiphos (Fallang et al. 2004). Recently, the scientific group of Prof. Tor Einar Horsberg (NMBU) has found a genetic marker that seems to be specific for resistance to azamethiphos, and the group has also developed a rapid method to identify this marker in sea lice (Kaur *et al*. 2014).

Pyrethroids act by binding to the sodium channel and blocking sodium transfer. This affects the nerve cell membranes and thereby changes the transmission of nerve impulses. Resistance is caused by a mutation in the sodium channels that changes the binding site of pyrethroids. This mechanism was first found in houseflies and named "**k**nock**d**own **r**esistance" (kdr), and it has also been found in sea lice (Fallang et al. 2005). The scientific group of Frank Nilsen (Sea Lice Research Centre) has found a genetic marker that is specific to pyrethroid resistance and a method of identifying this in sea lice has been developed.

Combination treatments
Combination treatments against sea lice were first developed and used in Scotland (Excis (Novartis) + Salmosan (FVG). The low cis-cypermetrin product Excis was the only pyrethroid on the market at that time. Excis alone was becoming less effective against adult female lice and the combination with Salmosan (azamethiphos) was developed. This combination was highly effective against all stages. By combining these two products, better efficacy was therefore achieved. The procedure was to begin with full dose Excis (40 minutes) followed by full dose azamethiphos (20 minutes). Total treatment time was 1 hour, as recommended for both products alone (Chris Wallace, Marine Harvest Scotland pers com).

A modified method was later developed in Norway, with the recommended doses of pyrethroid and azamethiphos administered to the fish at the same time, with a total treatment time of 60 minutes. Recommended doses were 2 ppb deltamethrin and 100 ppb azamethiphos.

The Ecolice method has also been used by some fish farming companies in Norway since 2009. This method is based on pre-treatment with a low dose of azamethiphos for a relatively short period (10-15 min) followed by a normal dose of pyrethroid. The recommended concentrations for this method are a pre-treatment dose with azamethiphos at only 5 – 10 % of the full dose (ie. 5-10 ppb) followed by a pyrethroid at 50 – 100 % of full dose (1-2 ppb with deltamethrin, 7.5-15 ppb with cypermethrin). Ecolice claim a synergetic effect[1] of the combined products. According to Ecolice, it is important that the azamethiphos dose is kept low to both avoid mortality and to avoid resistance development towards azamethiphos (Baard Johannesen, Ecolice, pers com).

A variety of these methods have been used, both the Ecolice- and the combination with recommended concentrations of pyrethroid and azamethiphos. A survey was performed in 2012 asking fish health services along the coast about the resistance situation. At that time,

[1] Synergetic effect = an effect arising between two or more agents, entities, factors, or substances that produces an effect greater than the sum of their individual effects.

good effect was being achieved with pyrethroids, while resistance to azamethiphos seemed to have increased. Of greater concern was that in some specific areas resistance to both substances appeared to be developing where combinations of azamethiphos and pyrethroids were being used. Details regarding the specific concentrations of pyrethroids, azamethiphos and exposure times used for such treatments were not known.

Resistance development to azamethiphos and pyrethroids, as well as the focus on implementing product rotation in general, has led to an increase in the use of hydrogen peroxide (H_2O_2) (Paramove). In recent times, and in some specific areas, a reduction in sensitivity to hydrogen peroxide has also been observed and seems to be developing. There is a possibility that some of the same resistance mechanisms for pyrethroids and azamethiphos (enhanced multi-function oxidases (MFO) or esterase activity) could function as a resistance mechanism for hydrogen peroxide. Treatment with hydrogen peroxide was therefore included in the present study to investigate if some of the previous treatment regimes had changed the sensitivity to hydrogen peroxide.

Objective

The objective of the initial study was to observe and investigate resistance development to azamethiphos and pyrethroids by exposing sea lice over 4 generations to sub-lethal doses of either; azamethiphos (single treatment), pyrethroid (single treatment), Ecolice method (low dose azamethiphos and 100 % pyrethroid) or combined treatment method (100 % pyrethroid + 100% azamethiphos mixed). Summary from the initial study: The frequency of the genetic marker to azamethiphos resistance was investigated by sampling surviving sea lice. The genetic marker for pyrethroid resistance was however unavailable for the study because it required further development. Two separate attempts of exposure to select for 4. generations were made on two population of lice - population 1 from Southern Norway (first attempt) and population 2 from Northern Norway (second attempt). Both studies failed to produce 2nd generation sea lice.

The objective for a 3rd follow-up study was therefore redefined as follows; observe and investigate resistance development of a laboratory designed sea lice population with a defined frequency of resistance markers to azamethiphos and pyrethroid (deltamethrin) resistance, after exposure to either; azamethiphos (single treatment, recommended dose), pyrethroid (single treatment, recommended dose), Ecolice method (pre-treatment with low dose azamethiphos followed by pyrethroid exposure using the recommended dose) and combined method (mixture of azamethiphos and pyrethroid, both used simultaneously at the recommended dose). The frequency of genetic markers to azamethiphos and pyrethroid resistance were determined on both inactivated/ dead lice during treatment and on surviving lice. The study was terminated by hydrogen peroxide exposure to investigate if any of the treatment regimes had changed sensitivity to hydrogen peroxide compared to an untreated control group.

Evaluation of the possible synergetic effects of the Ecolice method was not an aim in the present study. The specific aim was to investigate if there was a difference in the drive to resistance between the investigated treatment regimes under laboratory conditions. The results

are intended to be used for making recommendations on the optimal use of azamethiphos and pyrethroid treatments.

Materials and methods.

Study compounds

Table 1. Description of investigational veterinary products.

Vet. Product	Active substance	Conc.	Treatment method	Recomm. dose
AlphaMax	Pyrethroid, deltamethrin	10 mg/ml	Bath, single treatment.	2 ppb (delt.)
Salmosan	Organophosphate; azamethiphos	0.5mg/mg	Bath, single treatment.	100 ppb(aza)
Paramove	Hydrogen Peroxide		Bath, single treatment.	1300 mg/L

Laboratory designed sea lice population

The population was developed by mixing copepodids from 3 different populations differing in resistance status: one sensitive population; one population resistant to both pyrethroids and organophosphates; one multi-resistant (including H_2O_2) population. These were mixed to give a proportional frequency of approx.: 25:50:25. The number of copepodids from each population was estimated to be:

- 728 sensitive copepodids
- 1604 double resistant copepodids
- 838 multi-resistant copepodids

The copepodids were produced and delivered by Lars Atle Hamre at the Sea Lice Research Centre in Bergen, Norway.

