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1. Innledning og oppsummering

OPPDRAG:
Veterinærinstituttet fikk 30. mars 2010 en bestilling fra Fiskeri- og kystdepartementet der det ble bedt om "forslag til standardiserte og validerte metoder for telling og beregning av lakselus i oppdrett som tar hensyn til biomassen på lokaliteten, og andre forhold på lokaliteten som har betydning for spredning av lakselus til villfisk". I tillegg skulle Veterinærinstituttet "videreutvikle standardiserte og validerte metoder for påvisning av resistens hos lakselus".

GJENNOMFØRING:

1. Standardiserte og validerte metoder for telling og beregning av lakselus i oppdrett.
Veterinærinstituttet har tatt utgangspunkt i de endringene norsk lakseoppdrett nå går igjennom på utstyrssiden, med større merder og mer fisk, og de gjeldende forskrifter mht. lakselusbekjempelse. Disse forholdene gir rammebetingelsene for telling av lus og rapportering av lusetall til Mattilsynet, men har også betydning for når og hvordan undersøkelse av lakselus for resistens mot medisiner gjøres. Nye oppdrettsanlegg har ofte sirkulære merder med omkrets omkring 160m, og største dybde rundt 40m. I hver av disse står omkring 200 000 fisk. Det finnes lite kunnskap om hvordan lakselus fordeler seg i slike anlegg, og hvordan man bør gå frem for å best beregne gjennomsnittlig antall lus der. Veterinærinstituttet har derfor valgt å undersøke spesielt hvordan man kan få det beste estimat for gjennomsnittlig antall lakselus på lokaliteter med stormerder. Fordeling av lus er analysert i innsamlede data, og disse er brukt til å simulere uttak av forskjellig størrelse. Vi har slik evaluert eksisterende telleprotokoll (Luseforskriften). Det beskrives også her hvordan andre uttaksstørrelser vil virke, og det blir presentert en kost-nytteanalyse for undersøkelse av ytterligere økning av utvalgsstørrelse for å bedre nøyaktigheten. Arbeidet er beskrevet i denne rapportens punkt 2-7.

2. Videreutvikling av standardiserte og validerte metoder for påvisning av resistens hos lakselus.
Lakselusas følsomhet for avlusningsmidler måles av fiskehelsetjenester i et "bioassay". Bioassay med lakselus ble utviklet for laboratoriedyrkede lus, mens fiskehelselaboratoriene tester direkte på innsamlede lus fra oppdrettsanlegg. Fordi disse lusene er av varierende kvalitet og ulike stadier og alder, har det vært nødvendig å videreutvikle testene slik at de blir sikrere for lakselus samlet inn i felt. Andre grunner til variasjon i resultater er forskjeller i fremgangsmåte og evaluering av respons på laboratoriene. Det var nødvendig å kvalitetssikre metodene hos de laboratoriene som tester i Norge slik at den eksakt samme fremgangsmåten for testing benyttes. Bioassayresultater som ikke er i samsvar med observert behandlingseffekt forekommer. Det finnes ingen systematiske registreringer av dette, så data er samlet inn for å undersøke størrelsen og hyppigheten av problemet. Videre er grenseverdiene som er satt for å kategorisere lakselus som følsom – nedsatt følsomhet og resistent, vurdert i forhold til målte EC_{50} verdier og behandlingsresultat ved behandling. Arbeidet med resistens er beskrevet i rapporten "Følsomhet hos lakselus, standardisering og kvalitetssikring av bioassay" (Vedlegg 1).

OPPSUMMERING AV RESULTATER

1. Standardiserte og validerte metoder for telling og beregning av lakselus i oppdrett.
Innsamlede lusedata fra flere oppdrettsanlegg med stormerder viser at det er klare forskjeller på lusenivå mellom merder. Dette kan forklares ved forskjellig utsettstidspunkt og/eller størrelse på fisken, men kan også ha noe med strømforhold innen anlegget å gjøre.

Det anbefales derfor at alle merder i anlegget undersøkes. Ved å regne gjennomsnittlig abundans (antall lus/fisk) på 100 fisk som det sanne gjennomsnitt, og sammenligne utvalgsstørrelsene 5, 10, 20 og 50 fisk/merd med dette, ble ideell utvalgsstørrelse analysert. For hver fisk ekstra som blir undersøkt opp til 10 fisk/merd øker sikkerheten for å beregne riktig gjennomsnitt markert, det er så frem til 20 fisk/merd noe bedre sikkerhet. Fra omtrent 20 fisk/merd vil ikke selv 10 ekstra fisk øke sikkerheten i særlig grad. Effekten av de forskjellige utvalgsstørrelsene ble modellert i et scenario der målet var å komme frem til om anlegget hadde gjennomsnittlig abundans over bestemte grenser. Det ble her vist at selv om liten utvalgsstørrelse gir liten sikkerhet for å beregne riktig gjennomsnitt, vil man ha mindre tendens til å underestimere antall lus/fisk. Gitt forutsetningene i våre modeller vil man ved å undersøke 10 fisk/merd eller flere beregne gjennomsnittlig abundans for lavt i ca. 10 % av tellingene.

Den økonomiske konsekvensen for noen av de forslåtte telleprotokoller, i form av medgått arbeidstid, ble derfor undersøkt. Det ble laget en modell, på basis av observert tidsbruk, for innfanging og bedøvelse av

fisk, telling av lus og transport mellom merdene. Resultatene viste at utvalgsstørrelser over 20 fisk/merd vil gi en uforholdsmessig ekstra kostnad i forhold til oppnådd økning i nøyaktighet.

Veterinærinstituttet vurderer den nåværende telleprotokoll (Luseforskriften) som god til rutinemessig overvåking av lusebestanden i oppdrettsanlegg med mye fisk i store merder. Problemene med denne forskriftens tellinger er knyttet til innrapportering av tall. For å sikre standardisert rapportering bør lusetallene fra enkeltfisk rapporteres til Lusedata og Altinn. Nødvendige gjennomsnitt bør der bli automatisk utregnet og sendt til oppdretter og forvaltning. Rådata vil på denne måten også bli tilgjengelig for forskning.

2. Videreutvikling av standardiserte og validerte metoder for påvisning av resistens hos lakselus.
Følsomheten for pyretroider (deltametrin – AlphaMax og cypermetrin – Betamax), emamektin benzoat (Slice) og azametifos (Salmosan) er undersøkt hos ulike stadier av lakselus. Nye bioassayprotokoller, som er bedre tilpasset felt innsamlede lakselus, er skrevet på bakgrunn av resultatene og etter behov for spesifisering av prosedyrer avdekket under kvalitetssikringsarbeidet.

Fiskehelsetjenestene gjennomfører bioassay før behandling med flere midler, og behandler deretter med et middel som testen viser god følsomhet for. Det er gjennomført en undersøkelse basert på EC_{50} verdier samlet inn før behandling og det aktuelle behandlingsresultatet. Det har vært vanskelig å få inn resultater fra fiskehelsetjenestene, både fordi disse ikke systematiserer sine data, men også fordi oppdrettsfirmaene ikke ville gi oss adgang til dem. Derfor kan ingen klare konklusjoner trekkes på grunnlag av innsendte data for pyretroider og emamektin benzoat, men analysene indikerer at en grenseverdi på 10 ppb for azametifos kan være noe for høy.

Ni av fiskehelselaboratoriene som utfører bioassay i Norge er blitt kvalitetssikret ved at minst ett bioassay ble gjennomført og eventuelle avvik fra protokoll ble registrert. Det ble også holdt et bioassay-seminar og en ringtest der lakselus fra samme anlegg ble testet under like forhold av 3 laboratorier. Tidligere tester hadde gitt forskjellig resultat for følsomhet for emamektin hos lus fra anlegget. Resultatene fra ringtesten viste sammenlignbare resultater.

2. Introduction to statistical part

Control of sea lice infections in farms rely on periodical monitoring of sea lice levels on farmed fish, and mandatory delousing once certain thresholds are exceeded. Monitoring is assessed by counting sea lice in a sample of fish for a given number of pens. The number of fish and pens used in sampling protocols has varied over time as well as between salmon producing countries (1). The majority of studies on sea lice counting have been carried out in other countries where the characteristics of farmed salmon production differ from Norway. We have tested and validated the current protocol for monitoring sea lice ("Luseforskriften") in fish farms with large pens and biomass of fish. The protocol was validated using field data from farms with 120-157m circumference pens.

This is first study to provide a scientific basis for sampling methods used in farms with large pens. Due to the increasing attention to the sea lice problem and its association with the increase in fish biomass in recent years, a complete evaluation of the counting procedure was demanded.

The amount and source of variation determines sample size and the best sampling method. Recent research from Canada, Scotland and Norway (1) has shown that the distribution of the fish in pens introduces a significant source of variation in infection of fish in a farm, but the magnitude of this effect was not known for large pen farms. Another factor producing variation is the distribution of lice between the fish. To evaluate these sources, data on lice infections in several farms were collected and analyzed. In the last part, we present a cost-benefit analysis which analyses the increase in precision achieved by sampling more fish, relative to the total cost in time. The cost for sampling few fish is similar to the time used for making the preparations in the pen prior to counting fish. Sampling 10 fish is the most efficient design among those studied, and gains in performance from sampling more than 20 fish were modest in this model.

