SEA LICE RESISTANCE TO CHEMOTHERAPEUTANTS:

A HANDBOOK IN RESISTANCE MANAGEMENT

Prepared by the consortium involved in the EU-funded project SEARCH (QKK2-CT-00809): Sea lice resistance to chemotherapeutants: Diagnosis, mechanisms, dynamics and control

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THE SEARCH PROJECT

The SEARCH project is an EU-funded R&D project (QLK2-CT-2000-00809, 2001 – 2003) with the following aims:

Resistance in the parasite *Lepeophtheirus salmonis* is known to exist, and may become a serious threat to European aquaculture. This multi-disciplinary effort by scientists and the salmon industry will develop strategies to identify, monitor and control resistance.

It will provide:

i) protocols for bioassays determining the presence and scale of resistance to all agents in current use,
ii) protocols for biochemical and molecular diagnostics of resistance mechanisms,
iii) monitoring for sensitivity in sea-lice populations, documenting patterns, and the impact of control practices upon them,
iv) seasonal and temporal data on the genetic structure of *L. salmonis* sub-populations
v) management recommendations based upon correlation of toxicological and mechanistic resistance patterns, genetic, environmental and operational factors,
vi) a framework for disseminating the output to salmon farmers, manufacturers and regulatory authorities.

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GENERAL ASPECTS REGARDING RESISTANCE IN ARTHROPODS

Introduction

Resistance to xenobiotics develops through the same process as any other evolutionary adaptation. Undirected mutations generate unstructured diversity, which can then be shaped by natural selection. In eukaryotes, the timescales over which these forces operate are usually too large to observe in situ, but the development of resistance to chemical control agents provides a notable exception. In this case, the overall factors driving selection and adaptation are well understood. Their study and manipulation can reveal much about how diversity originates, and how it is possible to influence the evolutionary process to the benefit of crop and animal welfare.

Genes conferring resistance probably arise repeatedly by mutation, but in the absence of exposure to chemical toxins are expected to remain at very low frequencies in pest populations. With the onset of such exposure, individuals possessing such genes are selectively favoured and increase in frequency. During the early stages of selection, the number of resistant survivors may be too small to have any discernible impact on the quality of control. If resistance is unchallenged, however, these individuals eventually reach a level at which control difficulties are readily apparent. The speed at which resistance increases, and the degree of resistance that can be tolerated, depend on many inter-related factors including the nature of damage inflicted by a pest, the potency of resistance mechanisms, the frequency of chemical use, and the biology of the pest itself. For many pests (including salmon lice), key aspects of biology such as dispersal capacity and the temporal and spatial stability of populations are still poorly understood. This makes an assessment of resistance risk a complex and difficult task.

There are now well over 500 species of arthropod reported to have developed resistance to at least one class of chemical toxins. The majority of these cases relate to insects (Class Insecta), as pests of crops, livestock and human health and animals. In any one species, resistance may be limited to a few closely related compounds in a single chemical class, and/or be restricted to a small part of their geographical range. At the other extreme are pests with a cosmopolitan distribution that resist most or all of the insecticides available for their control. Examples of these include anopheline mosquitoes, e.g *Anopheles* and *Culex* spp., the diamondback moth, *Plutella xylostella* (L.), the Colorado beetle, *Leptinotarsa decemlineata* (Say), and the cotton whitefly, *Bemisia tabaci* (Gennadius). The most extensively used insecticide classes - organophosphates, carbamates and pyrethroids – have generally been the most seriously compromised by resistance but, in recent years, there has also been a worrying increase in resistance to other classes. These include the benzoylphenylureas, (e.g. diflubenzuron and teflubenzuron), which attack developmental pathways in arthropods, and the macrocyclic lactones (e.g. abamectin), which bind to receptors associated with the transmission of nerve impulses.

Mechanisms

The most important and powerful mechanisms of resistance involve either an increased ability to detoxify a chemical, or a structural alteration to its target site within the pest. Other mechanisms that have been demonstrated or hypothesised include reduced penetration of toxins through the cuticle, and behavioural changes that enable pests to avoid them in the first place. Behavioural resistance in salmon lice is very unlikely due the uniformity of chemical applications and the limited mobility of chalimus and adult stages. Based on experience with other pests, reduced penetration is best considered as a potential 'modifier' of increased detoxification or target-site resistance rather than a major mechanism in its own right.

Mechanisms of OP and pyrethroid resistance based on both increased detoxification and altered target sites are well documented for agricultural pests. These are also the most likely to occur in sea lice, and are summarised below.

Increased detoxification of insecticides
Three major detoxification systems have been implicated in causing resistance:

(i) Compounds of many chemical classes are vulnerable to enhanced oxidative breakdown by enzymes known as mixed function oxidases or MFOs. Evidence for this type of resistance may be indirect, based on the ability of known inhibitors of MFOs to overcome resistance when co-applied with toxins in laboratory assays, or direct, based on enzyme binding or metabolism studies.
(ii) Enhanced activity of glutathione-S-transferases (GSTs), which assist with cleaving active sites from toxic molecules, is potentially important in resistance to OPs, but for biochemical reasons is unlikely to affect tolerance to pyrethroids.
(iii) Many toxins, including most pyrethroids and all OPs, contain ester bonds that are vulnerable to cleavage by esterases, resulting in non-toxic metabolites. Of the three possible detoxification routes, enhanced esterase activity is by far the best characterised biochemically.

Alterations to target-sites
Since OPs and pyrethroids attack different sites in the insect nervous system, mechanisms of target site resistance to these classes are obviously distinct.

OPs exert their toxicity by inhibiting the enzyme acetylcholinesterase (AChE), which plays a key role in the transmission of nerve impulses across gaps ('synapses') between nerve cells. Forms of AChE showing reduced inhibition by OPs have been selected in several arthropod pests of agriculture, livestock and public health. Advances in methodology for characterising resistant AChE now enable this mechanism to be diagnosed rapidly and precisely in individual insects, and offer scope for implicating and monitoring this mechanism in sea lice as well.

Pyrethroids act by binding to and blocking ion channels in nerve cell membranes that are also vital for the transmission of nerve impulses. A mechanism based on the insensitivity of such channels to pyrethroids was first identified in houseflies and termed knockdown resistance or kdr. In recent years, analagous forms of kdr resistance have been confirmed in a diverse range

of insect pests. As with insensitive AChE (see above), there are now sophisticated techniques available for diagnosing this type of resistance more accurately than was possible in the past.

Other chemotherapeutants available for sea lice control include the avermectins (also nerve poisons, but acting differently to OPs and pyrethroids) and teflubenzuron (a benzoylphenylurea affecting the moulting of immature stages). To date, these have tended to have very specialised uses against other pests, and cases of resistance are therefore less common than for OPs and pyrethroids. The underlying mechanisms are also less well established but are most likely also based on some form of increased detoxification and/or target-site modification.

Cross-resistance

Resistance mechanisms seldom if ever affect just one toxin. For combating resistance, it is therefore important to anticipate how the phenomenon of cross-resistance might influence the effectiveness of management strategies based, for example, on the alternation of products to avoid continuous selection for the same mechanism.

Unfortunately, cross-resistance patterns are inherently difficult to predict in advance, since mechanisms based on both increased detoxification and altered target sites may differ substantially in their specificity. The most commonly encountered patterns of cross-resistance tend to be limited to compounds within the same chemical group (equivalent to the term ‘side-resistance’ as used by parasitologists). However, even these patterns can be very idiosyncratic. As an example, OP resistance based on increased detoxification or target-site alteration can be broad-ranging across this group or highly specific to a few chemicals with particular structural similarities. The effectiveness of azamethiphos in overcoming resistance selected previously by dichlorvos implies that the latter was relatively restricted in its breadth and, fortuitously, did not rule out the use of OPs entirely. Similarly complex patterns have been demonstrated within the pyrethroids.

It is also possible for single mechanisms (especially ones based on increased detoxification) to confer cross-resistance across chemical groups. For example, mechanisms based on enhanced MFO or esterase activity have the potential to affect both OPs and pyrethroids. MFO-based systems selected by OPs or pyrethroids could conceivably extend to other unrelated compounds including teflubenzuron and the avermectins, whose molecules are also vulnerable to oxidative attack. As far as cross-resistance is concerned, the only 100% certainty is that we can never be 100% sure, highlighting the importance of detecting and characterising resistance mechanisms at the earliest possible stage.

Factors promoting resistance

Based on experience with agricultural pests, some of the factors that conspire to promote the rapid selection of resistance, and the successive accumulation of resistance mechanisms, are as follows:

- a continuous availability of host plants, enabling pest populations to build up to large numbers and to persist at these levels for much of the year

- the relative genetic isolation of populations resulting from a lack of mixing of pests from treated and untreated areas
- lack of untreated 'refuges' in which selection for resistance is avoided and susceptible individuals are preserved
- low damage tolerance thresholds, promoting the frequent use of chemicals with little attention to pest scouting
- a limited diversity of compounds available for inclusion in control strategies, leading to over-reliance on single products or chemical classes.

To what extent do such considerations apply to the ecology and treatment of sea lice on salmon farms? Due to their host specificity, the proportion of *L. salmonis* individuals exposed to chemotherapeutants is clearly much higher, and selection therefore much stronger, than for the more 'polyphagous' louse, *Caligus elongatus*. As a consequence, concerns over resistance management need to be focussed on the former species. In some areas salmon are grown all year round, and successively from year to year. Even where fallowing occurs, some fallow sites are so close to production ones that the life-cycle of *L. salmonis* may never be effectively broken.

Approaches to combating resistance

The over-riding principle for resistance management can be summarised neatly as 'variety is the spice'. Rather than relying solely on any single product for pest control, farmers should be encouraged to exploit the maximum diversity of control measures available. Such diversity includes both operational diversity, taking full advantage of non-chemical tactics (biological control by natural enemies, physical exclusion of pests, host-free periods etc), and chemical diversity, exploiting a range of products least likely to encounter problems from cross-resistance.