Infestation of fish

Infestation of fish was performed at VESO Vikan 06.06.14, according to VESO Vikan SOP S-1056-08. Atlantic salmon postsmolts (255) were infected with a total of 3170 copepodids at the proportions indicated above. This gave approximately an average of 5 lice per fish, with an infestation success of 40%. Sea lice were not counted on fish in this study, but summarizing all registered lice during the study the actual average must have been higher than 5.2 per fish.

Selective treatments

Selective treatments were performed on 25.06.14 according to Table 2 when the sea lice had reached the pre-adult mobile stage. Recommended treatment concentrations were used for all treatment regimes.

Table 2. Selective treatment regimes

Treatment	Exposure method	Time of exposure	Recomm. dose
AlphaMax	Bath	30 min	2 µg/L deltamethrin
Salmosan	Bath	60 min	100 µg/L azamethiphos
Ecolice method	Bath (Salmosan)	15 min+30 min	10 µg/L azamethiphos
	Bath (AlphaMax)	30 min	2 µg/L deltamethrin
Combined method	Bath (Salmosan)	60 min	100 µg/L azamethiphos
	Bath AlphaMax	60 min	2 µg/L deltamethrin
H_2O_2	Bath	20 min	1300 mg/L

The fish were divided into groups of 50 in each of 5 tanks. Each tank was exposed to one of the treatment regimes (Table 2) in 200 litres of seawater.

H_2O_2 treatment was performed one week (7 days) after the selective treatments, 02.07.14. The same dose (1300 mg/L for 20 minutes) was applied to all groups /tanks including the control.

Sampling

Mortality of sea lice was registered at 10, 20, 30, 60, 240, 1440 minutes (24h) post treatment and then each day from the start of treatment, until H_2O_2 treatment. The sampling was done by tapping out 33 litres from the bottom of the tank through a filter. The water was added back to the tank so that the exposure volume remained at 200 litres. The sea lice that had detached from the fish and were lying on the bottom were collected at each timepoint. After exposure, normal flow of water was established but the outlet water left the tank through the filter. The sampling at time 240 and 1440 min post-treatment, and then each day before H_2O_2 treatment, were performed by collecting sea lice from the filter. Total number of lice was registered after H_2O_2 treatment. A maximum of ten randomly selected lice were analysed at timepoints 10, 20, 30, 60, 240, 1440 min. post-treatment from each treatment regime as well as prior to and after H_2O_2 treatment. All lice were analysed if < 10lice were dead/moribund. Lice sampled in the period 48 – 144 hours (2 – 6 days) were registered and sampled but not analysed.

The sampling after H_2O_2 treatment was performed by collecting lice from the filter 30 minutes after start of treatment to collect all sea lice that fell off the fish during the treatment period of 20 minutes + 10 minutes of water exchange. The study was terminated within 1 hour after H_2O_2 treatment. The fish were killed with an overdose of benzocaine and the number of lice left on the fish was registered as surviving lice. Lice that fell off the fish during anaesthesia were also registered as surviving lice.

Total number of lice was registered after termination of the study by adding the number of dead and moribund lice at each sampling point, dead and moribund lice after H_2O_2 treatment and lice surviving lice after H_2O_2 treatment. The survival of each genotype was estimated by the equation: Total no of dead x % dead per genotype analysed / 100.

Identification of genetic markers.
The analysis of genetic markers was performed by PatoGen Analyse AS, Ålesund Norway.

Organophosphate (OP) sensitivity
Each louse was separated in three categories for OP sensitivity on the basis of the type allele; SS – homozygote sensitive, RS – heterozygote (one resistant- and one sensitive allele) and RR homozygote resistant.

Deltamethrin sensitivity
Each louse was separated in two categories for deltamethrin sensitivity, as either R – resistant or S – sensitive.

Both resistance markers were only determined for the combined and Ecolice regimes. Only OP resistance markers were identified for the azamethiphos regime and deltamethrin resistance markers for the deltamethrin regime.

Statistics
Treatment efficacy was estimated by the equation: Tot. no of dead / Tot. no x 100.

Analysis of the genetic resistance markers for azamethiphos and pyrethroid sensitivity were performed on individual lice.

Mortality of genotypes at each time-point is estimated by the equation: No of dead x % dead pr genotype / Tot. no of lice.

The effect of treatment regimes (mortality or moribund lice after treatment) was investigated with contingency analysis (chi-square test) with JMP 7.0 (SAS Institute).

The survival of the genotypes from treatment to 1440 minutes post-treatment was estimated using survival analysis with JMP 7.0 (SAS Institute).

The effect of H_2O_2 on the treatment regimes is compared between treatment groups and the control group, and between treatment groups with contingency analysis (chi-square test) with JMP 7.0 (SAS Institute).

The frequency of genotypes before and after H_2O_2 treatment was analysed by contingency analysis with JMP 7.0 (SAS Institute).

Results

Laboratory population

The frequency of genotypes in the test population.
The frequency of genotypes was investigated by analysing sea lice in the control group before termination of the study, from both moribund and surviving lice after H_2O_2 treatment.

Table 3. Frequency of genetic markers for azamethiphos- and pyrethroid resistance in the test population of sea lice.

Sensitivity	Frequency of genetic markers for azamethiphos	Frequency of markers for pyrethroids
Sensitive	37.5 % (SS)	29.2 % (S)
Medium resistant (Heterozygote)	50 % (RS)	Not applicable
Resistant	12.5 % (RR)	70,8 % (R)

Effect of the different treatment regimes
Total number of surviving lice was registered after termination of the study by adding; the number of dead and moribund lice from each sampling point, dead and moribund lice after H_2O_2 treatment, and surviving lice after H_2O_2 treatment. Total number of lice was registered at termination, 1 hour after treatment.

The effect of the different treatment regimes are shown in Table 4 and Figure1.

Table 4. Effect of the treatment regimes; AlphaMax (deltamethrin – pyrethroid), Salmosan (azamethiphos – organophosphate), Combination and Ecolice method.