The work confirms the value of the current sampling protocol, but indicates that an increase in sample size per pen may be necessary to estimate the mean abundance accurately. The importance of counting in all pens is highlighted. We also want to emphasize the importance of quality data and urge both the industry and the competent authorities to standardize and improve the methods for collection and reporting.

2.1. Recommendations

The aim of this work has been to establish knowledge on occurrence and distribution of sea lice in large pen farms in order to evaluate the current sea lice counting protocol ("Luseforskriften") and provide recommendations to improve sampling and reporting.

We recommend that:

- A standardized sampling protocol for routine monitoring should be used at all sites to ensure that the data can be compared across sites and regions. Other protocols may be used for other purposes such as evaluation of treatment effect.

- The methods for catching fish for counting should be consistent and reproducible. Sea lice counts should be made on a random sample of fish from each pen, which is representative of the number and distribution of lice in the pen, as specified in the current protocol.

- A dedicated staff member on each site should be responsible for keeping and updating of the sampling protocol as well as farm records and obligatory recording of sea lice numbers. The sampling protocols should be written concisely in a simple language and available at all times. New staff must learn sampling protocols and permanent staff should re-read protocols at least once a year.

- Calculation of site mean abundance must be based on sea lice counts for 10 to 20 fish per pen and in all the pens. Sampling 10 fish per pen can be used to determine whether level of sea lice has exceeded the allowable threshold and treatments are required.

- Data recorded should, in addition to the data required in "Luseforskriften", also include: louse species (*C. elongatus* or *L. salmonis*), time since last delousing or specification of the date each pen was treated, and the name of the medication which was used in the last delousing of the pen.

- The reporting of data should be done for **individual fish**, on forms which feed the data to ALTINN and send mean abundance back to farmers and Mattilsynet. This will make possible a better resolution in data for research, and also give the possibility of extracting other measures describing the infections, such as prevalence. As effect of treatments against lice should be evaluated separately for each pen, such a reporting system will allow a better monitoring of the effect of treatments, fallowing and zonation on every farm by Mattilsynet and researchers. The arrangement would not demand more work by the farmer, who would have to enter the raw data in a computer anyway.

3. Materials and methods

3.1. Farm characteristics

To collect necessary input-data to use in our statistical work and models, we visited five farms from August to October 2010. The farms were located in Hordaland and Sør-Trøndelag counties, within areas with high density of farms.

A summary of the farm and production characteristics is provided in Table 1. Four farms had only fish in the first year of the production cycle. Differences in the stocking times ranged between less than one month and six months. The average weight of fish in a pen was used as a proxy for the length of time since fish were first stocked. All pens had wrasse in a proportion varying from 1 to 7.5%. According to our records, none of the stocked fish at the time of the visit had previously been deloused.

Table 1. Summary of the characteristics of the salmon farms included in this study (Fish were not weighted or measured on sampling day).

Characteristics	Farm1	Farm2	Farm3	Farm4	Farm5
Visit date	17/08/2010	18/08/2010	13/10/2010	12/10/2010	11/10/2010
Location of farm	Roan	Hitra	Kvinherad	Austevoll	Austevoll
Number of sampled pens (total)	8(13)	7(7)	3(4)	2(2)	3(3)
Species	*Salmo salar*	*Salmo salar*	*Salmo salar*	*Oncorhynchus mykiss*	*Oncorhynchus mykiss*
Total number of fish in farm (millions)	1.97	2.0	0.83	0.45	0.68
Average number of fish per sampled pen	140.000	290.000	210.000	220.000	230.000
% wrasse	1.2	4.0	7.5	3.5	4
Time from stocking the first pen to our sampling	16 months	4 months	3 months	15 days	2 months
Most recent month of fish stocking	May 2010	May 2010	May 2010	Sep 2010	Sep 2010
Average weigh of fish (kg) in sampled pens	2.8	0.7	1.2	0.5	0.2
Largest difference in the average weight of fish between among two pens in the farms (kg)	4.2	0.47	0.93	0.05	0.4
Pen circumference (m)	157	120-157	157	157	157
Pen depth (m)	35	12/35	45	45	45

3.2. Procedure for calculation of input variable for sampling method

We determined to sample 100 fish per pen based on first hand calculation from the farmer and the practical limitations that exists on a production site. This sample size (n) was calculated using formula: $n \geq (1.96/p)^2 * (CV)$, where n is the sample size, the critical value for the 95% level of confidence is 1.96, p is the margin of error (difference estimate versus true mean), and CV is the coefficient of variation. The confidence interval and margin of error are decided by the user and the CV is calculated from field data.

The margin of error *p* was set to give 10%, 20% and 30% accuracy of the mean with a probability of 0.95 (level of confidence). The CV is a measure of the variance present in the data.

The method used for catching and sampling fish was similar among farms. This method is used by farmers to best obtain a random sample of fish. Briefly, fish were fed 5-10 minutes prior catching to attract fish close to the surface. Then a seine was set at the largest width of the pen and pulled across to crowd the fish. A small sample of fish was caught with the help of a dip net or a lifting net operated from the boat. Approximately 5 to 7 fish was placed in a container for anesthetization. After counting, fish were allowed to recover in a separate container with sea water before being returned to the pen.

In total, 23 pens were sampled. Data collected included counts for both *Caligus elongatus* (*C. elongatus*) and *Lepeophtheirus salmonis* (*L. salmonis*). We also count chalimus, pre-adult, adult male and adult female stages of *L. salmonis*. Sampling and counting were done with the assistance from staff and veterinarians. In most instances, counting was done by two people while a third person measured length and weight of the fish and entered all data in a form. Other personnel in the farm helped in the operations for setting the seine, and capturing fish. The time required in each sampling step was recorded and includes: moving between pens, setting and releasing the seine and capturing fish, anesthetizing fish, and the time for counting sea lice until fish were returned to the pen. These data were used to calculate the cost in time for sampling sea lice.

3.3. Statistical analysis

Data collected were entered into a spreadsheet (Excel™). Statistical calculations were done using Excel™, the publicly available software "Quantitative Parasitology" and "R". The bootstrapping, mean abundance and 90% confidence intervals were done in R. Figures were plotted in Excel and R.

Bootstrapping was used to show that mean abundance calculated from a random group of pens differs from the "true" mean abundance from all pens in the farm.

The mean abundance calculated from re-sampling the original dataset (n=100 fish per pen) was compared with sample size n=5, 10, 20 and 50 fish to evaluate the accuracy and precision achieved using these small samples.

4. Results and discussion

4.1. Statistics

4.1.1. *Description of sea lice infections*

Two sea lice species were recorded: skottelus *C. elongatus* and lakselus *L. salmonis*. In general, *C. elongatus* were less prevalent than *L. salmonis*. The proportion of *C. elongatus* in four of the farms was less than 5%, with the exception of farm 2 (Table 2). These parasites have different host preferences, life cycles and biology such that levels of infection and the efficacy of mitigation strategies and treatments are not the same. Infections with adult *C. elongatus* may come from wild fish such as saithe and cod living in the immediate surroundings of the farms (2)(6), as these parasites are good swimmers, but they also of course infect in the copepodid stage. However, large increases in adult or preadult stages may appear to have happened if all mobile lice are thought to belong to one species. This may be serious in the case of evaluation of results after treatments. A recent infection with adult *C. elongatus* may be judged to be survivors from the treatment, and therefore give the impression of reduced sensitivity. Our results demonstrate that *C. elongatus* may constitute 50% of the parasitic copepods in a farm, and it would seem appropriate to count the adults of this louse species in regular countings.

Table 2. Proportion of the two species of sea lice (*L.salmonis* and *C.elongatus*) found in the 5 farms sampled during this study. The proportion is calculated from sea lice counts from 100 fish in each pen. Chalimus larvae were not included.

Site	% *L. salmonis*	% *C.elongatus*
Farm1 (Atlantic Salmon)	96.3	3.7
Farm2 (Atlantic Salmon)	51	49
Farm3 (Atlantic Salmon)	99.8	0.2
Farm4 (Rainbow trout)	98.7	1.3
Farm5 (Rainbow trout)	99.4	0.6

The distribution of sea lice in a fish population is called *aggregated*, if the majority of fish have none or few lice whilst some fish carry large numbers of lice. All farms had mean abundance of mobiles or adult females close to the required thresholds. The *median* values are often lower than the *mean*, which indicates that some of the fish may have large extreme values (Figure 1).

To fully describe aggregated distributions a combined use of several statistics, such as prevalence, mean abundance and variance to mean ratio (VMR) are required (Tables 3 and 4). The statistic mean abundance is alone a poor descriptive measure of the level of infection for research or scientific studies. However, mean abundance is useful as a sole statistic for monitoring and control of sea lice and it is commonly used by the worlds' main salmon producers. If the raw data were reported, measures of variation could be calculated, and give the possibility of estimating the variance around the mean.