As always, this principle is far easier to extol in theory than to implement in practice. Nonetheless, opportunities for reducing reliance on chemotherapeutants through the use of cleaner wrasse, mechanical delousing methods, year-class separation, net cleaning and fallowing undoutedly represent the front-line of defence against resistance, and deserve considerably more research to render them more accessible, effective and affordable.

Providing farmers with access to sufficient chemical diversity, and enabling them to exploit this effectively raises a different set of problems. There has been and still is a marked tendency in the salmon industry to rely on single products. The difficulty in registering new compounds, and in gaining consents from the authorities for their discharge, has forced this situation upon them. Nonetheless, when a choice has been available, it has often been overlooked. A far more desirable approach would be to base sea-lice control on at least two (preferably more) unrelated compounds applied in pre-planned alternations.

CURRENT KNOWLEDGE ABOUT RESISTANCE IN SEA LICE

Methods to identify resistant sealice populations

There are several methods available to diagnose resistance in arthropods. When the mechanism behind is known, the most cost-effective method is to study the expression of this mechanism, e.g. the activity of detoxifying enzymes, or the presence of mutations associated with resistance. However, these diagnostic tools can normally only be carried out in a laboratory environment. For field use, **bioassays** are the most effective diagnostic tools. Detailed protocols for several bioassays are presented in Part IV of this handbook.

Pyrethroids

Deltamethrin and cis-cypermethrin are important delousing agents in Norway (AlphaMax™ and Betamax™). In Scotland and Ireland, cypermethrin (Excis™) is the dominating product. The bioassays are performed on preadult II sealice, collected from infested fish, in a bath exposure. Full bioassays using 5 different concentrations and one control have been developed to determine the exact sensitivity in the tested population. Field bioassays, designed to be used by local fish health services to distinguish between "sensitive", "moderately insensitive" and "resistant" populations have also been developed.

Azamethiphos

Even though this organophosphorus compound (Salmosan™) has lost importance as a delousing agent, it is used to a minor extent in Scotland and Ireland. The bioassay is performed on preadult II sealice, collected from infested fish, in a bath exposure. A full bioassay using 5 different concentrations and a control has been developed.

Emamectin

Emamectin benzoate (Slice™), an in-feed compound, has during the last few years become the most important delousing agent in Canada, Ireland and Scotland, and it is also of great importance in Norway. The bioassay is performed on preadult II sealice, collected from infested fish, in a bath exposure. Full bioassays using 5 different concentrations and one control have been developed. However, as no clinical treatment failures have been verified, it has not been possible to validate the bioassay fully.

Teflubenzuron

This chitin synthesis inhibitor was to some extent used in Norway and Scotland during the late 1990s, but has now lost much of its importance. Bioassays with this compound are difficult, as the lethal effect of the compound is only seen when the parasite moults. Moulting is only possible when the parasite is attached to its host. The developed assay is based in administration of the test compound to sealice-infested fish, and recording of the number of parasites capable of developing into the next stage.

Resistance situation for sealice in Norway, Scotland, Ireland and Canada

Organophosphate resistance

Organophosphates were, until the mid 90's, the most frequently used control agents. In the late 80s and early 90s, dichlorvos (Aquaguard™, Nuvan™) was the dominating compound; this was later replaced by azamethiphos (Salmosan™). Until 1995 more than 80% of all delousing in Norway was done using organophosphates. There are several reports of treatment failure due to the development of resistance in Norway and Scotland, as well as anecdotal reports from Canada. In several areas in mid-Norway, the organophosphate dichlorvos totally lost its effect against sea lice in 1991 – 1992 and the same happened in mid- and southern Norway with azamethiphos (Salmosan™) in 1995 – 1996, rendering this compound virtually ineffective as an anti-sealice agent. Since then, this compound has been used to a minor extent only, and clinical cases with treatment failures have not been reported during the period 2001 - 2003.

Pyrethroid resistance

Treatment failures against sealice with the pyrethroids deltamethrin (AlphaMax™) high-cis-cypermethrin (Betamax™) and cypermethrin (Excis™) have been anecdotally reported from Norway, Scotland and Ireland. In the SEARCH project, a total of 18 Norwegian, 9 Irish and 4 Scottish sealice strains collected in the period 2001 - 2003 were investigated for sensitivity to pyrethroids using bioassays and probit modelling. The sensitivity was expressed as EC_{50} – the concentration (ppb) immobilizing 50 % sea lice in bioassays. Treatment failures with cypermethrin were reported in connection with ten of the samples, nine Norwegian and one Irish. Treatment failures with deltamethrin were reported in connection with six of the Norwegian samples. A total of 14 strains had significantly higher EC_{50} to cypermethrin compared to a control strain, while 7 strains had a significantly higher EC_{50} to deltamethrin, indicating reduced sensitivity. Of the samples associated with reported treatment failure to cypermethrin, 7 out of 10 showed significant reduced sensitivity, while only 2 out of 6 showed reduced sensitivity to deltamethrin. The resistance ratio (resistance ratio = recorded EC_{50} / control EC_{50}) to cypermethrin ranged from 0.6 to 7.0 among the investigated strains, while the resistance ratio for deltamethrin ranged from 0.7 to 11.4. The monitoring demonstrated that reduced sensitivity to pyrethroids occurred occasionally, but the frequency did not increase during the time-span for the project. The three-year monitoring period is, however, too short to make firm conclusions on this point.

Emamectin resistance

Emamectin benzoate (Slice™) has become the most popular delousing agent in Scotland, Ireland and Canada, and also plays a significant role in Norway. A bioassay method was developed for this agent. Some reports of clinical failures with emamectin benzoate were reported. However, no strains with reduced sensitivity towards this compound were identified.

Mechanisms

Through the SEARCH project, several basic mechanisms for resistance development known from other arthropods have been examined in sealice.

Molecular mechanisms in pyrethroid resistance
In many arthropods, specific mutations in the gene coding for the voltage gated sodium channel have been reported to result in an altered function of this channel, resulting in a decreased ability for pyrethroids to interfere with its function. As resistance towards pyrethroids was detected in several sea lice strains through the SEARCH project, molecular methods to detect these mutations were established (RT-PCR and direct PCR methods). Through these studies, none of the previously described mutations (kdr, super-kdr and others) could be detected in any of the sealice strains tested. However, a novel mutation in this gene was detected in several strains. This mutation was found in strains from sites with a reported reduced efficacy of pyrethroids only.

Enhanced detoxification capability in pyrethroid resistance
Through the SEARCH project, several sealice strains with a reduced sensitivity towards pyrethroids were detected. The mutation described previously was not found in all these strains. Another mechanism was suspected for these strains: an enhanced detoxification capability by the parasite. From other arthropods, increased activities of monooxydases and unspecific esterases have been identified as mechanisms in question. However, esterases seem to play an insignificant role in sealice, as the parasite has a very low background activity of these enzymes. On the other hand, unspecific monooxidases seem to play a significant role, indicated by a correlation between enzyme activity and reduced sensitivity. This observation was confirmed by studies using the specific oxidase inhibitor piperonylbutoxide.

Altered acetylcholinesterase in organophosphate resistance
Since the early 1990s, organophosphates have been reported to be ineffective against sealice in certain areas. One important mechanism described from other arthropods is the presence of an organophosphate-insensitive form of the target enzyme, acetylcholinesterase. In a large survey conducted in Norway and Canada using three different biochemical methods, the presence of an OP-insensitive form of acetylcholinesterase in a large number of individual sealice in all areas was demonstrated. Individuals with both a sensitive and an insensitive form of the enzyme were found. These results demonstrate that this is a latent resistance mechanism in sealice. It may well emerge as a problem within a short time-span if these substances again gain popularity as delousing agents.

Gene flow in sealice populations

The examination of gene flow between different sealice populations is very important, as heritable resistance mechanisms are assumed to spread together with other genes between strains. Through the SEARCH project, the gene flow has been studied using microsatellites as markers. This approach is commonly applied on a variety of organisms, among these several arthropod pests. Microsatellites are small, repetitive base sequences, which may vary in length between populations. This variation can be used in assessments of the genetic relationship between the populations. Eight sets of microsatellites were identified to display polymorphism between different strains and thus were suitable for the analyses. These were used in a study of sealice from Norway, Ireland, Scotland and Canada: Sealice from nine Canadian sites (eight Atlantic and one Pacific), five Scottish, nine Norwegian, and five Irish sites were examined. While all the samples from the 4 countries resulted in polymorphic products with at least 4 of the 8 polymorphic markers, the diversity was not enough to establish any genetic differentiation either within the countries themselves or between the

countries. Indeed, the F_{ST} value expressing genetic distance within the total dataset was not significantly different from zero, suggesting that sea lice across the North Altantic as a whole comprise a single panmictic population. Such extensive gene flow could account for the lack of more severe and localised resistance problems in sea lice, due to continued influx of susceptible genotypes to counter selection for resistance. However, it can also be detrimental since resistance genes selected in one area can subsequently be transferred over long distances to “infect” areas where they have not arisen by mutation in situ.

STRATEGIES FOR THE CONTROL OF RESISTANCE IN SEA LICE

Optimal strategies to control resistance situations will differ according to the situation:

- Resistance is not suspected in the farm or area
- A clinical failure of a treatment agent gives rise to a suspicion that resistance might be a problem
- Resistance has been confirmed in the farm or in the area

Resistance is not suspected

When there are no suspicions about resistance towards the chemotherapeutants used to combat sea lice infestations, care should be taken to try to maintain this optimal situation.

Avoid any unnecessary use of anti-sea lice agents
The development of resistance is driven by the use of anti-sealice agents, and any action taken to avoid unnecessary use will reduce the risk of an unfavourable development. The following management procedures have been proven to reduce the sea lice problem significantly, and thereby also reducing the use of chemotherapeutants and the risk of resistance development.

Monitoring the sealice situation. To be able to take correct actions at appropriate times, it is vital to monitor the infestation rate of sealice in several pens at the site regularly. A minimum control procedure is to count the number of sealice twice a month on at least 20 fish in several pens, as described in the protocol included later in this handbook.