Regime	Tot. no of dead 24 hours after treatment	% Efficacy 24 hours after treatment	Tot. no of dead from 48 – 144 hours (2 – 6 days) after treatment	Tot. no of surviving	Tot. no	% efficacy*
Deltamethrin	67	29.3	7	155	229	32.3
Azamethiphos	226	80.4	0	55	281	80.4
Combined	240	75.2	3	76	319	76.2
Ecolice	184	67.6	2	86	272	68.4
Control	10	3.8	13	238	261	5.0

* % treatment efficacy is calculated by: Tot. no of dead / Tot. no x 100

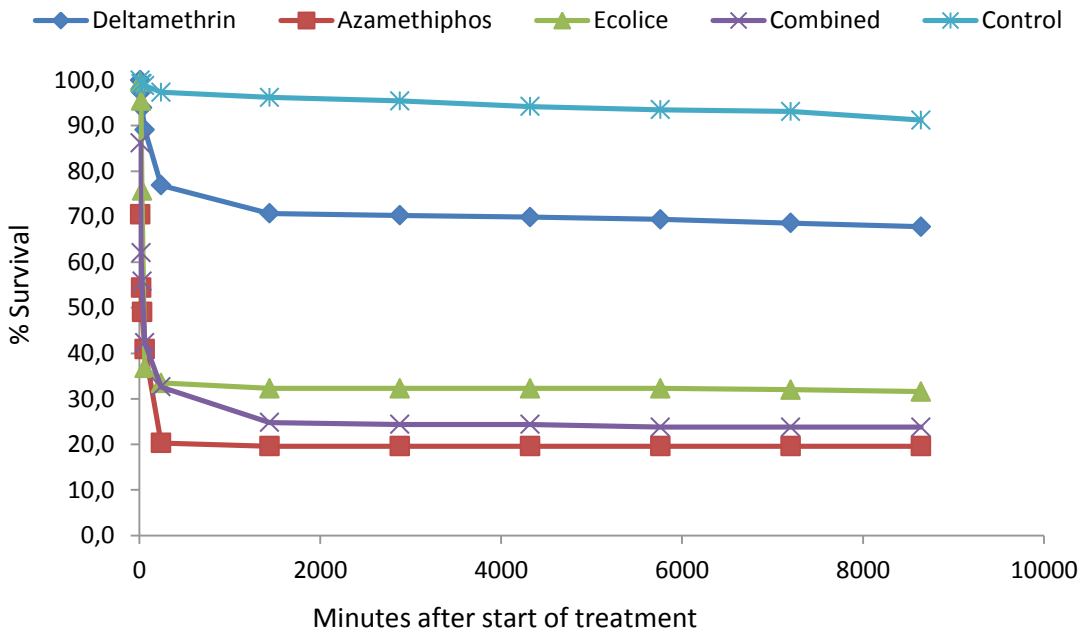

Figure 1. Percent Survival of sea lice exposed to the treatments azamethiphos (Salmosan), deltamethrin (AlphaMax), Combination and Ecolice methods, from start of treatment to 6 days post treatment (8640 minutes). Estimated by survival analysis (JMP 7.0) (Appendix c).

The difference in effect between the treatment regimes was investigated with contingency analysis. The difference in effect between control group and the treatment regimes was significant in all cases ($p < 0.0001$). The difference between azamethiphos and Combination was not significant, but the Combination method was significantly more efficacious than the Ecolice method ($p = 0.0343$) (Table 5, Appendix d and e).

Table 5. Level of significance in differences of effect between treatment regimes (contingency analysis, chi square test, JMP 7.0, SAS Institute)

Regime	Deltamethrin	Azamethiphos	Combined
Azamethiphos	S, p<0.0001	-	-
Combined	S, p<0.0001	N, p=0.2084	-
Ecolice	S, p<0.0001	S, p=0.0012	S, p=0.0343

S = Significant, N = Not Significant

Treatment with azamethiphos showed highest efficacy (80.4 %) 24 hour (1440 minutes) after start of treatment, followed by Combined method (75.2 %), Ecolice method (67.6 %) and deltamethrin treatment had an efficacy of (29.3 %). The mortality in the untreated control group was 3.8 % in the same period (24 h).

Frequency of genetic markers to pyrethroid resistance during and after treatment

Survival analysis as frequency of genotypes for pyrethroid sensitivity of dead and moribund lice, sampled at 10, 20, 30, 60, 240 and 1440 minutes post-treatment are shown in Figure 3, and the total number and number of each of the genotypes of surviving lice is shown in Figure 4.

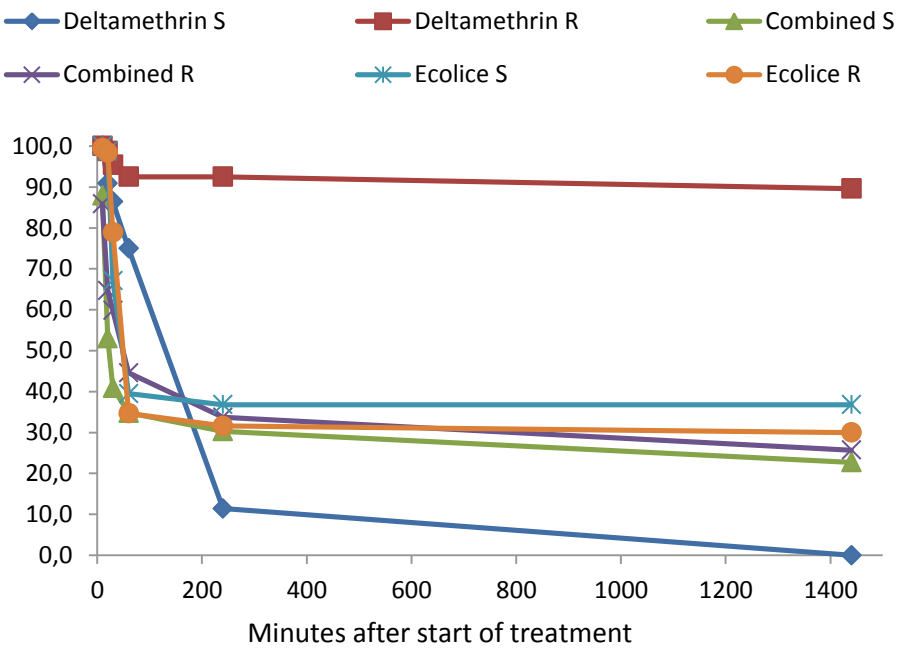

Figure 3. Percent cumulative survival of the genotypes S (sensitive) and R (resistant) to deltamethrin, from start of treatment to 1440 minutes (24 h). Treatment regimes; Deltamethrin, Combined and Ecolice. (Example: Deltamethrin S shows the survival of S genotypes after treatment with deltamethrin). Estimated by survival analysis (JMP 7.0)