Counting chalimus larvae is time consuming, and estimation of mean chalimus abundance has been shown to be inaccurate in field studies (3). However, counting and reporting these stages is important to understand the pattern of sea lice infections, plan treatments and to evaluate the efficacy of sea lice treatments.

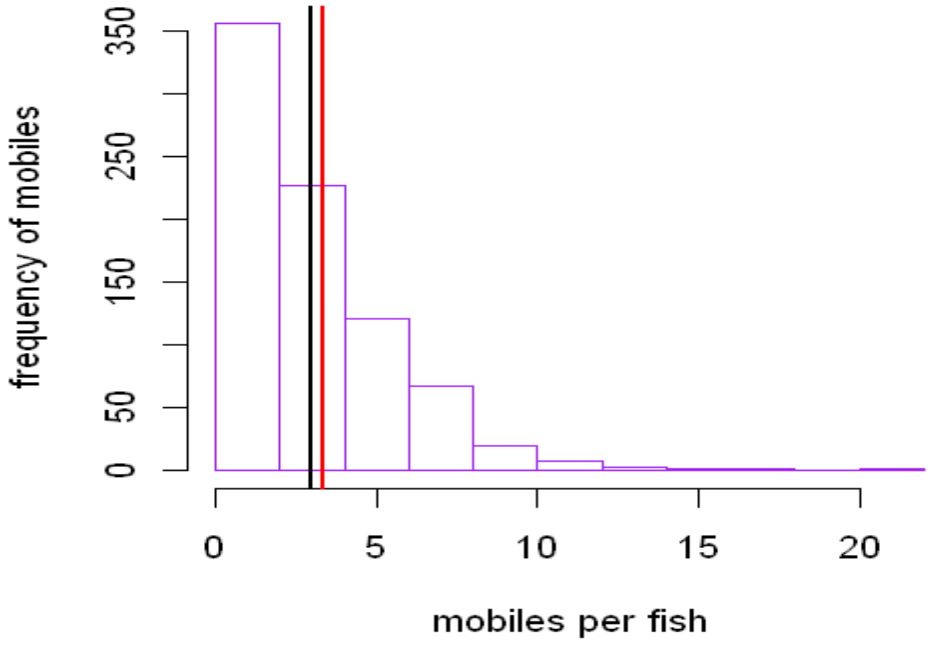

Figure 1. Frequency distribution of mobiles in farm 4. The median and mean are represented by the black and red vertical lines, respectively.

Table 3. Statistics for the level of sea lice infections in farms for mobiles, which include pre-adults and adult male sea lice.

Statistics	Farm 1	Farm 2	Farm 3	Farm 4	Farm 5
Prevalence	90.2	50.9	45.1	95	96.7
95% CI	87.9-92.1	47.2-54.6	39.5-50.8	91.1-97.3	93.9-98.2
Mean abundance	3.4	0.8	2.3	3.6	5.1
95% CI	3.2-3.5	0.7-0.9	1.9-2.7	3.3-3.9	4.7-5.5
Min number lice/fish	0	0	0	0	0
Max number lice/fish	21	8	16	12	18
Variance/mean ratio	2.1	1.3	5.2	1.5	2.1

Table 4. Statistics for the levels of sea lice infestation in farms for adult females.

Statistics	Farm 1	Farm 2	Farm 3	Farm 4	Farm 5
Prevalence	75.1	1.1	34.3	8.0	15.3
95% CI	72.0-78.0	0.9-1.5	29.1-40.1	4.9-12.7	11.6-19.9
Mean Abundance	2.1	0.2	2	0.1	0.21
95% CI	1.9-2.2	0.1-0.3	1.6-2.4	0.04-0.2	0.1-0.3
Min number lice/fish	0	0	0	0	0
Max number lice/fish	14	3	25	2	3
Variance to Mean ratio	1.9	1.3	6.4	1.0	1.4

As shown in Figure 1, the characteristic distribution of sea lice is skewed, which means that it is asymmetrical in the shape. In this type of distribution a larger dispersion of values around the central tendency (i.e. mean) is observed compared to a normal distribution where most data are closer to the mean. The amount of dispersion can be calculated as standard deviation, variance or the variance to mean ratio.

For sea lice distributions, a linear and positive relationship is seen between standard deviation and mean abundance. However, a larger variance is observed with low mean abundance in adult females compared to adult males (red circle, Figure 2). This observation may be explained by the greater tendency of adult females to aggregate compared to other stages (4).

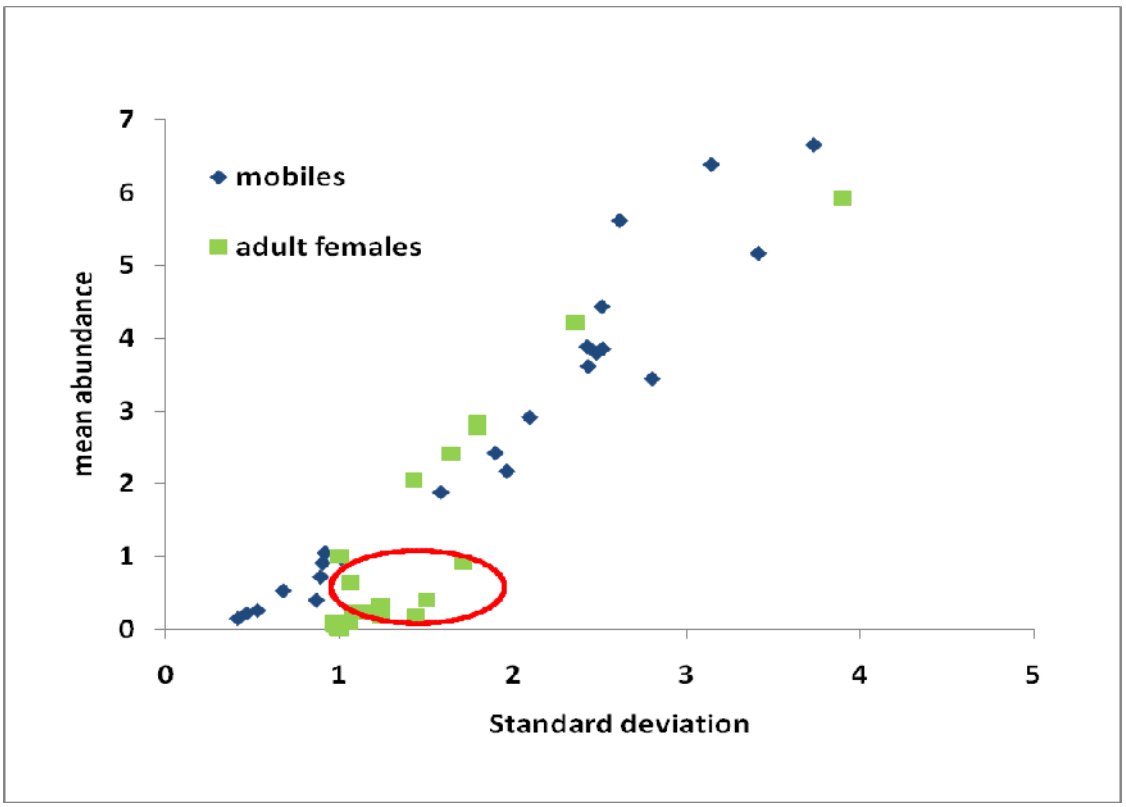

Figure 2. The relationship between mean abundance and standard deviation of mobiles and adult female sea lice in salmon and rainbow trout pens. The circle in red shows pens with large variance and low mean abundances of adult females.

We believe that the average standard deviation for the full production cycle (from stocking to slaughter) is larger than the ones shown in this report. In four out of five farms, all fish stocked were in the first production year and had not been deloused. However, mean abundance of sea lice is higher in the second year of production, and delousing treatments may increase the variation. Larger variation may be due to non-homogeneous treatments, resulting in some fish with many lice being exposed to suboptimal levels of treatment.

Result 4.1.1:

Mean abundance is a useful and commonly used statistic to monitor sea lice levels on farmed salmon in large pens. Mean abundance cannot be used alone to fully explain the complex distribution of sea lice populations. If the raw data were reported, measures of variation could be calculated, and give the possibility of estimating the variance around the mean.

4.1.2. *Sources of variation in the farm*

Variation in the farm is largely influenced by the hierarchical organization of farm. Fish are grouped in pens and multiple pens are located at one site. This means that the total variance in sea lice counts has two components: one resulting from fish within the same pen and the other from fish in different pens.

The variance occurring in each of these components can be compared by calculating the intracluster correlation coefficient (ICC). The intracluster correlation coefficient (ICC) is a measure of similarity of clustered data. An ICC value of zero indicates that there is no difference between fish within a pen or between pens. In contrast, an ICC close to 1 indicates that the variation seen is mostly between pens and little within pens, and the opposite produce an ICC close to -1.