Year – class separation. Keeping separate yearclasses of fish on separate sites delays the onset of sealice problems, as the smolts are not infected by parasites on larger fish at the site. The all in – all out operations where new cages are added when the fish grows, and where all fish are harvested during a short time-span generally experience fewer problems with sealice than sites with a continuous production cycle.

Fallowing between production cycles. During a fallowing period, there is no production of sealice at the site. When a new yearclass is introduced, there is a considerable time-span before the sealice burden builds up again, with parasites origining from outside the site.

Clean nets. Copepodides (the infective stage of the sealice) tend to grab on to any debris and cling to it until a host passes by. Extensive growth on the nets increases the surface they can attach to significantly. Regular and thorough cleaning of the nets reduces this opportunity for the copepodids, resulting in a larger proportion being transported away from the site with the currencies.

Removal of losers. Diseased , deformed or sexually mature fish move slowly in the water and are much more susceptible to attack by sealice than normal fish. Such fish should be removed instantly; otherwise they will act as a constant sealice hatchery in the netpens.

Cleaner fish (wrasses). Whenever possible, cleaner fish should be used to keep a favourable sealice situation in the farm. Wrasses are traditionally mainly used on smaller fish, but there are species that can be used on larger fish as well. The correct use of cleaner fish in a fish farm is an art, but it is an art that any sincere site manager can learn. It is recommended to download the following document from the internet, and to follow the instructions given: http://www.leppefisk.no/no/EngelskLeppefisk.pdf

Targeted use of anti-sealice agents

Winter actions. The reproduction of sealice during the cold season is normally low, but with increasing daylight the hatching of new parasites explodes ("spring rise"). It is therefore important to reduce the number of gravid females to an absolute minimum during the winter using targeted treatments with effective anti-sealice agents, as this hurts the parasite in its' most vulnerable period, effectively cutting off the spring rise and thereby also the infection potential.

Synchronized actions. During the summer and fall, synchronized actions to reduce the number of sealice in adjacent farms will effectively reduce the infection pressure within an area.

Correct use of anti-sealice agents. Any treatment should be carried out according to recommended procedures. Incorrect use of an agent increases the risk of a sub-optimal result, and may favour a selection of resistant parasites. The most common pitfalls to avoid for bath treatments are

- Too short exposure time
- Incorrect calculation of the water volume
- Insufficient mixture of the agent in the netpen
- Insufficient oxygenation of the netpen during treatment
- Insufficient reduction of pen-volume during treatment
- Improper use of tarpaulin "skirts" resulting in a leakage of the treatment solution

Some common pitfalls to avoid for orally administered agents are

- Errors in dosage rate as a result of erroneous calculation of biomass
- Premature termination of the treatment
- Splitting of the daily dose on several meals instead of administration as a single meal
- Insufficient removal of losers

Rotation in the use of chemicals. The use of only one anti-sealice agent for a prolonged period of time increases the selection pressure for this compound, and may speed up resistance development. Although only a limited number of compounds are licensed in several countries, there is normally a potential for rotation. Orally administered agents (emamectin benzoate, teflubenzuron) are most conveniently used on smaller fish (up to 1 kilogram), while larger fish can be treated using bath treatments (preferably pyrethroids).

Monitoring of efficacy. The efficacy of each treatment should be monitored and recorded, by comparing lice counts conducted prior to treatment and lice counts conducted 14 days post treatment. For some agents, the sealice may stay attached to the fish for such a long period, without being able to recover. When the water temperature is low, another week may be

necessary before the result is evaluated. The time-point of reinfestation with viable lice should also be monitored.

Suspicion of resistance due to a clinical failure of a treatment

A clinical failure of a treatment against sealice may have a number of possible causes. Many of these have nothing to do with resistance development. It is very important to rule out treatment errors as a cause. The most common pitfalls were described earlier, and include a too short exposure time, incorrect calculation of the water volume, insufficient mixture of the agent in the netpen, insufficient reduction of pen-volume during treatment, improper use of tarpaulin "skirts" resulting in a leakage of the treatment solution, errors in dosage rate as a result of erroneous calculation of biomass, premature termination of oral treatments, treatment by small doses over the whole day instead of in a single meal, premature evaluation of treatment efficacy.

Sensitivity tests

If any of these events are unlikely as the cause, efforts should be made to conduct a sensitivity test of the population for the agent in question. Later in this handbook, the protocols for field tests and full bioassays for deltamethrin, cypermethrin, cis-cypermethrin, azamethiphos, emamectin benzoate and teflubenzuron are described in detail. Most tests are relatively easy to perform, but require some experience when the results are evaluated. With exception of the teflubenzuron test, they are all carried out in a bath exposure of preadult II sealice, and can be carried out in any laboratory with a minimum of equipment.

Selection of new treatment

An immediate re-treatment using the same agent that resulted in the treatment failure is *not* advised, unless a treatment error is strongly suspected. If the failure was caused by resistance, a moderately elevated dose might result in a better efficacy, but the selection of resistant parasites may be accelerated and cause more severe problems in the future. If an immediate re-treatment is unavoidable, an agent from a different class of chemicals should be chosen.

Confirmed resistance problems

When resistance has been confirmed by bioassays or biochemical / molecular assays, actions should be taken to combat the particular sealice population and to prevent the resistance mechanism from spreading.

Not all resistance mechanisms are heritable. This can be checked by performing bioassays both on the field population and on the next generation, hatched from eggstrings of surviving female lice. If not heritable, no particular actions beyond an alternative strategy to combat the sealice infestation are necessary.

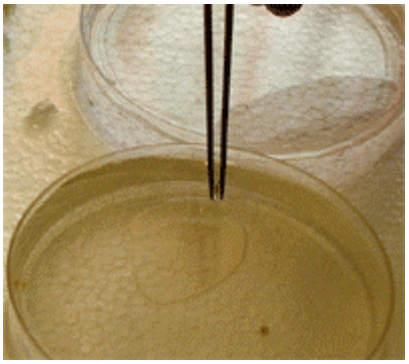
Some low-grade resistance mechanisms involve enhanced metabolism of the agent in question. These normally involve a "cost" for the parasite in terms of increased production of metabolising enzymes and/or reduced survival rates when the selection pressure has been removed. In such cases, alternative strategies to combat the sealice infestation are necessary, combined with local actions in the close proximity to the site.

The most serious resistance mechanisms involve specific mutations in genes coding for biochemical targets of various treatments. Some of these, e.g. kdr-mutations in the gene coding for the voltage-gated sodium channel, seem to have little or no costs for the parasite, and may rapidly become endemic. Such mechanisms may, if they are allowed to spread, compromise a whole group of effective therapeutic agents and pose a great risk for the sealice control by the industry. In such cases, a plan to minimize the negative impact should be worked out together with the fish health authorities.

PROTOCOLS FOR BIOASSAYS WITH SEA LICE

General considerations

Sampling
Whenever possible, pre-adult II salmon lice should be used in the bioassays to determine the sensitivity towards a treatment agent. Pre-adult I salmon lice or adult male lice can be used if the number of preadult II lice is too small. Pre-adult I lice have the same sensitivity to treatment agents, but they are smaller and more difficult to handle, and in some cases an increased mortality can be observed in the control group. Adult male lice may vary more in sensitivity between individuals due to variations in age. The bioassays are standardized by the use of the pre-adult II stage. This stage represent one defined stage in the development and then all tests performed with this stage can be compared. Bioassays can only be performed with normal, healthy individuals. It is therefore important that the lice are treated with the greatest care. The salmon lice are handled with anatomical forceps. Place the forceps with the arms on each side of the cephalothorax and press it together until the louse can be moved. The arms of the forceps must never be squeezed completely together. It is important to use a good forceps were the arms are of equal length. After handling, the lice must have normal behaviour; be able to suck to the surface (- and stay attached) or swim in a straight line (- and not in circles).



Sampling in the field: The best method to collect salmon lice is to find moribund fish. Moribund fish will often swim in the surface or stay in the corners of the net pen. After netting, kill the fish with a blow to the head. Collect all mobile stages (- both sexes of pre-adult I, pre-adult II and adults) with a forceps as described above. The lice must be placed in a bucket of fresh seawater until the bioassays can be performed. A maximum storage period of 6 hours is recommended.
Sampling from a culture in the lab: Use the same method as described above, but the development of the lice in the culture must be watched closely. At high water temperatures the development towards the pre-adult stage will be rapid. It is important that the lice are collected when the majority have reached the pre-adult II stage.

Number of lice in each group/ bioassay
Put 10 lice in each bioassay-box (b-box) (emamectin; petri-dish) after collection. A standard bioassay is performed to determine the EC_{50} (EC_{50} – the concentration immobilizing 50 % of

the target organism (moribund + dead)) or LC_{50} (LC_{50} – the concentration that kills 50 % of the target organism). A standard bioassay is performed with 6 concentrations (including one control group) in duplicates (- thus, it requires 120 lice). A field bioassay is performed with 3 concentrations (including one control group) in duplicates (- thus, it requires 60 lice).

Table 1. Number of lice in each bioassay

Bioassay	No in each group	No of groups/ conc.	Duplicates	Total no.
Complete bioassay	10	6	Yes	120
Field bioassay	10	3	Yes	60

Holding period

The salmon lice are kept in b-boxes and placed in seawater with air supply (- with an aquarium pump), before and after exposure to treatment agents.

Diluting and performing the bioassay

The correct amount of seawater for the different exposure baths and to the stock- and work solutions are prepared first, and thereafter put in the water baths. When the temperature has reached 12 °C, prepare the stock – and work solutions. Then, make the different exposure concentrations, one by one. Start with exposing the control group in pure seawater. Thereafter, make the lowest concentration from the work solution. Expose one group to this concentration. Then make the next concentration (- second lowest), expose one group to this concentration. Finally, make the highest concentration. Use 10 min between start of each exposure. Write the time of start and end of each exposure on the “Evaluation of response” scheme. The periods of exposure for the different agents are described in each separate protocol. When controlling the water temperature in each exposure bath, move the thermometer only from low to higher concentration, and never use tools that have been in contact with the treatment agent in the control bath.