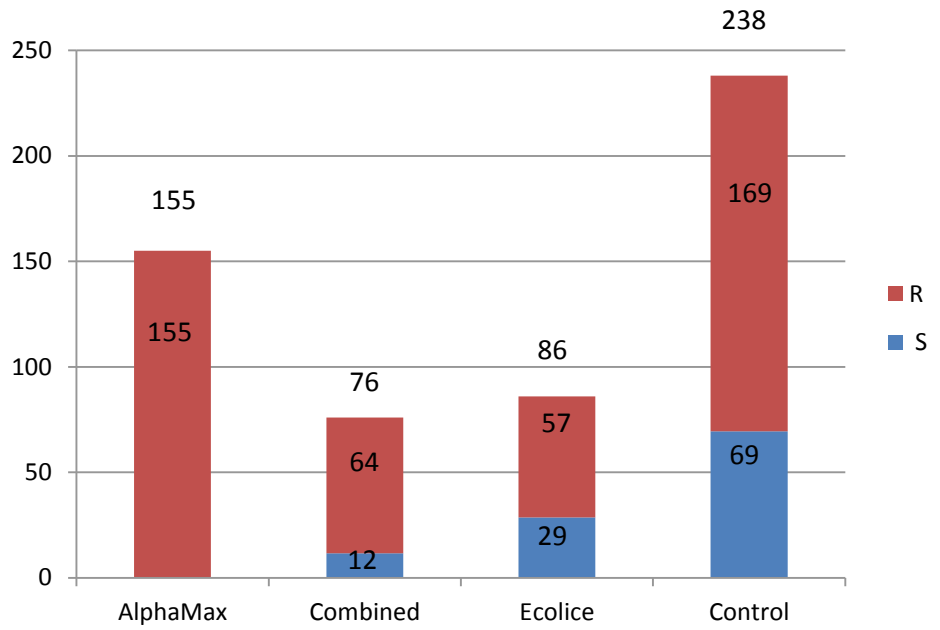

Figure 4. Total number of surviving lice and number of lice of each genotype (S – sensitive and R – resistant) after treatment with deltamethrin (AlphaMax), Combination or Ecolice regimes against the control. The number of genotypes is estimated by: number of lice survived x frequency of genotypes in analysed survived lice / 100. The number of samples and frequencies of genotypes in survived lice is described in appendix 1a.

All samples were not analysed and the survival of genotypes through treatments and number of genotypes in surviving lice are estimated. The results demonstrated that single treatment with deltamethrin is highly effective to sensitive lice. No sensitive lice were estimated to survive treatment with deltamethrin, while survival of deltamethrin resistant lice were estimated to be high (figure 3). This was confirmed because only deltamethrin resistant lice were found in the analysed samples of surviving lice (figure 4). Both Combined and Ecolice method showed higher total efficacy than deltamethrin, and sensitive lice survived treatment.

Frequency of genetic markers to azamethiphos sensitivity during and after treatment

Percent survival as frequency of genotypes for organophosphate sensitivity of dead and moribund lice, sampled at 10, 20, 30, 60, 240 and 1440 minutes post-treatment are shown in Figure 5, and the total number and numbers of each genotype of surviving lice is shown in Figure 6.

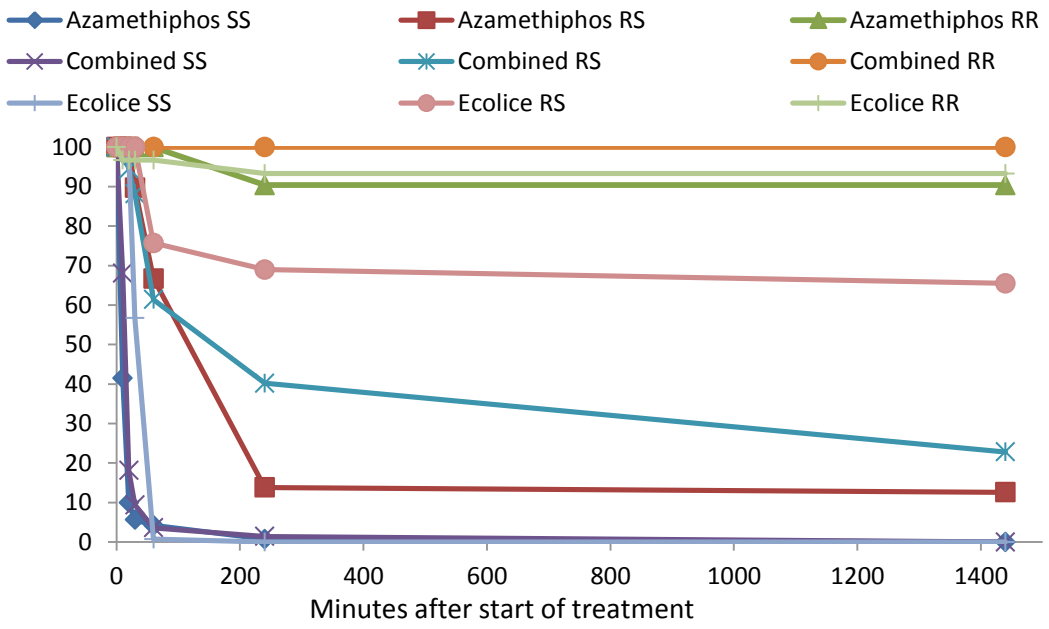

Figure 5. Percent cumulative survival of the genotypes SS (Homozygote sensitive), RS (Heterozygote) and RR (Homozygote resistant) to azamethiphos after treatment with azamethiphos (Salmosan), Combined or Ecolice regimes (Example: Azamethiphos RS shows the survival of RS genotypes after treatment with azamethiphos). Estimated with survival analysis (JMP 7.0).