ICC values calculated for mobiles and adult females are shown in table 5 and 6, respectively. Most ICC values are positive, which supports the hypothesis that fish within the same pen have similar levels of infection and that much of the variation is due to differences between pens. ICC values are larger for mobiles compared to adult females as the number of mobiles are also larger. ICC values are close to zero for farm 4, which had two pens stocked within the last 3 months and low counts of sea lice. Similarly, farms 2 and 5 had very low counts and ICC values close to zero for adult females. These results are in agreement with the findings of Revie et al. (5), which showed that low estimates of ICC correspond to farms where mean abundance is less than 0.5 mobiles or adult females per fish.

The ICC results agree with the boxplots in Figures 3 and 4, where it is shown that a large variation exist between pens in each farm. In farm 4, the counts of mobiles are similar between the two pens; while, in farms 2, 4, 5 the counts of adult females are very close to zero.

Table 5. Intracluster correlation coefficient values (ICC) values for each farm for mobiles.

Location	ICC	†95% LCI	†95% UCI
Farm 1	0.16	0.013	0.313
Farm 2	0.15	0.007	0.285
Farm 3	0.77	0.492	1.000
Farm 4	0.07	-0.073	0.206
Farm 5	0.31	-0.043	0.658

†LCI and UCI correspond to the lower and upper confidence intervals

Table 6. Intracluster correlation coefficient values (ICC) for each farm for adult females.

Location	ICC	†95% LCI	†95% UCI
Farm 1	0.35	0.112	0.602
Farm 2	0.07	-0.008	0.143
Farm 3	0.69	0.359	1.000
Farm 4	-0.004	-0.0151	0.005
Farm 5	0.11	-0.0602	0.280

†LCI and UCI correspond to the lower and upper confidence intervals

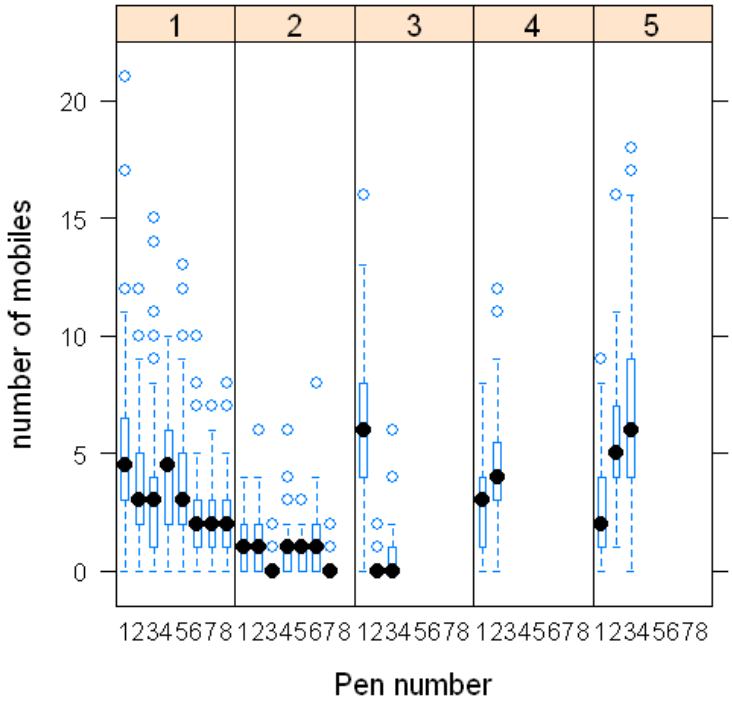

Figure 3. Boxplots of counts mobile lice for all pens in each farm. Connecting data for each pen (max and min counts per fish), the box extends from Q1 (first quartile) to Q3 (third quartile). The black dot in the center of the box is the mean abundance and the round blue circles above the line are outliers. Note the wide variation in the means from pen to pen at most sites. Counts are for *n*=100 fish per pen.

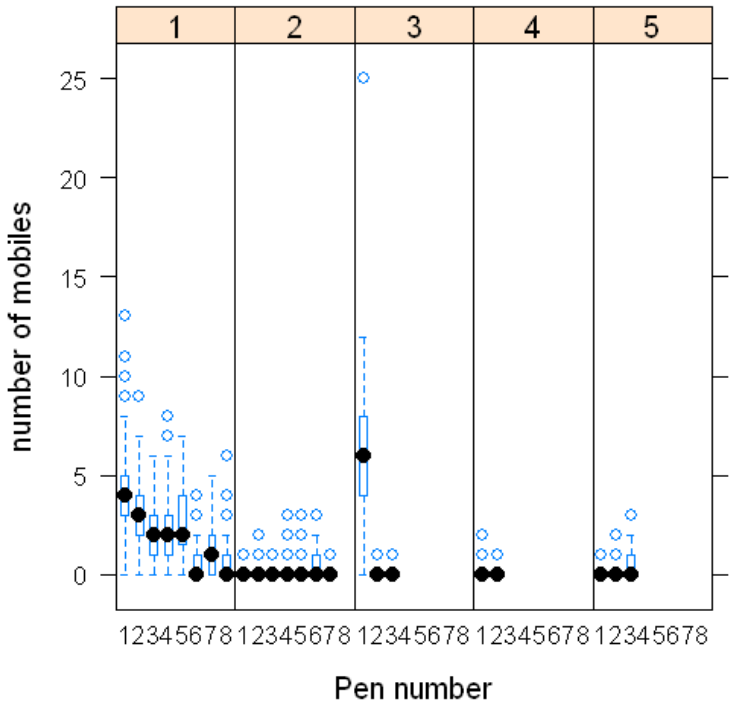

Figure 4. Boxplots of counts of adult females for all pens in each farm. Connecting data for each pen (max and min counts per fish), the box extends from Q1 (first quartile) to Q3 (third quartile). The black dot in the center of the box is the mean abundance and the round blue circles above the line are outliers. Note the wide variation in the means from pen to pen. Counts are for *n*=100 fish per pen.

Result 4.1.2:

The variation in sea lice counts is larger between fish in different pens than for fish within the same pen. This is here shown for first time in the case of farms with large pens (circumference 120-157 metres).

4.1.3. *Causes of variation in the level of sea lice infection between pens*

The causes of variation have a temporal and spatial component. The temporal component is explained by differences in the time for stocking fish in the sea for one production cycle. The spatial component is due to the exposure to different levels of risk factors as a result of the geographical position of the pen in the farm. In this section, we have not evaluated the spatial effects since this is beyond the scope of this study and little or no data on this issue were available.

In Figures 5 and Figure 6, we show that there is a positive association between stocking time and the level of infection (i.e. mean abundance of mobiles and adult females). Since the exact date of fish stocking was not available for all farms, we have selected the average weight of the fish as a proxy for the length of stocking time and plotted against mean abundance of sea lice. Pens with large fish had higher mean abundance compared to other pens with smaller fish. However, the increase in mean abundance relative to fish weight varied among farms most likely as a result of differences in the exposure to other risk factors (i.e. treatments).

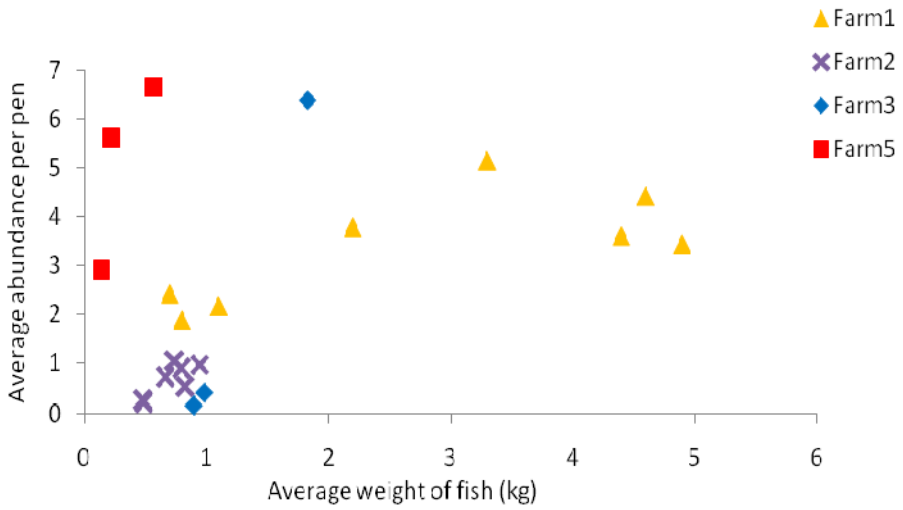

Figure 5. Relationship between mean abundance of mobile sea lice and fish weight (kg) in different pens in four farms.