After the exposure, each box should be rinsed thoroughly with clean seawater. The b-boxes should be kept in a container with clean, aerated seawater for the whole observation period.

A cooling box is suitable for field bioassays.

Table 2. Exposure periods

Treatment agent	Active agent	Exposure period	Observation period
AlphaMax	Deltamethrin	30 min	24 hours
Betamax	Cis-cypermethrin	30 min	24 hours
Excis	Cypermethrin	60 min	24 hours
Salmosan	Azamethiphos	60 min	24 hours
Slice	Emamectin	24 hours	None

Evaluation of response

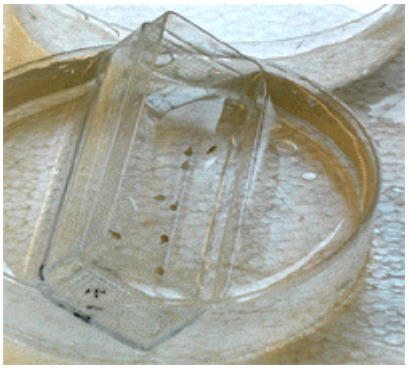
After 24 hours the response should be evaluated after the following criteria:

Live: Normal behaviour, swimming activity in a straight line or the lice are capable of suck to the wall of the b-box and stay attached.

Moribund: Not normal swimming activity, swims in circles, problems with sucking to the wall, - not capable of attach at all, or they fall dawn immediately. All lice with some kind of movements, but not classified as normal, belongs to this group.

Dead: No movements, either in extremities after touching or in the gut or other organs.

Perform the evaluation with the b-box (without the lid) placed in a petri-dish with seawater with the bottom in 45 ° to the bottom of the petri-dish. Place lice attached to the lid carefully in the bottom part of the b-box.



Start with evaluation of lice classified as live: With the 45 ° angle of the b-box, the moribund and dead lice will be collected in the lowest point and the live lice will stay attached to the wall. In that way, the lice collected in the corner, can easily be inspected. Each of them must be placed with the ventral side against the wall by the forceps, giving them a change to attach. Lice, that shows normal behaviour when they are touched or sucks to the wall and stays attached, shall be classified as live.

Distinguishing between moribund and dead lice: Turn the salmon lice lying in the corner with the ventral side up. Inspect each of them with a stereo-microscope by touching them with the tips of the forceps. If there are no movements, the lice are classified as dead.

Write down the number of lice in each category on the evaluation scheme.

Evaluating bioassay with emamectin can be performed directly in the exposure baths in the petri-dishes. A white sheet of paper should be placed under the dishes during evaluation.

Note: It is not necessary to distinguish between dead and moribund lice for estimation of EC_{50}. It is only for estimation of LC_{50} that this must be done.

Cleaning
All equipment used in the bioassay must be cleaned with hot water and a detergent, and then rinsed thoroughly with hot water.

1) Protocols for full bioassays – pyrethroids

TITLE
Bioassays with salmon lice (*Lepeophtheirus salmonis* K.) for the diagnosis of reduced sensitivity to AlphaMax (deltamethrin) and Betamax (cypermethrin)

OBJECTIVES
Performing bioassays with salmon lice strains to detect reduced sensitivity to AlphaMax (deltamethrin) and Betamax (cypermethrin).

IDENTIFICATION OF TEST SUBSTANCES
Test substance 1

Pesticide:	ALPHAMAX (deltamethrin)
Type:	Pyrethriod
Batch no.:	.....
Producer:	ALPHARMA AS, Oslo Norway

Test substance 2

Pesticide:	BETAMAX (cypermethrin)
Type:	Pyrethriod
Batch no.:	.....
Producer:	Vericore Limited

IDENTIFICATION OF TEST – AND REFERENCE STRAINS OF SALMON LICE
Test strain

Locality:	.....
Previous treatments:	.....
Results:	.....

Reference strain

Locality :	.....
Treatments:	.....
Results:	.....

STUDY DESIGN
Salmon lice will be collected from Atlantic salmon (*Salmo salar* L.) in seawater. The fish will be anaesthetized or killed by a blow to the head before collection. Pre-adult salmon lice will be collected (preferable pre-adult II). The lice will be placed in a container with seawater after sampling. Before performance of the bioassay, 10 lice, 5 of each sex will be placed in each bioassay-box (b-box). Only individuals with normal behaviour will be used in the bioassay. The bioassays will be performed with at least 5 concentrations and 30 minutes of exposure with each pesticide. The result will be evaluated 24 h. after end of treatment. The sensitivity as EC_{50} (EC_{50} – the concentration immobilizing 50 % of the target organism (moribund + dead)) will be estimated with probit analysis, and it will be compared statistically to the sensitivity of the reference strain.

Adult female salmon lice will also be sampled for biochemical analysis.

TIME-SCHEDULE (DATES)
Test strain
Collecting pre-adults: .....
Start of bioassays: .....
End of bioassays: .....
Reference strain .....
Collecting pre-adults: .....
Start of bioassays: .....
End of bioassays: .....

PERSONS INVOLVED
.....
.....

MATERIALS AND METHODS
Fish
Salmon lice will be collected from Atlantic salmon (*Salmo salar* L.) in seawater. The fish will be anaesthetized or killed by a blow to the head before collection.

Salmon lice
Pre-adult salmon lice will be collected (preferable pre-adult II). The lice will be placed in a container with seawater after sampling. Before performance of the bioassay, 10 lice, 5 of each sex will be placed in each b-box. Only individuals with normal behaviour will be used in the bioassay. If possible, a total of 120 salmon lice in 12 b-boxes will be prepared for each pesticide. If 120 salmon lice are not available, for each pesticide, as many as possible will be used, divided in groups as mentioned above.

Water quality
Seawater of 12 °C with a salinity ranging from 30 – 33 $^{o}/_{oo}$ will be used. The water will be kept stable during exposure in water baths, and for 24 hours in a thermo box. The temperature will be registered every 6 h.

Exposure
The bioassays will be performed duplicates. The exposure time will be 30 minutes. The following concentrations of each pesticide will be used:

Table 1. Concentrations

Substance	Excepted mortality and concentrations of each pesticide (ppb)					
	0 %	10 %	25 %	50 %	75 %	100 %
Deltamethrin	0	0.03	0.1	0.3	1.0	3.0*
Cypermethrin	0	0.15	0.5	1.5	5.0	15.0*

* Recommended concentration for treatment

Evaluation of the response

The response to each pesticide will be evaluated 24 hours after end of exposure.

The response criteria will be as follows:

- Dead, - no movements, neither extremities nor gut or other organs.
- Moribund, - not capable of attaching to a surface using the flat body as a "sucking disc", neither salmon skin nor the walls of a b-box. Movements of extremities or internal organs could still be observed.
- Live, - attached to salmon skin or the walls of the b-box or actively swimming behaviour.

SAMPLING

Adult or pre-adult salmon lice will be sampled and transported live in cooled seawater to the laboratory. The salmon lice will be frozen in liquid nitrogen and stored at –75 °C until analysis.

STATISTIC

The date will be analysed by Probit analysis (POLO PC, LeOra Software Inc. Berkeley, Ca and the following parameters will be estimated:

- EC50 – The concentration that immobilize 50 % of the target organism (moribund + dead)
- EC90 – The concentration that immobilize 90 % of the target organism (moribund + dead)
- LC50 – The concentration that kills 50 % of the target organism
- LC90 – The concentration that kills 90 % of the target organism

Slope, intercepts and 90 % confidence limits will be estimated for each parameter when possible.

REPORT

A report from this study will be written within 2 months after termination. The report will contain:

- The results with statistics
- Discussion
- Conclusion

APPENDICES

1. List of necessary equipment
2. SEARCH evaluation scheme

SIGNATURES

This protocol is read and approved by

_____ Date: _____

_____ Date: _____

Appendix 1
List of necessary equipment for performing bioassay

Category	Equipment	No.	Note1	Note 2
Div. cloths	Lab. Coat.	1		
	Latex gloves	Min. 20		
Div. equipment	Bioassay-boxes	60	Enough to 4 bioassays	
	Petri-dishes	4		
	Rubber bands	Min. 60		
	Containers, 1.0 L.	12		
	Plastic bucket	1		
	Forceps	2		
	Bottles 1.0 L.	6	Pyrex	
	Pens	2		
	Graded cylinder 0.5 L.	1		
	Pippette, auto	1	100 – 1000 µl	
	Pippette tips	Min. 100		
	Timer	1		
	Thermometer	1		
	Thermo box, 30 l	1		
	Containers with/ lids (watertight)	2		
	Cooling elements	5		
Chemicals	Alphamax		Deltamethrin	
	Betamax		Cypermethrin	
Div. paper	Evaluation scheme	15		
	Protocol book (A-4)	1		
	Protocols	4		
Div.	PC (laptop)	1		
	Polo program	1		

Appendix 2

SEARCH - Evaluation of response

Pesticide: Site:

Date: Performed by:

Dish No.	Dose ppb	Treatment		No. in each category 24 h. after end of treatment				No. in each category 48 h. after end of treatment				Female/ male
		Start	End	Time	L	M	D	Time	L	M	D	

L = Live, M = Moribund and D = Dead

Dilutions:

Pesticide	**Formu-lation**	**Stock solution**		**Work solution**		**Dilutions**		
		conc. (ppm)	**preparation**	**conc. (ppm)**	**preparation**	**conc. (ppb)**	**ml. work s.**	**ml. seawater**
deltamethrin	AlphaMax	1.0	100 µl AlphaMax + 999.9 ml seawater	0.01	10 ml stock + 990 ml seawater	0	0	1000
						(0.01)	1	999
						0.03	3	997
						0.1	10	990
						0.3	30	970
						1	100	900
						3	300	700
cypermethrin	Betamax	5.0	100 µl Betamax + 999.9 ml seawater	0.05	10 ml stock + 990 ml seawater	0	0	1000
						(0.05)	1	999
						0.15	3	997
						0.5	10	990
						1.5	30	970
						5	100	900
						15	300	700

2) Protocols for full bioassays – cypermethrin (Excis)

TITLE
Bioassay with salmon lice (*Lepeophtheirus salmonis* K.) for the diagnosis of reduced sensitivity to Excis vet. (Vericore Limited).