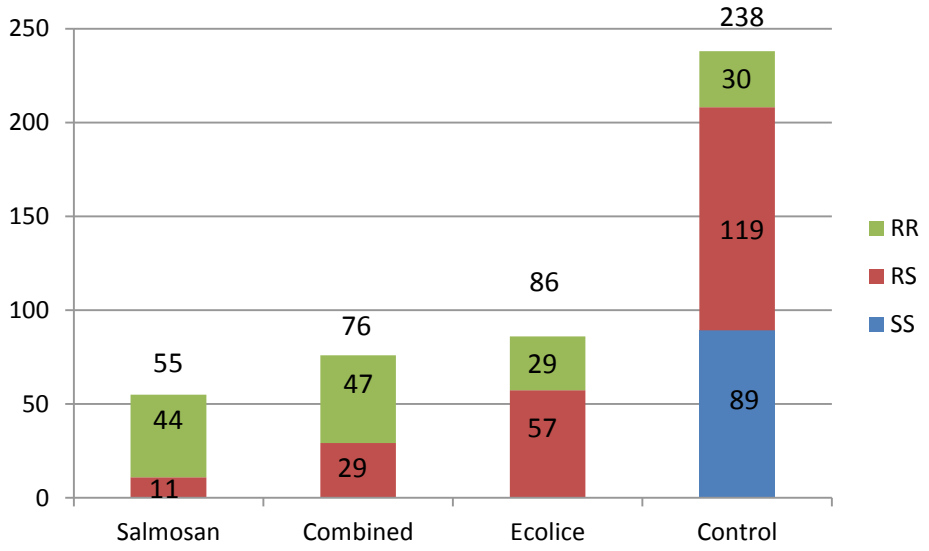

Figure 6. Total number of surviving lice and number of lice of each genotype (SS – homozygote sensitive, RS – heterozygote and RR – homozygote resistant) after treatment with azamethiphos (Salmosan), Combination or Ecolice regimes against the control. The number of genotypes was estimated by: number of lice survived x frequency of genotypes in

analysed survived lice / 100. The number of samples and frequencies of genotypes in survived lice is described in appendix 1b.

All samples were not analysed and the survival of genotypes through treatments and number of genotypes in surviving lice are estimated. Treatments were estimated to be 100 % effective to homozygote sensitive lice (SS) (figure 5). This was confirmed by the analysed samples from the survived lice. No sensitive (SS) were found in lice that survived treatments, while 37.5 % were found in the control group (Estimated no of lice 87, figure 6, appendix 1b). Medium resistant heterozygote (RS) lice had low survival to single azamethiphos treatment (figure 5). This was confirmed by the analysis of surviving lice. Only 11 of 55 lice (20 %) were estimated to be heterozygote lice, while 119 (50 %) were heterozygotes (RS) in the control group (figure 6, appendix 1b). The estimated effect of the treatments to homozygote resistant lice was low (figure 6). The lowest number of homozygote resistant (RR) lice, and highest number of heterozygote (RS) lice were found in lice that survived Ecolice treatment (figure 6). The results also show some uncertainty in the estimated frequency of the genotypes. The number of RR genotypes was estimated to be 30 in the control group with totally 261 lice (12.5 %), while the number was estimated to be 44 in the group treated with azamethiphos. This group had totally 281 lice. Thus, the frequency of RR genotypes was estimated to be 15.7 % (Estimated no of genotype/ total no of lice x 100).

The sensitivity to hydrogen peroxide (H_2O_2)

The effect of H_2O_2 treatment on the surviving lice from deltamethrin, azamethiphos, Combined and Ecolice treatments were compared to the effect on the untreated control group with contingency analysis (chi-square test) (JMP 7.0). Significant differences are listed in table 6.

Table 6. Difference in effect of H_2O_2 surviving lice from the deltamethrin, azamethiphos, Combined or Ecolice regimes compared to the untreated control group.

Regime	Tot. no before H_2O_2 treatment	No detached during H_2O_2 treatment	No surviving H_2O_2 treatment	% efficacy*	Different from control
Deltamethrin	155	73	82	47	No (p=0.14)
Azamethiphos	55	19	36	35	Yes (p=0.007)
Combined	76	30	46	39	Yes (p=0.02)
Ecolice	86	31	55	36	Yes (p=0.003)
Control	238	130	108	55	-

* % treatment efficacy is calculated by: No of lice detached during H_2O_2 treatment / Tot. no of lice before H_2O_2 treatment x 100

Exposure to 1300 mg/L H_2O_2 for 20 minutes had highest efficacy on the control group, followed by the effect on sea lice that had survived deltamethrin treatment, then Combined and Ecolice. The lowest effect was on sea lice that had survived azamethiphos treatment. However, the differences in effect of H_2O_2 between the treatment groups were not significant ($p > 0.05$).

Difference in genotypes between lice killed by H_2O_2 and surviving lice

The effect of H_2O_2 on the different genotypes for deltametrin sensitivity (S and R) was investigated. Totally 30 samples from dead/ moribund lice of all groups (deltamethrin: 7, Combined: 5, Ecolice: 5 and control: 13) sampled 30 minutes after start of H_2O_2 treatment, and totally 37 samples from survived lice of all groups (deltamethrin: 8, Combined: 8, Ecolice: 10 and control: 11). The results were pooled in dead/ moribund – and surviving lice and are shown in table 7. The frequency of genotypes in each category was investigated with contingency analysis (chi-square test, JMP 7.0). The results are shown in figure 8.

Table 7. Effect of H_2O_2 on the different genotypes. This is based on samples taken from lice killed by H_2O_2 and surviving lice. The frequency of genotypes at start is based on all samples pooled, i.e. both killed and survived lice.