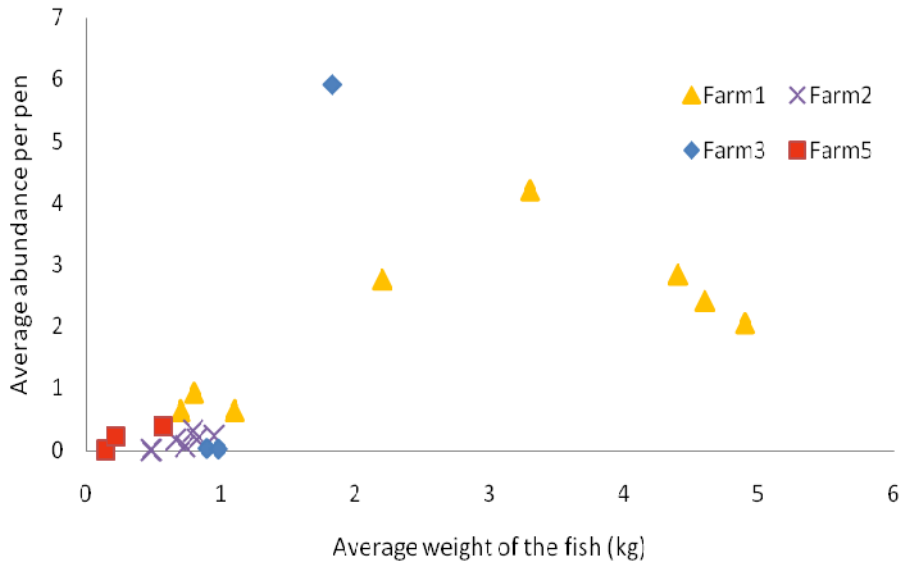

Figure 6. Relationship between mean abundance of adult female sea lice and fish weight (kg) in the different pens in four farms.

Result 4.1.3:

Differences in the time of stocking fish can partly explain the variation in sea lice counts between pens. Variance has a direct impact on sample size so the sampling strategy should target the component with higher variance. The total numbers fish sampled is based on the number of pens and fish counted per pen. So, by sampling more pens relative to fish, the variance is reduced and estimates of mean abundance improved.

4.2. Sample size

4.2.1. *Calculation of sample size*

Counts of mobile and adult female lice collected in the farms were used to calculate the ideal sample size. The sample size is dependent on the variation in the population. For example, if we have a population of 100 fish and 100% of the fish are infected with one louse, we will only need to sample one fish to determine the "true" mean abundance of sea lice (100 lice/100 fish=1 louse per fish). But if we have 100 fish and 50% of the fish had no lice while the other 50% of fish had two lice, we would need more than one fish to calculate the mean abundance (i.e. by sampling one fish we will get a wrong estimate of the mean abundance: either 0 or 2). In our tests, coefficient of variation (CV) at farm level (Table 7) varied from 0.7 to 3.5.

Table 7. Variance calculated with the coefficient of variation (CV*) at farm level for mobile and adult female lice. An=100 fish were counted in each pen.

Stages	Farm 1	Farm 2	Farm 3	Farm 4	Farm 5
Mobiles	0.79	1.28	1.51	0.67	0.64
Adult females	0.95	2.93	1.81	3.49	2.61

*standard deviation was calculated using a bootstrapping technique with r=2000 iterations.

To calculate sample size, we have used three margins of error (10, 20 and 20%) and the range of CV obtained from field data (Table 8). The increase in sample size is proportional to increase in variance (CV). For example, for a CV of 0.7, as the lowest level in our test farms, a sample size of 189 fish will be required to give an accuracy of ±10% (above and below) the true mean in the population. Hence, most sampling protocols will require a large sample size for the lowest CV values obtained from our field data.

Table 8. Sample size in relation to coefficient of variation (CV) in a population, and demands on accuracy to the true value.

	Accuracy		
CV	**10%**	**20%**	**30%**
0.25	16	4	2
0.5	97	25	11
0.7	*189*	*48*	21
1	*385*	*97*	*43*
1.5	*865*	*217*	*96*
2	*1537*	*385*	*170*

Result 4.2.1:

The large variation in sea lice counts requires a sample size that is often impractical (n>100 fish) to use in the field

4.3. Number of pens to sample

To demonstrate the effect of not sampling all pens on a site, we established various groups of pens using data on mobile counts from farm 1. We selected farm 1 because it had many pens. The sampling design includes two groups of 2 and 4 pens. For each group, three sets were made by random combination of pens as shown in Table 9.

Table 9. Pen composition (subgroup) for each set within the group of 2 and 4 pens.

	Set 1	**Set 2**	**Set 3**	**Set 4**	**Set 5**	**Set 6**
Pens	5,7	5,8	1,6	1,2,3,5	2,3,4,7	2,3,6,8

Mean abundance and the 90% confidence intervals of mobile lice were calculated for each set using a sample size of n=100 fish per pen. The results show than none of the estimates of mean abundance for the different subgroups (circle in the center) is the same as the mean abundance (red line) of the farm (8 pens)(Figure 7).

A box plot was made to better illustrate the distribution of lice (Figure 8). For three groups, we observed overlapping between the median abundance of the subgroup with the farm median. This overlapping is explained by the median being a more stable statistic than the mean, as the median is not affected by outliers.

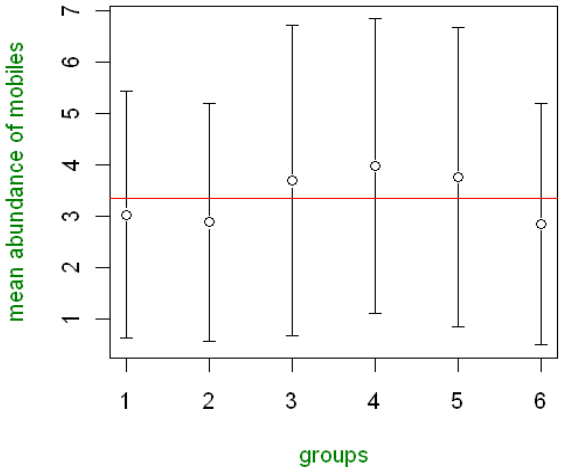

Figure 7. Plot of lice mean abundance on fish and 90% confidence interval of the mean abundances for the each of the 6 replicates. Groups 1 to 3 had two pens and Groups 4 to 6 had 4 pens. The red line is the mean abundance calculated from a sample of 100 fish from all the pens. The 90% confidence intervals were calculated with 2,000 bootstrapping iterations for each replicate.

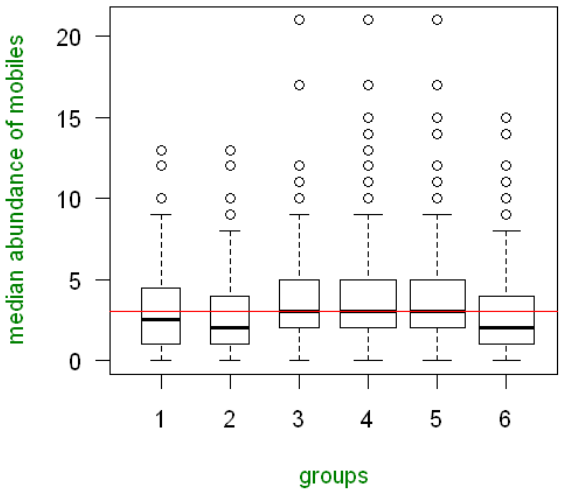

Figure 8. Box plot of the counts of number of mobile lice in each group. The line within the box is the median and the lower and upper sides of the box represent the 25 and 75% percentiles, respectively. The circles are outliers. Groups 1 to 3 had two pens and Groups 4 to 6 had 4 pens. The red line is the median abundance (=3) of all sampled pens in farm 1.

Result 4.3:

Calculation of mean abundances based on one pen or a group of pens will produce very inaccurate estimates of mean abundance as most of the variation is explained by differences between pens. Consequently, in order to make appropriate inferences on mean abundance at farm level it is necessary to use a sample of fish from all pens.

4.4. Relationship between sample size and variance

Previously, we have shown that the total variance has two components; one is the variance between fish in the same pen and the other is the variance among pens. Using data from farm 1, we have examined the relationship between variance and sample size. Figure 9 shows how variance decreases with larger sample size. The largest reduction in total variance (exponential phase) is seen for sample sizes below 10 fish while increments above 10 fish result in a slow linear reduction in variance (linear phase). This means that for every fish examined up to 10 fish, the variance is markedly reduced, but that from additional fish above 10 the variance is not much reduced. At about 20 fish sample size, the curve is almost horizontal showing that 10 more fish will not increase the coverage of the variance appreciably.

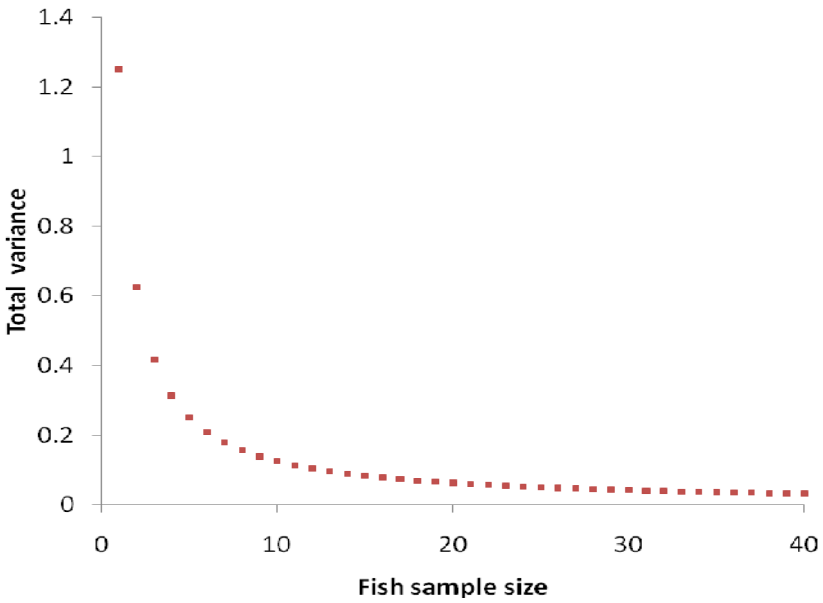

Figure 9. Calculation of total variance in lice numbers calculated by increment in the number of fish sampled per pen. The variance was calculated as the *MSW*/(*n***m*), where *m* is the number of pens, *n* the subsample of fish and *MSW* is the mean square error within pens obtained from an ANOVA table.