OBJECTIVES
Performing bioassay with salmon lice strains to detect reduced sensitivity to Excis vet.

IDENTIFICATION OF TEST SUBSTANCES
Test substance 1

Pesticide:	Excis vet.
Type:	Pyrethriod (cypermethrin)
Batch no.:	.....
Producer:	Vericore Limited

IDENTIFICATION OF TEST – AND REFERENCE STRAINS OF SALMON LICE
Test strain

Locality:	.....
Previous treatments:	.....
Results:	.....

Reference strain

Locality:	.....
Treatments:	.....
Results:	.....

STUDY DESIGN
Salmon lice will be collected from Atlantic salmon (*Salmo salar* L.) in seawater. The fish will be anaesthetized or killed by a blow to the head before collection. Pre-adult salmon lice will be collected (preferable pre-adult II). The lice will be placed in a container with seawater after sampling. Before performance of the bioassay, 10 lice, 5 of each sex will be placed in each bioassay-box (b-box). Only individuals with normal behaviour will be used in the bioassay. The bioassay will be performed with at least 5 concentrations and 60 minutes of exposure with the pesticide. The result will be evaluated 24 h. after end of treatment. The sensitivity as EC_{50} (EC_{50} – the concentration immobilizing 50 % of the target organism (moribund + dead)) will be estimated with probit analysis, and it will be compared statistically to the sensitivity of the reference strain. Adult female salmon lice will also be sampled for biochemical analysis.

TIME-SCHEDULE (DATES)
Test strain

Collecting pre-adults:	.....
Start of bioassays:	.....

End of bioassays: .....

Reference strain
Collecting pre-adults: .....
Start of bioassays: .....
End of bioassays: .....

PERSONS INVOLVED
.....
.....

MATERIALS AND METHODS
Fish
Salmon lice will be collected from Atlantic salmon (*Salmo salar* L.) in seawater. The fish will be anaesthetized or killed by a blow to the head before collection.

Salmon lice
Pre-adult salmon lice will be collected (preferable pre-adult II). The lice will be placed in a container with seawater after sampling. Before performance of the bioassay, 10 lice, 5 of each sex will be placed in each b-box. Only individuals with normal behaviour will be used in the bioassay. If possible, a total of 120 salmon lice in 12 b-boxes will be prepared for the pesticide. If 120 salmon lice are not available, as many as possible will be used, divided in groups as mentioned above.

Water quality
Seawater of 12 °C with a salinity ranging from 30 – 33 $^{o}/_{oo}$ will be used. The water will be kept stable during exposure in water baths, and for 24 hours in a thermo box. The temperature will be registered every 6 h.

Exposure
The bioassay will be performed duplicates. The exposure time will be 60 minutes. The following concentrations of each pesticide will be used:

Table 1. Concentrations

Substance	Excepted mortality and concentrations of each pesticide (ppb)					
	0 %	10 %	25 %	50 %	75 %	100 %
Excis vet.	0	0.05	0.15	0.5	1.5	5.0*

* Recommended concentration for treatment

Evaluation of the response
The response to the pesticide will be evaluated 24 hours after end of exposure. The response criteria will be as follows:

- Dead, - no movements, neither extremities nor gut or other organs.
- Moribund, - not capable of attaching to a surface using the flat body as a “sucking disc”, neither salmon skin nor the walls of a b-box. Movements of extremities or internal organs could still be observed.

- Live, - attached to salmon skin or the walls of the b-box or actively swimming behaviour.

SAMPLING
Adult or pre-adult salmon lice will be sampled and transported live in cooled seawater to the laboratory. The salmon lice will be frozen in liquid nitrogen and stored at –75 °C until analysis.

STATISTIC
The date will be analysed by Probit analysis (POLO PC, LeOra Software Inc. Berkeley, Ca and the following parameters will be estimated:

- EC_{50} – The concentration that immobilize 50 % of the target organism (moribund + dead)
- EC_{90} – The concentration that immobilize 90 % of the target organism (moribund + dead)
- LC_{50} – The concentration that kills 50 % of the target organism
- LC_{90} – The concentration that kills 90 % of the target organism

Slope, intercepts and 90 % confidence limits will be estimated for each parameter when possible.

REPORT
A report from this study will be written within 2 months after termination. The report will contain:

- The results with statistics
- Discussion
- Conclusion

APPENDICES
1. List of necessary equipment
2. SEARCH evaluation scheme

SIGNATURES
This protocol is read and approved by

_____ Date: _____

_____ Date: _____

Appendix 1
List of necessary equipment for performing bioassay

Category	Equipment	No.	Note1	Note 2
Div. cloths	Lab. Coat.	1		
	Latex gloves	Min. 20		
Div. equipment	Bioassay-boxes	60	Enough to 4 bioassays	
	Petri-dishes	4		
	Rubber bands	Min. 60		
	Containers, 1.0 L.	12		
	Plastic bucket	1		
	Forceps	2		
	Bottles 1.0 L.	6	Pyrex	
	Pens	2		
	Graded cylinder 0.5 L.	1		
	Pippette, auto	1	100 – 1000 µl	
	Pippette tips	Min. 100		
	Timer	1		
	Thermometer	1		
	Thermo box, 30 l	1		
	Containers with/ lids (watertight)	2		
	Cooling elements	5		
Chemicals	Excis		Cypermethrin	
Div. paper	Evaluation scheme	15		
	Protocol book (A-4)	1		
	Protocols	4		
Div.	PC (laptop)	1		
	Polo program	1		

Appendix 2

SEARCH - Evaluation of response

Pesticide: Site:

Date: Performed by:

Dish No.	Dose ppb	Treatment		No. in each category 24 h. after end of treatment				No. in each category 48 h. after end of treatment				Female/ male
		Start	End	Time	L	M	D	Time	L	M	D	

L = Live, M = Moribund and D = Dead

Dilutions:

Pesticide EXPOSURE	Formu-lation	Stock solution		Work solution		Dilutions		
		conc. (ppm)	preparation	conc. (ppm)	preparation	conc. (ppb)	ml. work s.	ml. seawat er
Cypermethrin NB. 60 MIN EXPOSURE	Excis 10 mg/ml	1.0	100 µl Excis + 999.9 ml seawater	0.01	10 ml stock + 990 ml seawater	0	0	1000
						(0.025)	(2.5)	997.5
						0.05	5	995
						0.15	15	985
						0.5	50	950
						1.5	150	850
						5.0	500	500

3) Protocols for full bioassays – azamethiphos

TITLE
Bioassay with salmon lice (*Lepeophtheirus salmonis* K.) for the diagnosis of reduced sensitivity to SALMOSAN (azamethiphos)

OBJECTIVES
Performing bioassay with salmon lice strains to detect reduced sensitivity to SALMOSAN (azamethiphos).

IDENTIFICATION OF TEST SUBSTANCES
Test substance

Pesticide:	SALMOSAN
Type:	azamethiphos
Batch no.:	.....
Producer:	Novartis

IDENTIFICATION OF TEST – AND REFERENCE STRAINS OF SALMON LICE
Test strain

Locality:	.....
Previous treatments:	.....
Results:	.....

Reference strain

Locality :	.....
Treatments:	.....
Results:	.....

STUDY DESIGN
Salmon lice will be collected from Atlantic salmon (*Salmo salar* L.) in seawater. The fish will be anaesthetized or killed by a blow to the head before collection. Pre-adult salmon lice will be collected (preferable pre-adult II). The lice will be placed in a container with seawater after sampling. Before performance of the bioassay, 10 lice, 5 of each sex will be placed in each bioassay-box (b-box). Only individuals with normal behaviour will be used in the bioassay. The bioassay will be performed with at least 5 concentrations and 60 minutes of exposure with the pesticide. The result will be evaluated 24 h. after end of treatment. The sensitivity as EC_{50} (EC_{50} – the concentration immobilizing 50 % of the target organism (moribund + dead)) will be estimated with probit analysis,and it will be compared statistically to the sensitivity of the reference strain. Adult female salmon lice will also be sampled for biochemical analysis.

TIME-SCHEDULE (DATES)
Test strain
Collecting pre-adults: .....

Start of bioassays: .....
End of bioassays: .....

Reference strain
Collecting pre-adults: .....
Start of bioassays: .....
End of bioassays: .....

PERSONS INVOLVED
.....
.....

MATERIALS AND METHODS
Fish
Salmon lice will be collected from Atlantic salmon (*Salmo salar* L.) in seawater. The fish will be anaesthetized or killed by a blow to the head before collection.

Salmon lice
Pre-adult salmon lice will be collected (preferable pre-adult II). The lice will be placed in a container with seawater after sampling. Before performance of the bioassay, 10 lice, 5 of each sex will be placed in each b-box. Only individuals with normal behaviour will be used in the bioassay. If possible, a total of 120 salmon lice in 12 b-boxes will be prepared for the pesticide. If 120 salmon lice are not available, as many as possible will be used, divided in groups as mentioned above.

Water quality
Seawater of 12 °C with a salinity ranging from 30 – 33 $^{o}/_{oo}$ will be used. The water will be kept stable during exposure in water baths, and for 24 hours in a thermo box. The temperature will be registered every 6 h.

Exposure
The bioassay will be performed duplicates. The exposure time will be 60 minutes. The following concentrations of each pesticide will be used:

Table 1. Concentrations

Substance	Excepted mortality and concentrations of each pesticide (ppb)					
	0 %	10 %	25 %	50 %	75 %	100 %
Salmosan vet.	0	3	10	30	100	300*

* Recommended concentration for treatment

Evaluation of the response
The response to the pesticide will be evaluated 24 hours after end of exposure. The response criteria will be as follows:

- Dead, - no movements, neither extremities nor gut or other organs.
- Moribund, - not capable of attaching to a surface using the flat body as a “sucking disc”, neither salmon skin nor the walls of a b-box. Movements of extremities or internal organs could still be observed.