Status	Genotype	Deltamethrin genotypes	% Effect on deltamethin genotypes	Azamethiphos Genotypes	% Effect on azamethiphos genotypes
At start	R / RR	53	-	28	-
	RS	-	-	30	-
	S / SS	14	-	9	-
	Total	67	-	67	-
Killed by H_2O_2	R / RR	23	**43.4** [1]	6	**21.4** [1]
	RS	-	**-**	16	**53.3** [1]
	S / SS	7	**50.0** [1]	6	**66.7** [1]
	Total	30	**44.8** [2]	28	**41.8** [2]
Surviving	R / RR	30	-	22	-
	RS	-	-	14	-
	S / SS	7	-	3	-
	Total	37	-	39	-

[1] Number of genotype killed / Tot no of genotype x 100
[2] Total killed / Total x 100

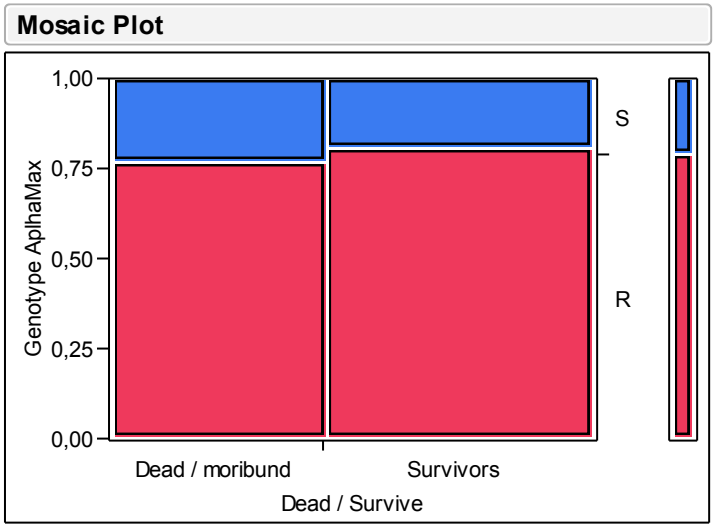

Figure 8. Contingency analysis of frequency of sensitive (S) and resistant (R) genotypes to deltamethrin of lice killed / moribund by H_2O_2 and surviving lice. All groups are pooled in two categories; dead/ moribund during treatment and survived treatment. The width of each bar represents the number of samples (dead/ moribund: 30, Survivors: 37). The bar to the right shows total average frequency of genotypes. Differences are not significant (appendix f).

The effect of H_2O_2 on the different genotypes for azamethiphos sensitivity (SS, RS and RR) was investigated. Totally 28 samples from dead/ moribund lice of all groups (azamethiphos: 5, Combined: 5, Ecolice: 5 and control: 13) sampled 30 minutes after start of H_2O_2 treatment, and totally 39 samples from survived lice of all groups (azamethiphos: 10, Combined: 8, Ecolice: 10 and control: 11). The results were pooled in dead/ moribund – and surviving lice and are shown in table 6. The frequency of genotypes in each category was investigated with contingency analysis (chi-square test, JMP 7.0). The results are shown in figure 9.

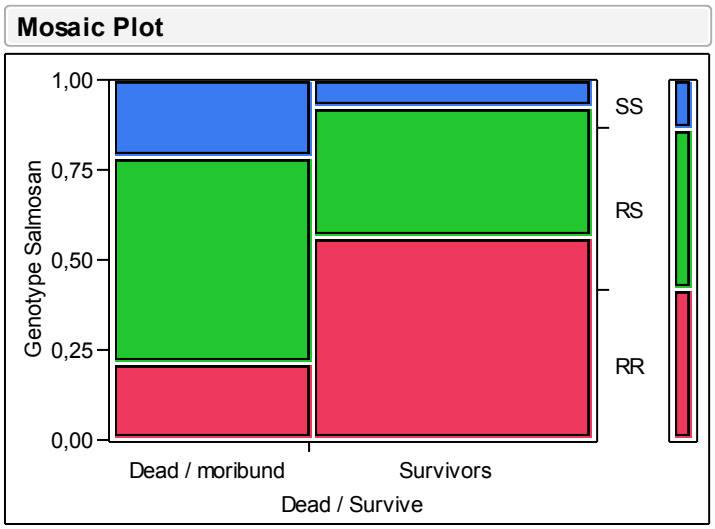

Figure 9. Contingency analysis of frequency of sensitive (SS), medium resistant (RS) and resistant (RR) genotypes to deltamethrin of lice killed/ moribund by H_2O_2 and surviving lice. All groups are pooled in two categories; dead/ moribund during treatment and survived treatment. The width of each bar represents the number of samples (Dead/ moribund: 28, Survivors: 39).The bar to the right shows total average frequency of genotypes. Differences are significant ($p = 0.01$).

There was no difference in H_2O_2 efficacy on the two different genotypes of deltamethrin sensitivity (Figure 8). The frequency of the resistant genotype to azamethiphos (RR) was higher in lice that survived H_2O_2 treatment (Figure 9). The frequency of heterozygote to azamethiphos resistance (RS) and sensitive (SS) were higher in sea lice that was found dead or moribund after H_2O_2 treatment. The difference is also significant ($p<0.01$, appendix e).

Discussion

This study was performed with recommended concentrations of treatment products for all regimes. A laboratory designed sea lice population was used to ensure survival of sea lice. The initial goal of reaching a frequency of genotypes (25 % sensitive, 50 % cross resistant and 25 % multi-resistant) was more or less obtained, but the mixed population ended up with a relatively low number of azamethiphos resistant (RR) lice at 12.5 % by the genetic method used in the study (Table 3).

The selective treatments were effective in the following order: Azamethiphos ≥ Combined > Ecolice > deltamethrin. The total mortality demonstrated highest efficacy with azamethiphos and Combined regimes. High efficacy will result in longer intervals between treatments and fewer treatments and therefore less selective pressure for resistance.

Not all lice were analysed in this study. A maximum of ten randomly selected lice were analysed at time-points 10, 20, 30, 60, 240, 1440 minutes post-treatment and prior to and after H_2O_2 treatment. All were analysed if < 10 lice were dead/ moribund. Whether the estimated frequency represents the actual or correct frequency of genotypes will depend on the size of the analysed fraction, meaning a degree of uncertainty must be applied. The results regarding the frequency of genotypes are therefore indications and cannot be regarded as absolute. However, the estimated survival of each genotype following treatment was confirmed by the analysis of genotypes in the lice that survived treatment. The results corresponded well with the frequency of genotypes that survived.