Result 4.4:

The largest reduction in variation occurs with increments in sample size below 10 fish. In contrast, the reduction in the variation is negligible for samples larger than 20 fish.

4.5. Calculation of the number of fish per pen using field data

By counting 100 fish per pen in farm 1, the mean abundance was found to be 3.3 for mobiles (counting all pens) and 0.8 for adult females (counting pens 12, 17 and 18). Both slightly exceeded the lowest threshold limits set in the regulations. These numbers were used as "true values" for the farm to evaluate accuracy and precision in mean abundances calculated from different sample sizes. Samples size of *n*=5, 10, 20 and 100 fish were used and confidence intervals were calculated on the basis of a model doing a theoretical 2000 times re-sampling procedure (i.e. catching 5 fish 2000 times) (Figures 10 and 11).

Mean abundance of sea lice for all sample sizes (circle in the center) overlaps with the mean abundance calculated for n=100 fish (accuracy) and the confidence interval gets narrower as sample size increases

(precision). The overlapping in mean abundance is because the mean abundance of each sample size is calculated from 2000 iterations. So, when using a group with a small sample size, the sample mean abundance will approximate the “true” mean abundance.

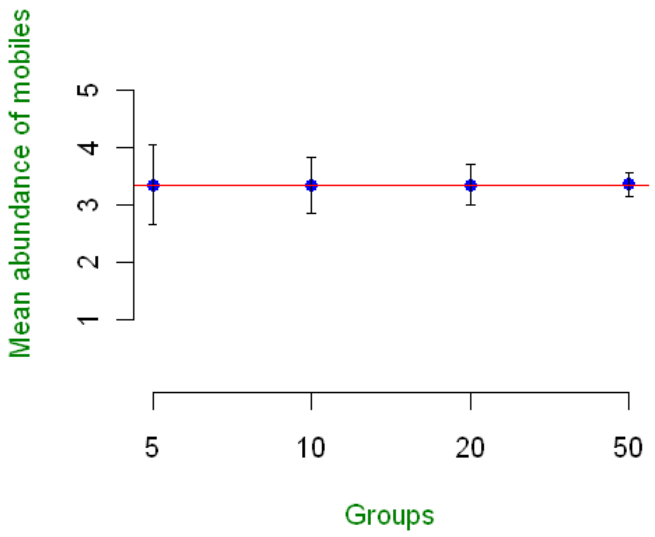

Figure 10. Mean abundance of mobile lice for sample size of n=5 (Group 1), 10 (Group 2), 20 (Group 3) and 50 fish (Group 4) collected from each of the 8 pens sampled. The red line represents the “true” mean abundance=3.36 (3.183-3.549, 90% CI) for mobiles in farm 2. The mean abundance and 90% CI is calculated from 2000 samples for each sample size group obtained by bootstrapping.

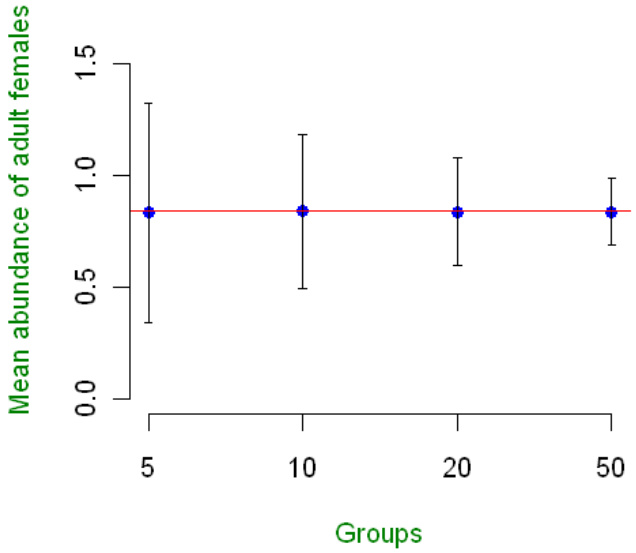

Figure 11. Mean abundance of adult female lice for sample size of n=5 (Group 1), 10 (Group 2), 20 (Group 3) and 100 (Group 4) collected from each of the 3 selected pens. Adult female counts from three pens (pens 12,17,18) were combined to get a mean abundance close to 0.5. The red line represents the mean abundance=0.84 (0.7200-0.9733, 90% CI). The mean abundance and 90% CI is calculated from 2000 samples for each sample size group obtained by bootstrapping.

In addition, we determine the proportion of samplings within each sample size group that would fall outside the calculated 90% confidence interval for the "true" mean abundance. As shown in table 11 and 12, a large proportion of samples within each group (*n*=5, 10, 20) fall outside the 90% CI (i.e. up to 36% in mobiles of *n*=20). A large number of samples fall outside the 90% CI because the CI calculated for a sample of *n*=100 fish with bootstrapping is very small (Table 2). In addition, the distribution of mobiles and adult females are skewed. So, by using a small sample size we are less likely to include fish with more lice in one sampling. For example, in Figure 1, 51% of the fish will have less than 3 mobiles but less than 10% of the fish will have more than 6 mobiles. Assuming that the proportion of fish with more than 5 mobiles is 0.1, then the probability of sampling at least one fish with more than 5 mobiles in each sampling will be: 41% (for *n*=5), 65% (for *n*=10), 87% (for *n*=20) and 99.9% (for *n*=100). It is therefore not surprising that a large number of estimates from samples of small size will fall outside the 90% C.I of the "true" mean abundance of the farm.

We then tested the proportion of samplings where mean abundance was above the threshold limit. For this, we have two scenarios using field data where the "true" mean abundance exceeds the minimum threshold of 0.5 and 3 adult females and mobiles, respectively. The mean abundance of mobiles in all pens in farm 1 is 3.4 (table 10); while by combining three pens (12, 17 and 18) is 0.8 for adult females (table 11). In the case of adult females, we could not create a scenario in which mean abundance of adult females for a farm was closer to 0.5, since in most instances, counts of adult females in each pen were either too small or too big.

We see that for sample size of 10 or more fish, the proportion of sampling below the threshold limits of 0.5 adult females and 3 mobiles is below 10%. This means that for every 100 times we do a sampling of 10 fish, the mean will be underestimated in less than 10% of the estimates. In conclusion, small sample size of at least 10 fish per pen is a reliable indicator that the minimum threshold of mobiles or adult females has been exceeded.

Table 10. Number and proportion of mean abundance estimates calculated from samples sizes *n*=5,10,20 and 100 that are not included within the 90% confidence interval calculated for mean abundance of 3.3 mobiles achieved from counting 100 fish in all pens in farm 1. The number of samplings in each group is 2000.

Group sample size	Number of samples with mean abundance outside 90% CI	% samples with mean abundances outside 90% CI	Number of samples with mean abundance below 3 mobiles	% of samples with mean abundance below 3 mobiles
5	1282	64.1	346	17.3
10	1005	50.3	170	8.5
20	714	35.7	68	3.4
100	60	3	0	0

Table 11. Number and proportion of mean abundance estimates calculated from samples of sizes m=5,10,20 and 100 that are not included within the 90% confidence interval calculated for mean abundance of 0.8 female adults achieved from counting 3 pens in farm 1 (pens 12,17,18). The number of samplings in each group is 2000.

Group sample size	Number of samples with mean abundance outside 90% CI	% samples with mean abundances outside 90% CI	Number of samples with mean abundance below 3 adult females	% of samples with mean abundance below 3 adult females
5	1279	63.95	224	11.20
10	1006	50.30	51	2.55
20	781	39.05	3	0.15
100	109	5.45	0	0

Result 4.5:

Using a small sample size gives an unacceptably large number of wrong estimates of "true" mean abundance. However, mean abundance calculated from counting at least 10 fish from each of all pens on a site gives a valid estimate for judging whether the site is exceeding the maximum threshold of mobiles or adult females set by current Norwegian regulations.

4.6. **Cost benefit analysis**

The goal of any sampling strategy is to get quality data (precision) at the least cost and inconvenience. However, as accuracy and precision increases with larger sample size, cost will do as well. The inflexion point is reached when the cost of sampling is balanced by the acceptable level of precision.