- Live, - attached to salmon skin or the walls of the b-box or actively swimming behaviour.

SAMPLING
Adult or pre-adult salmon lice will be sampled and transported live in cooled seawater to the laboratory. The salmon lice will be frozen in liquid nitrogen and stored at –75 °C until analysis.

STATISTIC
The date will be analysed by Probit analysis (POLO PC, LeOra Software Inc. Berkeley, Ca and the following parameters will be estimated:

- EC_{50} – The concentration that immobilize 50 % of the target organism (moribund + dead)
- EC_{90} – The concentration that immobilize 90 % of the target organism (moribund + dead)
- LC_{50} – The concentration that kills 50 % of the target organism
- LC_{90} – The concentration that kills 90 % of the target organism

Slope, intercepts and 90 % confidence limits will be estimated for each parameter when possible.

REPORT
A report from this study will be written within 2 months after termination. The report will contain:

- The results with statistics
- Discussion
- Conclusion

APPENDICES
1. List of necessary equipment
2. SEARCH evaluation scheme

SIGNATURES
This protocol is read and approved by

_____ Date: _____

_____ Date: _____

Appendix 1
List of necessary equipment for performing bioassay

Category	Equipment	No.	Note1	Note 2
Div. cloths	Lab. Coat.	1		
	Latex gloves	Min. 20		
Div. equipment	Bioassay-boxes	60	Enough to 4 bioassays	
	Petri-dishes	4		
	Rubber bands	Min. 60		
	Containers, 1.0 L.	12		
	Plastic bucket	1		
	Forceps	2		
	Bottles 1.0 L.	6	Pyrex	
	Pens	2		
	Graded cylinder 0.5 L.	1		
	Pippette, auto	1	100 – 1000 µl	
	Pippette tips	Min. 100		
	Timer	1		
	Thermometer	1		
	Thermo box, 30 l	1		
	Containers with/ lids (watertight)	2		
	Cooling elements	5		
Chemicals	Salmosan		Azamethiphos	
Div. paper	Evaluation scheme	15		
	Protocol book (A-4)	1		
	Protocols	4		
Div.	PC (laptop)	1		
	Polo program	1		

Appendix 2

SEARCH - Evaluation of response

Pesticide: Site:

Date: Performed by:

Dish No.	Dose ppb	Treatment		No. in each category 24 h. after end of treatment				No. in each category 48 h. after end of treatment				Female/ male
		Start	End	Time	L	M	D	Time	L	M	D	

L = Live, M = Moribund and D = Dead

Dilutions:

Pesticide	**Formu-lation**	**Stock solution**		**Work solution**		**Dilutions**		
		conc. (ppm)	**preparation**	**conc. (ppm)**	**preparation**	**conc. (ppb)**	**ml. work s.**	**ml. seawater**
azamethiphos		333	5 mg azamethiphos + 15 ml ethanol	1.0	3 ml stock + 997 ml seawater	0	0	1000
						(1)	1	999
						3	3	997
						10	10	990
						30	30	970
						100	100	900
						300	300	700

4) Protocols for full bioassays – emamectin

TITLE
Bioassay with salmon lice (*Lepeophtheirus salmonis* K.) for the diagnosis of reduced sensitivity to Slice (emamectin benzoate)

OBJECTIVES
Performing bioassay with salmon lice strains to detect reduced sensitivity to Slice (emamectin benzoate).

IDENTIFICATION OF TEST SUBSTANCES
Test substance

Pesticide:	Emamectin benzoate (Slice)
Type:	Avermectin
Batch no.:	.....
Producer:	Schering Plough Animal Health

IDENTIFICATION OF TEST – AND REFERENCE STRAINS OF SALMON LICE
Test strain

Locality:	.....
Previous treatments:	.....
Results:	.....

Reference strain

Locality :	.....
Treatments:	.....
Results:	.....

STUDY DESIGN
Salmon lice will be collected from Atlantic salmon (*Salmo salar* L.) in seawater. The fish will be anaesthetized or killed by a blow to the head before collection. Pre-adult salmon lice will be collected (preferable pre-adult II). The lice will be placed in a container with seawater after sampling. Prior to exposure, one litre of each concentration will be made and 40 ml of each should be placed in a petri dish. Thus, 6 (-7) Petri-dishes will be used for each replicate, At exposure, 10 lice, 5 of each sex will be placed in each petri-dish. Only individuals with normal behaviour will be used in the bioassay. The bioassay will be performed with at least 5 concentrations and 24 hours of exposure with the pesticide. The result will be evaluated after end of treatment. The sensitivity as EC_{50} (EC_{50} – the concentration immobilizing 50 % of the target organism (moribund + dead)) will be estimated with probit analysis, and it will be compared statistically to the sensitivity of the reference strain. Adult female salmon lice will also be sampled for biochemical analysis.

TIME-SCHEDULE (DATES)
Test strain

Collecting pre-adults: .....
Start of bioassays: .....
End of bioassays: .....

Reference strain
Collecting pre-adults: .....
Start of bioassays: .....
End of bioassays: .....

PERSONS INVOLVED
.....
.....

MATERIALS AND METHODS
Fish
Salmon lice will be collected from Atlantic salmon (*Salmo salar* L.) in seawater. The fish will be anaesthetized or killed by a blow to the head before collection.

Salmon lice
Pre-adult salmon lice will be collected (preferable pre-adult II). The lice will be placed in a container with seawater after sampling. Prior to exposure, one litre of each concentration will be made and 40 ml of each should be placed in a petri dish. Thus, 6 (-7) petri-dishes will be used for each replicate, At exposure, 10 lice, 5 of each sex will be placed in each petri-dish. Only individuals with normal behaviour will be used in the bioassay. If possible, a total of 120 salmon lice in 12 petri-dishes will be prepared for the pesticide. If 120 salmon lice are not available, as many as possible will be used, divided in groups as mentioned above. Sufficient oxygen will be taken up directly from the liquid surface.

Water quality
Seawater of 12 °C with a salinity ranging from 30 – 33 $^{o}/_{oo}$ will be used. The water will be kept stable during exposure in a thermo box or incubator cabinet. The temperature will be registered every 6 h.

Exposure
The bioassay will be performed duplicates. The exposure time will be 24 hours.
The following concentrations of each pesticide will be used:

Table 1. Concentrations

Substance	Excepted mortality and concentrations of each pesticide (ppb)					
	0 %	10 %	25 %	50 %	75 %	100 %
Emamectin benzoate	0	5	15	25	50	100

Evaluation of the response
The response to the pesticide will be evaluated after 24 hours of exposure.
The response criteria will be as follows:

- Dead, - no movements, neither extremities nor gut or other organs.

- Moribund, - not capable of attaching to a surface using the flat body as a “sucking disc”, neither salmon skin nor the walls of a petri-dish. Movements of extremities or internal organs could still be observed.
- Live, - attached to salmon skin or the walls of the petri-dish or actively swimming behaviour.

SAMPLING
Adult or pre-adult salmon lice will be sampled and transported live in cooled seawater to the laboratory. The salmon lice will be frozen in liquid nitrogen and stored at –75 °C until analysis.

STATISTIC
The date will be analysed by Probit analysis (POLO PC, LeOra Software Inc. Berkeley, Ca and the following parameters will be estimated:

- EC_{50} – The concentration that immobilize 50 % of the target organism (moribund + dead)
- EC_{90} – The concentration that immobilize 90 % of the target organism (moribund + dead)
- LC_{50} – The concentration that kills 50 % of the target organism
- LC_{90} – The concentration that kills 90 % of the target organism

Slope, intercepts and 90 % confidence limits will be estimated for each parameter when possible.

REPORT
A report from this study will be written within 2 months after termination. The report will contain:

- The results with statistics
- Discussion
- Conclusion

APPENDICES
1. List of necessary equipment
2. SEARCH evaluation scheme

SIGNATURES
This protocol is read and approved by

_____ Date: _____

_____ Date: _____

Appendix 1
List of necessary equipment for performing bioassay

Category	Equipment	No.	Note1	Note 2
Div. cloths	Lab. Coat.	1		
	Latex gloves	Min. 20		
Div. equipment	Petri-dishes	60	Enough to 4 bioassays	
	Forceps	2		
	Bottles 1.0 L.	8	Pyrex	
	Pens	2		
	Graded cylinder 0.5 L.	1		
	Pippette, auto	1	100 – 1000 µl	
	Pippette tips	Min. 100		
	Timer	1		
	Thermometer	1		
	Thermo box, 30 l	1		
	Containers with/ lids (watertight)	2		
	Cooling elements	5		
Chemicals	Emamectin benzoate			
Div. paper	Evaluation scheme	15		
	Protocol book (A-4)	1		
	Protocols	4		
Div.	PC (laptop)	1		
	Polo program	1		

Appendix 2

SEARCH - Evaluation of response

Pesticide: Site:

Date: Performed by:

Dish No.	Dose ppb	Treatment		No. in each category 24 h. after end of treatment				No. in each category 48 h. after end of treatment				Female/ male
		Start	End	Time	L	M	D	Time	L	M	D	

L = Live, M = Moribund and D = Dead

Dilutions:

Pesticide	**Formu-lation**	**Stock solution**		**Work solution**		**Dilutions**		
		conc. (ppm)	**preparation**	**conc. (ppm)**	**preparation**	**conc. (ppb)**	**ml. work s.**	**ml. seawater**
emamectin benzoate		100	5 mg emamectin + 50 ml methanol	1.0	10 ml stock + 990 ml seawater	0	0	1000
						5	5	995
						(10)	(10)	(990)
						15	15	985
						25	25	975
						50	50	950
						100	100	900

5) Protocols for full bioassays – teflubenzuron

TITLE
Bioassay with salmon lice (*Lepeophtheirus salmonis* K.) for the diagnosis of reduced sensitivity to teflubenzuron.

OBJECTIVES
Performing bioassays with salmon lice strains to detect reduced sensitivity to the chemotherapeutant teflubenzuron. Teflubenzuron is an in-feed compound and the effect is only observed during a moult. Chalimus (stage 3 - 4) larvae will be exposed to 6 different doses (- including one control group) of teflubenzuron by injection of the salmon host. The numbers of pre-adult salmon lice developing from the treated chalimus larvae will be registered for each dose – group.