Single azamethiphos treatment showed 80 % efficacy. No sensitive (homozygotes – SS) and few medium sensitive (heterozygotes – RS) were found in the analysed lice that survived treatment. This indicates that sensitive lice (homozygotes – SS) and a high portion of medium sensitive (heterozygotes – RS) lice were killed by azamethiphos alone. The high effect corresponds well with the low frequency of homozygote resistant (RR) of 12.5% estimated from the control population. The highest effect was achieved with single azamethiphos treatment. The effect was somewhat higher, but the difference was not significant compared

to the Combined method (table 5). The same concentration of azamethiphos was used for both regimes. This could indicate that the same genotypes survived both treatments, and that this genotype is multi-resistant to both azamethiphos and deltamethrin. The relatively poor efficacy of deltamethrin (32 %) corresponds with the estimated frequency of sensitive genotypes (29 %). No sensitive (S) genotypes were found in the analysed surviving lice, indicating that single deltamethrin treatment kills the sensitive genotype effectively. The Combined and Ecolice regimes both had higher effect on the test population compared to single deltamethrin treatment and did not kill all sensitive lice. This demonstrates that the effect treatment will depend on the resistant status of the sea lice population in question.

Pyrethroid resistant sea lice are defined as R and sensitive are defined as S. Several factors are probably involved in resistance, and not only one single mutation / allele or genetic marker (Frank Nilsen, UiB, pers.com.). The resistance measured for organophosphate resistance is caused by a resistant type of acetylcholine esterase (AChE). The identified marker for resistance (R) corresponds 100 % with the allele for resistant AChE. Thus, sea lice can be either homozygote sensitive (SS), heterozygote (RS) with intermediate sensitivity or homozygote resistant (RR). This means further that the genotypes of the offspring will follow Mendelian genetics, and will be expressed equally if there are no dominant or recessive alleles. Example: If two heterozygote sea lice mates. The offspring will have the following frequency of genotypes: 25 % RR, 50 % RS and 25 % SS. If one homozygote resistant mates with one heterozygote, the frequency of offspring genotypes will be: 50 % RR and 50 % RS. The important implication of that is that as long as some heterozygotes survive, the sensitive allele (S) will not be eradicated from the population. Theoretically, if the sensitive allele or gene is eradicated, it will not be possible to reverse the process of resistance. The sensitive gene will not be extinct as long as heterozygotes are left in the population. This is probably more complex regarding resistance to pyrethroids, as there seems to be several factors involved (Frank Nilsen, UiB, pers.com.).

The eradication / survival of genotypes will also depend on frequency, migration of lice from other sites with different treatment regime, wild fish and / or if the resistance mechanism is associated with metabolic costs that reduce fitness. Metabolic costs or reduced fitness have been found in resistant arthropods (Lee et al. 2014), but was not found in sea lice for emamectin benzoate resistance (Espedal et al. 2013) or pyrethroid resistance (P. G. Espedal, pers. com.). Fallang et al. 2005 found sea lice with resistant AChE in sea lice from areas where organophsphates had not been used for 5 years, indicating that low fitness cost may be associated with this resistance mechanism. Metabolic costs for resistance have not yet been found in sea lice (Frank Nilsen, UiB pers.com). Without the selective pressure of treatments, the frequency of the resistant allele will decrease in the population with time, but it will probably not be totally gone. Resistance will develop again if azamethiphos is reintroduced, because the resistant allele is already present in the sea lice population.

The frequency of the resistant genotype to azamethiphos (RR) was higher in lice that survived H_2O_2 treatment. The frequency of heterozygote to azamethiphos resistance (RS) and sensitive (SS) were higher in sea lice that was found dead or moribund after H_2O_2 treatment. (Figure 9). The multi-resistant sea lice used initially as 25 % of the copepodids, was also resistant to

H_2O_2. The reason for high frequency of the RR genotype in H_2O_2 survivors could be that surviving lice from all regimes have a higher frequency of originally multi-resistant sea lice, due to higher survival of those through all treatment regimes.

Conclusions

The study demonstrated that the treatment regimes azamethiphos, deltamethrin, Combination and Ecolice method are most effective against sensitive sea lice and least effective against resistant sea lice under laboratory conditions. The frequencies of genotypes for azamethiphos and deltamethrin sensitivity and resistance change after treatment. This change resulted in higher relative frequency of the resistant genotypes to deltamethrin and azamethiphos, demonstrating that treatments are an active driving force towards a higher degree of resistance.

The effect of any treatment regime will depend on the resistance status of the sea lice population, i.e. frequency of resistant or sensitive individuals prior to treatment.

High treatment efficacy will most likely result in longer intervals between treatments and fewer treatments and therefore less selective pressure for resistance. Rotation between different chemical classes of treatments will stop the selection towards high frequency of one specific resistance genotype. At the same time, treatments that leave only a small proportion of, or eradicate the sensitive allele in the population can be a driving force towards a higher resistance level. These forces act in opposite directions, and the balance point has not been determined in the current study.

High treatment efficacy with single agents and rotation between different classes of chemicals should be used to avoid or delay the development of resistance. It is possible that the strategy should be different when resistance to several agents is established as it is now in some areas. The best will be to avoid chemical treatments or use new or alternative chemicals or methods until sufficient sensitivity has returned.

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Appendixes

Appendix a) Number of dead and genotypes for sensitivity to deltamethrin (AlphaMax): S – sensitive and R – resistant, and cumulative mortality (%) of each genotype and frequency of genotypes in survived lice.

Time		Control	%	Deltameth	%	Combined	%	Ecolice	%
10	Total	0		0		44		1	
	S:	-		-	-	2	**12,1**	0	**0**
	R:	-		-	-	8	**14,1**	1	**0,5**
	n=	-		-		10		1	
20	Total	2		6		77		3	
	S:	-		4	**9,1**	3	**47,0**	1	**1,3**
	R:	-		2	**1,2**	7	**35,3**	2	**1,6**
	n=	-		6		10		3	
30	Total	0		8		20		62	
	S:	-		2	**13,6**	4	**59,1**	4	**32,9**
	R:	-		6	**4,6**	6	**40,2**	6	**21,0**
	n=	-		8		10		10	
60	Total	1		11		43		106	
	S:	-		5	**25,0**	1	**65,1**	2	**60,5**
	R:	-		5	**7,5**	9	**55,4**	8	**65,3**
	n=	-		10		10		10	
240	Total	4		28		31		9	
	S:	-		10	**88,6**	1	**69,7**	2	**63,2**
	R:	-		0	**7,5**	9	**66,3**	5	**68,4**
	n=	-		10		10		7	
1440	Total	3		14		25		3	
	S:	-		4	**100,0**	2	**77,3**	0	**63,2**
	R:	-		6	**10,4**	8	**74,3**	3	**70,0**
	n=	-		10		10		3	
Survived	Total	238		155		76		86	
	S:	7	**29.2**	0	**0**	2	**15,4**	5	**33,3**
	R:	17	**70.8**	15	**100**	11	**84,6**	10	**66,7**
	n=	24		15		13		15	

Appendix b). Number of dead and genotypes to azametiphos (Salmosan) sensitivity: SS – sensitive, RS – heterozygote and RR – resistant and cumulative mortality (%) of each genotype and frequency of genotypes in survived lice.