The relative net precision (RNP) is calculated to measure the efficiency of the sampling method (6). Relative net precision considers both the precision of the sampling method and its cost, commonly it terms of labor time for sampling. RNP is calculated as the inverse of the product of the cost to process (C) times the mean relative variation RV_m). In this example, it was calculated as: $RNP=100*(1/RV_m*C)$. Where RV_m is calculated as the ratio of the standard error (SE) by the mean and multiplied by 100.

The economical cost of sampling fish was measured as the total amount of time used for sampling procedures (Table 12). The time spent for counting pre-adult and adult sea lice on a fish depends on several factors. The two critical factors are fish weight and abundance of sea lice. We have estimated that it will take approximately 15-30 seconds to count individual fish of small size (average weight 2 kg or less) and low infections (up to 5 sea lice per fish); while larger fish (average weight 2 kg or more) or high infections (5 or more sea lice per fish) will require 45-90 sec. We have used a conservative approach and chosen for our calculation the longest time of 30 and 90 sec, respectively. Other factors influencing the duration of sampling have not been considered, such as experience of the personal and the type of sea lice stages present (i.e. counting chalimus larvae is very time consuming).

Table 12. Time used in the different steps of the sampling/counting procedures.

Procedure	Length of time (minutes)
Moving between pens	3
†Setting and unsetting of seine, crowding fish to the hand netting of 5 fish into a tank with anesthetic	20
†Placing 5 fish in tank with anesthetic bath until fish is anesthetized	5 (5 min for 5 fish)
‡Counting preadult and adult sea lice	0.5 (fast) and 1.5 (slow)

†The number of fish in the tank with anesthetic bath will depend on the size of the tank and the size of the fish
‡Varied depending on the level of experience of individual, number of stages included, size of the fish, and level of infection

The total cost in minutes for sampling 5 fish in a pen with small fish and low mean abundance of sea lice is calculated as: 20(setting) + 5 (fish) *[1(anesthesia] +0.5 (counting)]=27.5 minutes; assuming a farm with 4 pens the total cost is 3*3 +(27.5*4)= 119 minutes.

For the applied sample sizes, maximum relative net precision is achieved with sample sizes between 5 and 20 fish per pen; while relative net precision decreases for sample size beyond 20 fish (Table 13, Figure 12). The optimal sample size gets smaller as more time is spent for sampling individual fish (Table 14, Figure 13). Note that the scale is different in figures 12 and 13. For sample sizes larger than 10, the decline in relative net precision is larger when it takes longer to sample individual fish.

Table 13. Relative net precision for all pens in farm 2 (*m*=7 pens) where the mean abundance of sea lice (pre-adult and adult) is below 1 and the average fish weight in the farm is below 1 Kg.

Number of fish sampled per pen	Mean (y) abundance of mobiles +adult females	SE (y)	Cost to sample one farm	Relative net precision
5	0.96	0.58	238	0.069
10	0.96	0.41	298	0.078
20	0.96	0.29	418	0.077
30	0.96	0.24	538	0.074
40	0.96	0.21	658	0.069
50	0.96	0.18	778	0.065

Table 14. Relative net precision for all pens in farm1 (*m*=8 pens) where the mean abundance of sea lice (pre-adult and adult) is above 5 and the average fish weight in the farm is above 3 Kg. Relative net precision assuming mean abundance of sea lice is high (equal or larger than 5 sea lice per fish).

Number of fish sampled per pen	Mean (y) abundance of mobiles +adult females	SE (y)	Cost to sample one farm	Relative net precision
5	5.45	1.68	249	0.130
10	5.45	1.19	336	0.135
20	5.44	0.84	511	0.126
30	5.46	0.59	686	0.114
40	5.46	0.53	861	0.105
50	5.43	0.38	1036	0.098

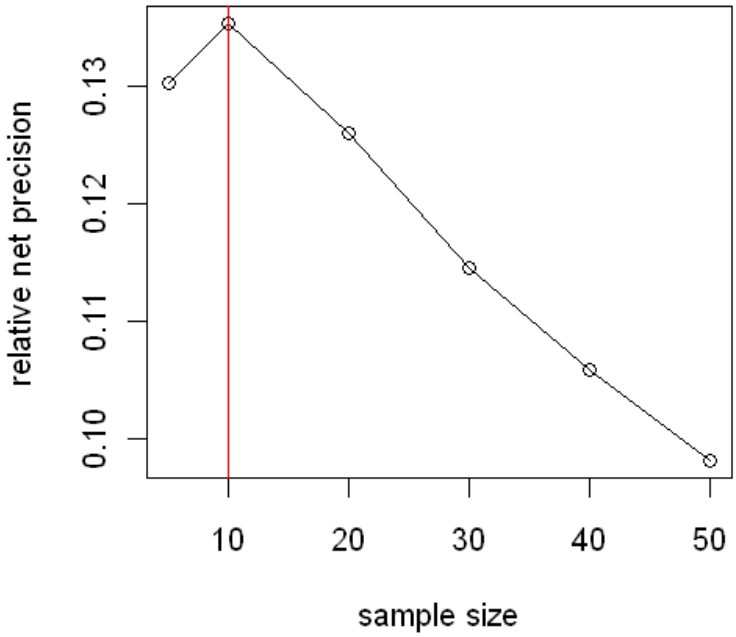

Figure 12. Change in the relative net precision relative to the sample size (*n*=5,10,20,30,40 and 50). Assuming that time for counting lice on individual fish is relatively short because of low level of infection and weight of the fish.

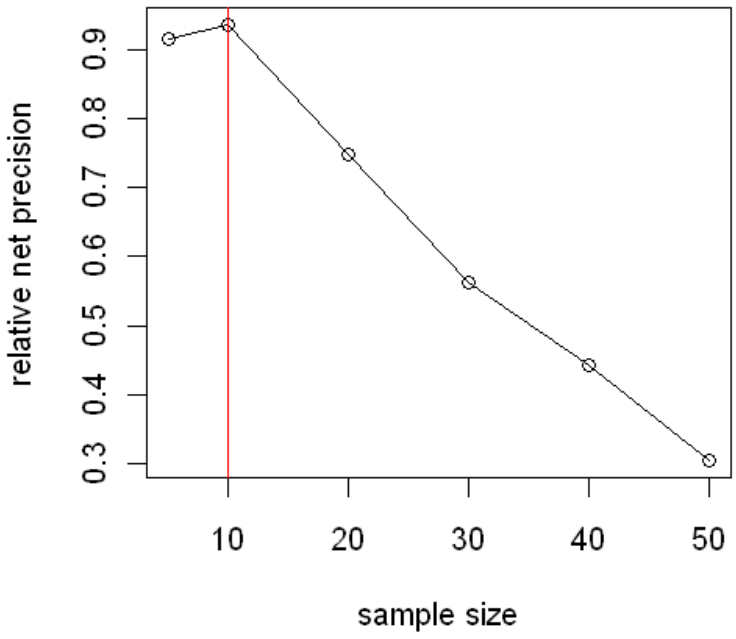

Figure 13. Change in the relative net precision relative to the sample size (*n*=5,10,20,30,40 and 50). Assuming that time for counting lice on individual fish is relatively long because of the high level of infection or weight of the fish.

Result 4.3:

By evaluating time spent on sampling/counting versus precision, we have shown that a sample size above 20 fish results in an excessive cost (time consumption) relative to the increase in precision gained.

5. Glossary

Accuracy (nøyaktighet): The degree of closeness to the true value in the farm compared the calculated estimate of a random sample from a group of fish.

Bootstrapping: Bootstrapping is a way of testing the accuracy of the sample estimates. It consist in the creation of pseudoreplicate datasets by randomly resampling the original dataset.

Coefficient of variation (CV) (Variasjonskoeffisient): The ratio of the standard deviation to the mean multiplied by 100. All variance, standard deviation and coefficient of variation are measures of dispersion of data points around the mean. Hence, the CV is a useful statistic for comparing the degree of variance from different data.

Intracluster correlation coefficient (intracluster korrelasjonskoeffisient): A measure of the relatedness of clustered data.

$ICC = \frac{Var_b}{Var_t}$; $Var_b = \frac{(n-1)*(MS_{between} - MS_{within})}{m*n}$ Var_b is the variance among pens and Var_t is the total variance (variance among pens and within pens) and MS is the mean square error.

Mean (gjennomsnitt): It is a type of average calculated by summing all the counts of sea lice and dividing by the number of fish counted.

Mean abundance (gjennomsnittlig abundans): Average number of sea lice in a group of fish.

$\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n_1}$ x_i is the parasite counts for fish *i* and *n* is the number of fish.

Median (Median): The middle value in an ordered range of values.

Precision (presisjon): How well the observed values (number of lice in a sample of fish) agree with each other. This is a measure of the repeatability.

Prevalence (prevalens): The percentage of fish with one or more sea lice. Prevalence=100*(n/N), where N is the total number of fish and n is the number of fish with lice.

Quartiles (kvartiler): Quartiles separate a quarter of data points from the rest. This roughly means that the first quartile is the value under which 25% of the data lie and the third quartile is the value over which 25% of the data are found. Half of all the values are between the 1st and the 3rd quartile.

Sample size (utvalgsstørrelse): The number of fish in the sample.