IDENTIFICATION OF TEST – AND REFERENCE SUBSTANCES
Test substance
Pesticide:
Type: teflubenzuron
Batch no.: .....
Producer: .....

Reference substance
Substance: Saline, 0.9 % NaCl
Type: Saline
Batch no.: .....
Producer: .....

IDENTIFICATION OF TEST – AND REFERENCE STRAINS OF SALMON LICE
Test strain
Locality: .....
Previous treatments: .....
Results: .....

Reference strain
Locality : .....
Treatments: .....
Results: .....

STUDY DESIGN
Salmon lice (adults and preadults) will be collected from Atlantic salmon (*Salmo salar* L.) in seawater. The fish will be anaesthetized or killed by a blow to the head before collection. Adult female salmon lice with eggstrings will be collected for cultivation. Eggstrings will be cultivated and salmon will be infected with copepodids. The copepodids will develop on the fish until they have developed to chalimus larvae, stages 3 and 4. The salmon with chalimus larvae will be anaesthetized, weighed and injected different concentrations (- 5 concentrations and one control group) of

teflubenzuron (mg teflubenzuron/ kg fish). The effect will be registered by observing the numbers of chalimus larvae developing to pre-adult sea lice in each dose – group. The control group will be treated the same way as the exposed groups. The development time from the chalimus stage to the pre-adult will depend on the temperature. Therefore, the test will be evaluated when the lice in the control group have developed into pre-adults. Only individuals with normal behaviour will be used in both tests. The results will be compared to the result of a test performed on a reference strain.

TIME-SCHEDULE (DATES)
Test strain
Collecting salmon lice in the field: .....
Start of cultivation: .....
Start of bioassays: .....
End of bioassays: .....

Reference strain
Collecting salmon lice in the field: .....
Start of cultivation: .....
Start of bioassays: .....
End of bioassays: .....

PERSONS INVOLVED
.....
.....

MATERIALS AND METHODS
Fish
Salmon lice (adults and preadults) will be collected from Atlantic salmon (*Salmo salar* L.) in seawater. The fish will be anaesthetized or killed by a blow to the head before collection.

Salmon lice
Eggstrings will be cultivated and salmon will be infected with copepodids. The copepodids will develop on the fish until they have developed to chalimus larvae, stages 3 and 4.

Water quality
Seawater of 10 - 16 °C with a salinity ranging from 30 – 33 $^{o}/_{oo}$ will be used. The temperature will be registered every 6 h.

Exposure
The salmon with chalimus larvae will be anaestetized, weighed and injected different concentrations (- 5 concentrations and one control group) of teflubenzuron (mg teflubenzuron/ kg fish). The control group will be treated the same way as the exposed groups.

Table 1. Concentrations of teflubenzuron (mg/ kg fish)

Group	Excepted mortality and concentrations teflubenzuron (mg/ kg fish)					
	0 %	10 %	25 %	50 %	75 %	100 %
dose	0	0.1	0.3	1.0	3.0	10.0

Evaluation of the response
The development time from the chalimus stage to the pre-adult will depend on the temperature. Therefore, the test will be evaluated when the lice in the control group have developed into pre-adults. The effect will be registered by observing the numbers of chalimus larvae developing to pre-adult sea lice in each dose – group.

STATISTIC
The response of the salmon lice will be analysed by Probit analysis (POLO PC, LeOra Software Inc. Berkeley, Ca and the following parameters will be estimated:

- EC_{50} – The concentration that immobilize 50 % of the target organism (moribund + dead)
- EC_{90} – The concentration that immobilize 90 % of the target organism (moribund + dead)
- LC_{50} – The concentration that kills 50 % of the target organism
- LC_{90} – The concentration that kills 90 % of the target organism

Slope, intercepts and 90 % confidence limits will be estimated for each parameter when possible.

REPORT
A report from this study will be written within 2 months after termination. The report will be presented to the partners of the project and to the EU-commission
The report will contain:

- The results with statistics
- Discussion
- Conclusion

SIGNATURES
This protocol is read and approved by

_____ Date: _____

_____ Date: _____

6) Protocols for field bioassays

Field bioassays are generally performed the same way as full bioassays, but with only two concentrations of the chemotherapeutant and a control group. Field tests are normally performed on sea lice collected directly at the fish farm.

The concentrations to be used in the field tests with deltamethrin (AlphaMax) and cis-cypermethrin (Betamax) are proposed on basis of a number of full bioassays, and generally correlate well with the clinical outcome of a treatment. However, for cypermethrin (Excis), azamethiphos (Salmosan) and emamectin (Slice), the number of full bioassays upon which the classification is based, are limited. The classification limits may change when more experience has been gained.

Dilutions:

Pesticide	**Formu-lation**	**Stock solution**		**Work solution**		**Dilutions**		
		conc. (ppm)	**preparation**	**conc. (ppm)**	**preparation**	**conc. (ppb)**	**ml. work s.**	**ml. seawater**
Deltamethrin Field-test 30 min exposure	AlphaMax	1.0	100 µl AlphaMax + 999.9 ml seawater	0.01	10 ml stock + 990 ml seawater	**0**	**0**	**1000**
						0.2	**20**	**980**
						0.6	**60**	**940**
Cis-cypermethrin Field-test 30 min exposure	Betamax	5.0	100 µl Betamax + 999.9 ml seawater	0.05	10 ml stock + 990 ml seawater	**0**	**0**	**1000**
						1.0	**20**	**980**
						3.0	**60**	**940**
Cypermethrin* Field-test 60 min exposure	Excis	1.0	100 µl Excis + 999.9 ml seawater	0.01	10 ml stock + 990 ml seawater	**0**	**0**	**1000**
						0.3	**30**	**970**
						1.0	**100**	**900**
Azamethiphos* Field-test 30 min exposure	Lab.	333	5 mg azamethiphos + 15 ml ethanol	1.0	3 ml stock + 997 ml seawater	**0**	**0**	**1000**
						30	**30**	**970**
						100	**100**	**900**
Emamectin* Field-test 24 h exposure	Lab.	100	5 mg emamectin + 50 ml methanol	1.0	10 ml stock + 999 ml seawater	**0**	**0**	**1000**
						30	**30**	**970**
						90	**60**	**910**

Evaluation
The field tests are evaluated by counting the number of live and immobilised lice in each b-box (emamectin; petri-dish) 24 hours after initiation of the test. The test can only be considered valid when the number of immobilized lice in the control group is less than 20 %.

PESTICIDE	**Immobilisation, low concentration**	**Immobilisation, high concentration**	**Evaluation**
Deltamethrin	More than 50 %	More than 50 %	Sensitive
Cis-cypermethrin			
Cypermethrin*			
Azamethiphos*			
Emamectin*			
Deltamethrin	Less than 50 %	More than 50 %	Moderately insensitive
Cis-cypermethrin			
Cypermethrin*			
Azamethiphos*			
Emamectin*			
Deltamethrin	Less than 50 %	Less than 50 %	Insensitive
Cis-cypermethrin			
Cypermethrin*			
Azamethiphos*			
Emamectin*			

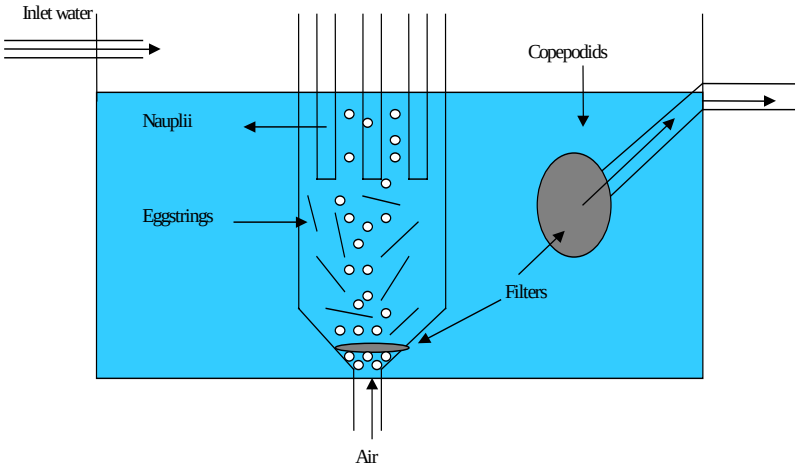
With other outcomes, the test is evaluated as inconclusive.

*These field tests are not yet fully validated, and the limits may change when more information has been gained.

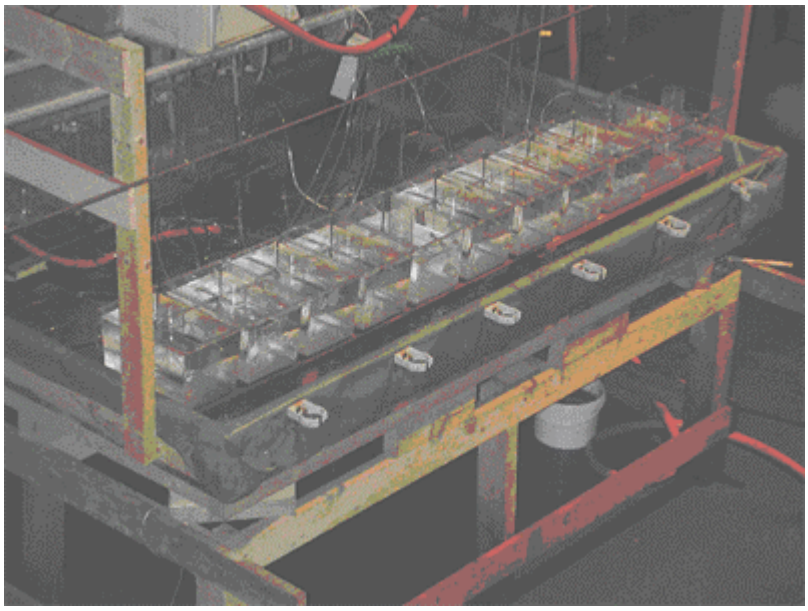
7) Cultivation of sea lice

When examining a particular sea lice strain, it is of importance for the handling of the case to examine whether or not the mechanism is inheritable. In such cases a new generation must be hatched from eggstrings of female adult sea lice, and the bioassays must be repeated on this generation. These procedures require access to a wet-lab with running seawater.