Time		Control	%	Azameth.	%	Combined	%	Ecolice	%
10	Total	0		83		44		1	
	SS:	-		10	**58,4**	10	**31,9**	0	**0**
	RS:	-		0	**0**	0	**0**	0	**0**
	RR:	-		0	**0**	0	**0**	1	**3,3**
	n=			10		10		1	
20	Total	2		45		77		3	
	SS:	-		9	**90,1**	9	**81,9**	3	**2,0**
	RS:	-		0	**0**	1	**5,5**	0	**0**
	RR:	-		0	**0**	0	**0**	0	**3,3**
	n=			9		10		3	
30	Total	0		15		20		62	
	SS:	-		4	**94,4**	6	**90,6**	10	**43,3**
	RS:	-		6	**10,3**	4	**11,8**	0	**0**
	RR:	-		0	**0**	0	**0**	0	**3,3**
	n=			10		10		10	
60	Total	1		23		43		106	
	SS:	-		1	**95,8**	2	**96,4**	8	**99,3**
	RS:	-		9	**33,3**	8	**38,6**	2	**24,1**
	RR:	-		0	**0**	0	**0**	0	**3,3**
	n=			10		10		10	
240	Total	4		58		31		9	
	SS:	-		1	**99,3**	1	**98,5**	1	**100**
	RS:	-		8	**86,2**	9	**59,8**	5	**31,0**
	RR:	-		1	**9,6**	0	**0**	1	**6,7**
	n=			10		10		7	
1440	Total	3		2		25		3	
	SS:	-		1	**100**	1	**100**	0	**100**
	RS:	-		1	**87,4**	9	**77,2**	3	**34,5**
	RR:	-		0	**9,6**	0	**0**	0	**6,7**
	n=			2		10		3	
Survived	Total	238		55		76		86	
	SS:	9	**37,5**	0	**0**	0	**0**	0	**0**
	RS:	12	**50**	3	**20**	5	**38,5**	10	**66,7**
	RR:	3	**12,5**	12	**80**	8	**61,5**	5	**33,3**
	n=	24		15		13		15	

Appendix c) JMP Survival analysis of the treatment regimes with azamethiphos (Salmosan), deltamethrin (AlphaMax), Combination and Ecolice methods. The p values indicate significant differences in the dataset (Log-Rank and Wilcoxon: $p < 0.0001$).

Summary

Group	Number failed	Number censored	Mean		Std Error
azamethiphos	226	55	354,306	Biased	33,1492
combination	243	76	1569,78	Biased	135,618
control	23	238	8208,2		107,452
deltamethrin	74	155	6164,8		249,153
ecolice	186	86	2844,3		243,219
Combined	752	610	4069,7		114,204

Quantiles

Group	Median Time	Lower95%	Upper95%	25% Failures	75% Failures
azamethiphos	30	20	60	10	240
combination	60	60	60	20	1440
control	.	.	.	.	.
deltamethrin	.	.	.	1440	.
ecolice	60	60	60	60	.
Combined	1440	240	1440	30	.

Tests Between Groups

Test	ChiSquare	DF	Prob>ChiSq
Log-Rank	461,9526	4	<,0001*
Wilcoxon	478,8565	4	<,0001*

Appendix d) Contingency analysis of differences in effect between azamethiphos and Combined regime

Tests

N	DF	-LogLike	RSquare (U)
600	1	0,79452113	0,0025

Test	ChiSquare	Prob>ChiSq
Likelihood Ratio	1,589	0,2075
Pearson	1,582	0,2084

Fisher's Exact Test	Prob	Alternative Hypothesis
Left	0,1231	Prob(Dead / survivor=Survived) is greater for Regime=Combined than Salmosan
Right	0,9128	Prob(Dead / survivor=Survived) is greater for Regime=Salmosan than Combined
2-Tail	0,2349	Prob(Dead / survivor=Survived) is different across Regime

Appendix e) Contingency analysis of differences in effect between Combined and Ecolice regime

Tests

N	DF	-LogLike	RSquare (U)
591	1	2,2359313	0,0064

Test	ChiSquare	Prob>ChiSq
Likelihood Ratio	4,472	0,0345*
Pearson	4,481	0,0343*

Fisher's Exact Test	Prob	Alternative Hypothesis
Left	0,9864	Prob(Dead / survivor=Survived) is greater for Regime=Combined than Ecolice
Right	0,0215*	Prob(Dead / survivor=Survived) is greater for Regime=Ecolice than Combined
2-Tail	0,0417*	Prob(Dead / survivor=Survived) is different across Regime

Appendix f) Contingency analysis of frequency of sensitive (S) and resistant (R) genotypes to deltamethrin (AlphaMax) of lice killed by H_2O_2 and surviving lice. All groups are pooled in two categories; dead during treatment and survived treatment. .

Tests

N	DF	-LogLike	RSquare (U)
67	1	0,09727775	0,0028

Test	ChiSquare	Prob>ChiSq
Likelihood Ratio	0,195	0,6592
Pearson	0,195	0,6585

Fisher's Exact Test	Prob	Alternative Hypothesis
Left	0,4422	Prob(Genotype AplhaMax=S) is greater for Dead / Survive=Dead / moribund than Survivors
Right	0,7720	Prob(Genotype AplhaMax=S) is greater for Dead / Survive=Survivors than Dead / moribund
2-Tail	0,7657	Prob(Genotype AplhaMax=S) is different across Dead / Survive

Appendix g) Contingency analysis of frequency of sensitive (SS), medium resistant (RS) and resistant (RR) genotypes to azamethiphos (Salmosan) of lice killed by H_2O_2 and surviving lice. All groups are pooled in two categories; dead during treatment and survived treatment. Differences are significant.

Tests

N	DF	-LogLike	RSquare (U)
67	2	4,5292127	0,0680

Test	ChiSquare	Prob>ChiSq
Likelihood Ratio	9,058	0,0108*
Pearson	8,705	0,0129*