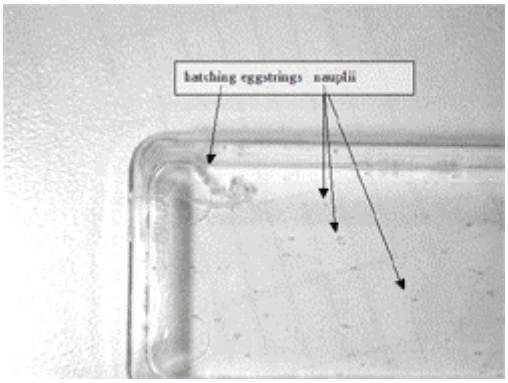
Eggstrings are removed from the adult females, counted and put in a “hatching chamber” in the cultivation system. By experience, this system gives optimal hatching results from salmon lice eggstrings. The inlet water must run very slowly (dripping).



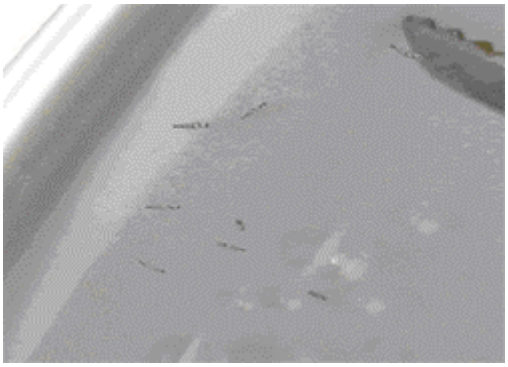
Groups of eggstrings can be cultivated separately.



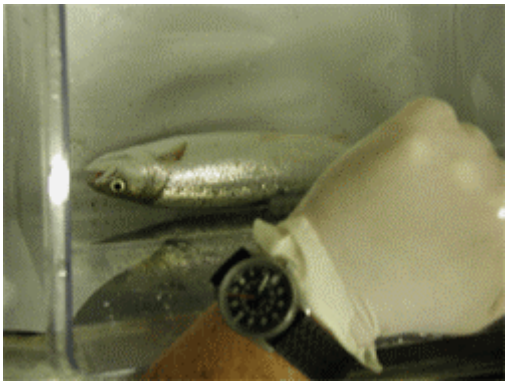
The hatching and survival of nauplii larvae must be monitored. Depending on water temperature, infective copepodides will be developed within 8 – 14 days.



When viable copepodides are evident,



anaesthetized Atlantic salmon must be infected with them after up-concentration.



It is very important not to allow too many copepodides to attach to the fish. This is regulated by the time the anaesthetised fish is submerged in the infection bath. One parasite per 10 grams of bodymass (15 parasites on a 150 grams fish) does not seem to be of any harm. With a density of 300 viable copepodides per liter, this is achieved by an exposure period of 30 seconds for each fish.

The fish is then transferred to a holding tank until the parasites develop into preadult II sealice.

8) Protocol for efficacy monitoring

AIM
This procedure is issued to prevent transmission of infectious agents, protect wild fish, prevent injures and reduced appetite, ensure effective delousing, prevent development of resistance towards delousing agents.

DESCRIPTION
Prevention (site manager, production manager, veterinary service)
The site manager must, in cooperation with the veterinary service, ensure that proper operating sites are selected (currence, distance to other sites). Nets and equipment in the sea must be kept free of algae, blue mussels and debris. Diseased / weakened fish must be removed. Wrasses should be used when appropriate.

Planning (site manager)
The site manager must continuously evaluate the need for delousing up against the levels set by the appropriate authorities and internal guidelines. The site manager must consult with the veterinary service once the level of lice approaches the maximum allowed limits, to ensure that appropriate delousing agents are available.

Delousing levels (site manager, veterinary service)
Delousing procedures must as a minimum be carried out when the limits set by the authorities are exceeded. Delousing procedures must only be carried out on basis of proper sea lice counts, and not according to subjective evaluations. The parameters to consider are the number of lice in different stages, the distribution between cages, the size of the fish, temperature, season, harvesting and other concurrent disease problems. Each site must comply with regional and national action plans.

Treatment (site manager, veterinary service)
The method of treatment (bath, oral, during sorting), delousing agent and treatment time must be evaluated together with the veterinary service. The treatment methods must be evaluated with appropriate considerations of possibilities for development of resistance. When performing bath treatments, the use of fully enclosed tarpaulins must be the first choice. The fish must be monitored carefully during the delousing procedure, and competent personnel must be available. Appropriate protection, as described in the product's safety sheet, must be used. The level of oxygen must be monitored before and during the delousing procedure. For pens larger than 70 m, two oxygen monitors must be used. If the oxygen level drops below 60 %, the delousing procedure must be aborted and the tarpaulin must be removed.

New treatment regimes (site manager, veterinary service)
New procedures must be evaluated together with the veterinary service. The risk for the fish and consumers, and the efficacy must be evaluated. The available information about the new procedure must be evaluated beforehand.

Follow-up work (site manager)
All equipment used must be repaired, cleaned and disinfected immediately after the procedure. The equipment must be stored in its designated space. Positive and negative incidents must be summarized, and discussed with the veterinary service / the production manager. In summer (temperatures > 10 ^{o}C), the sea lice levels must be recorded one week after treatment. Otherwise, normal counting procedures should be followed. If the sea lice counts indicate a treatment failure, the veterinary service must be consulted immediately.

Appendix

Norwegian regulation concerning actions against sea lice

Issued by the Ministry of Agriculture on February 1., 2000 under the act of December 19. 2003, no. 124 on food production and food safety etc. (the Food Act) § 33 first section, see also § 36 second section, see also delegation decision of December 19. 2003 no. 1790. Changed January 9. 2004 no. 104 (e.g. legal basis).

Chapter I. Aims, areas concerned and definitions

§1 Aims

The aim with this regulation is to set minimum actions to reduce the levels of sea lice, in order to minimize adverse effects on salmon and trout in fish farms and on wild populations.

§2 Areas concerned

This regulation is in effect for fish farms in the sea, and concerns the species salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*).

§ 3 Definitions

In the regulation, the following meaning of terms is applied:

1) Sea lice: *Lepeophtheirus salmonis*.
2) Adult female louse: Fully grown female louse with or without eggstrings
3) Mobile stages: Adult male lice and half-grown (preadult) stages.
4) Treatment: Delousing with medicinal products by bath or through the feed.
5) Site: Area where farms are located, in accordance with licence for aquaculture operations given in accordance with the Fish Disease Act and the Aquaculture Act. This term will also apply to sites operated by more than one owner.
6) Responsible subject: The subject(s) who, according to the Food Act and the Aquaculture Act hold a permit for aquaculture production on the site.
7) Sea temperature: The sea temperature measured at 3 meters depth.

Chapter II. Actions

§ 4 Counting, recording and reporting

At sea temperatures at or above 4 ° Celsius, the level of sea lice must be monitored al least every 14. days. This does not apply for sites from which all fish will be removed within 14 days after this date.

The number of adult female lice (sea lice), the number of mobile lice, the number of treatments, the sea temperature and the use of wrasses must be reported monthly to the Food Authorities within the 15. in the next month.

The Food Authorities issue guidelines for counting, recording and reporting of lice.

§ 5. Action levels for mandatory delousing

Whenever a counting in the period from December 1 to July 1 show 0.5 or more adult female lice, or a total of 5 or more of adult female lice and mobile stages, averagely on fish in individual cages, treatment must be carried out on the whole site. In the counties Troms and Finnmark, these limits will apply in the period November 1 to July 1.

Whenever a counting in the period July 1 to December 1 show 2 or more adult female lice, or a total of 10 or more adult female lice and mobile stages, averagely on fish in individual cages, treatment must be carried

out on the whole site. In the counties Troms and Finnmark, these limits will apply in the period July 1 to November 1.

Treatment of individual cages can be omitted if the average number of adult female lice and mobile stages is less than 0.1 per fish.

The treatment must be completed within 14 days after recording of sea lice levels exceeding the limits.

The Food Authorities may give a permit to postpone the treatment. Such a permit can be given when the limits are exceeded insignificantly, and a coordinated delousing action is planned within a short period of time, and this coordinated action is considered to meet the aims of this regulation better. The treatment can also be postponed in periods when wrasses show a satisfactory activity, when the limits are exceeded insignificantly and the use of wrasses is expected to reduce the levels of lice below the limit within a short period of time.

§ 6 Exceptions

The regulations in § 5 do not apply when the sea temperature is below 4 ° Celsius.

Chapter III. Final regulations

§ 7 Economy

The authorities will not compensate economical losses due to actions according to this regulation.

§ 8 Fines and enforcement

Violations of the regulations in § 4 can result in fines issued by the Food Authorities for each 10,000 fish at the site, at the level of the basic sum in the national pension plan divided through 36.5.

Violations of the regulations in § 5 can result in fines issued by the Food Authorities for each 10,000 fish at the site, at the level of the basic sum in the national pension plan divided through 36.5 per day.

Fines can be issued without further notice. Fines according to the second section will normally run from the day the fine is issued and until the situation mentioned in § 5 is corrected.

§ 9 Surveillance and decisions

The Food Authorities will conduct surveillance and make decisions to enforce the instructions given in this regulation.

The Food Authorities can also establish geographical zones for special actions whenever this is considered necessary for the reduction of sea lice levels.

§ 10 Dispensation

The Food Authorities can in special cases exempt from this regulation, provided that this will not violate the Norwegian international obligations, such as the EEA-agreement.

§ 11 Penalties

Violation of this regulation, unknowingly or deliberately, or of decisions made in agreement with it, can be penalized according to the Food Act, § 28.

§ Commencement

This regulation will come into force immediately.

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