Seine (orkastnot): A fishing net made to hang vertically in the water by weights at the lower edge and floats at the top. Pulled in by ropes, one in each corner, to catch the fish.

Standard deviation (standardavvik): Measure of variance and diversity used in statistics and probability theory. It measures the amount of variation of dispersion from the average (mean or expected values). Low variance indicates that data are close to the mean while high variance indicates that data are spread out.

Variance (Varians): Measure of variation and diversity. The variance is computed as the average square o deviation of each number from its mean. The variance is the square of the standard deviation (Var=sd^2).

Variance to mean ratio (VMR) (Varians-gjennomsnitt ratio): It is a measure of dispersion used to characterize the distribution of objects in the space (i.e. sea lice in fish). When the distribution is random then the VMR equals one meaning that the distribution of sea lice on fish is random; while VMR greater than one indicates that some fish are more likely to have sea lice than others.

VMR=$\frac{s^2}{\bar{x}}$ $s^2$is the variance of the sample and the $\bar{x}$ is the average number of sea lice per fish.

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7. Acknowledgements

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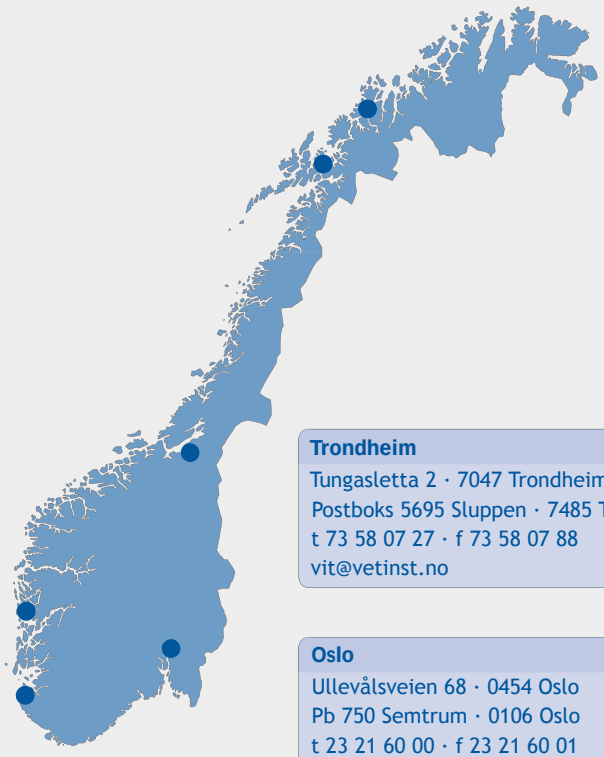

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Rapport
Oppdrag 1768

VESO Oslo Veterinary science opportunities

Følsomhet hos lakselus, standardisering og kvalitetssikring av bioassay

Sluttrapport 2011

Tittel: **Følsomhet hos lakselus, standardisering og kvalitetssikring av bioassay**	
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Utarbeidet av: VESOs hovedkontor, Oslo	Oppdragsgiver: Veterinærinstituttet, Oslo
Prosjektnummer: 1768	Oppdragsgivers referanse:
Prosjektleder: Sigmund Sevatdal	Kontaktperson: Peter Andreas Heuch

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Stikkord: Lakselus, *Lepeophtheirus salmonis*, resistens, bioassay, standardisering	

Engelsk sammendrag:
Standardized protocols for testing field sampled sea lice have been developed, The new protocols are based on the sensitivity of the different stages (pre-adults and adult males), and the need of specifying procedures detected during inspection of fish health laboratories.

The data collected regarding EC_{50} (Effective concentration inactivation 50 %) and treatment efficacy are too limited to investigate the threshold values to distinguish between sensitive and reduced sensitivity. It is important to collect more data of better quality. This work should continue.

Norsk sammendrag:
Nye standardiserte protokoller som er bedre tilpasset lakselus samlet inn i felt, er skrevet på bakgrunn av følsomheten til de ulike stadiene og etter behov for spesifisering av prosedyrer avdekket under kvalitetssikringsarbeidet.

Det er for lite datagrunnlag (EC_{50} og behandlingsresultat) til å vurdere om grenseverdiene som skiller mellom følsom og nedsatt følsomhet/ resistens, er bestemt riktig. Det er viktig å få flere data og bedre data til vurdering. Dette arbeidet bør derfor fortsette.

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Følsomhet hos lakselus, standardisering og kvalitetssikring av bioassay.

Sluttrapport 2011

Sigmund Sevatdal, mai 2011

Innhold

1. Innledning

1.1.Resistens

Den klassiske definisjonen på resistens er følgende: ”Utvikling av individer (populasjon) som overlever doser som er dødelige for majoriteten i en normal populasjon”. Et individ er ikke resistent før resistensratio (målt følsomhet / kontroll følsomhet) er økt 10 ganger. Dersom resistensratio er lavere, er det et tilfelle av nedsatt følsomhet. En annen definisjon på nedsatt følsomhet og resistens, er at ved nedsatt følsomhet kan effekt oppnås ved å øke dosene, mens ved resistens hjelper det ikke lenger å øke dosene. Dosene til pyretroider (deltametrin – AlphaMax og cypermetrin – Betamax) kan ikke økes mer enn ca 25 % før det oppstår toksiske effekter på fisk.

Årsaken til resistens er genetisk. Gjennom seleleksjon endres frekvensen av ett eller flere alleler som gir resistens i en populasjon (allel = variant av et gen). Dette betyr ikke at samtlige individer i populasjonen blir mindre følsomme, men at det blir en høyere frekvens av individer med redusert følsomhet. I litteraturen er det beskrevet andre typer resistens, som a) adferd, b) induksjon og c) endring av egenskapene til cuticula. Adferd: Denne typen resistens forutsetter at organismen kan smake / lukte eller på en annen måte oppdage behandlingsmiddelet og forandrer adferd for å unngå skadelig eksponering. Når det gjelder lakselus kan dette teoretisk være mulig med orale midler. Lakselus kan slutte å beite på laksen i perioder, eller flytte seg til laks som ikke spiser for å unngå eksponering. Induksjon: Lakselus kan regulere følsomhet når den utsettes for et lakselusmiddel, eksempelvis økt avgiftning med metabolisme ved å produsere mer enzym som detoksifiserer behandlingsmiddelet. Cuticula: Egenskapene, dvs gjennomtrengelighet, forandres slik at lakselusmiddelet ikke trenger like lett gjennom som tidligere. Ingen av disse mekanismene er dokumentert hos lakselus.

1.2 Bioassay

Et bioassay er enhver test som utføres på levende organismer. I dette tilfellet bestemmes følsomhet til lakselus ved å eksponere lakselus for forskjellige doser behandlingsmiddel med påfølgende registrering av effekt (respons). Følsomhet beregnes ut fra dose – respons kurven (probit analyse), som EC_{50} (”Effectiv Concentration” – dosen som inaktiverer 50 % av lusene). For å kunne estimere følsomhet er vi avhengige av at testen gir doserespons. Vi må derfor velge konsentrasjoner som gir lav respons ved lave doser, og høy respons ved de høyeste dosene.

Bioassay med lakselus ble opprinnelig utviklet for å teste følsomhet hos 1. generasjon laboratorieproduserte preadulte, fordi disse vil være like gamle, ha god kvalitet, det vil være kort tid mellom innsamling og testing, og en tester arvbare egenskaper. Testen blir altså utført med en ”homogen populasjon” som gir et sikkert resultatog relativt liten variasjon. Det har vist seg i praksis at når følsomhet skal bestemmes i felt for å finne riktig type behandling, er det mest praktisk å samle inn lakselus fra anlegget, og teste disse direkte. Produksjon av 1. generasjon lus på lab. er kostbart, risikofylt og tar lang tid. Kapasiteten er også begrenset (Det ble utført over 400 bioassay i 2009). Testing av direkte innsamlede lakselus gir andre utfordringer. Det er stor variasjon i det innsamlede materiale, god og dårlig kvalitet. Tiden fra innsamling til testing kan også variere. Det er tider på året når innsamling er ugunstig pga lav- eller høy temperatur. Det største problemet er som regel å finne nok preadulte lus. Antallet bevegelige lus pr fisk er sjelden høyere enn 0,5. Dette gjør innsamling arbeidskrevende, og resulterer i at adulte hannlus blir benyttet sammen med preadulte. Dette har ikke store konsekvenser for pyretroider og azametifos, fordi preadulte og adulte hannlus har lik følsomhet. Følsomhet for emamektin benzoat er imidlertid forskjellig for preadulte og adulte

hannlus. Det innsamlede materialet vil på mange måter gjenspeile den populasjonen som skal behandles, dvs samles det inn mye adulte lus, betyr det at det er mye av dette stadiet på anlegget, og det er denne lusa som skal behandles. I dette prosjektet er det laget nye protokoller som er bedre tilpasset testing av lakselus innsamlet i felten. Protokollene er også skrevet på bakgrunn av behovet for bedre spesifisering av prosedyrer, noe som ble avdekket ved kvalitetssikring av fiskehelselaboratoriene.

Det som leveres i dette prosjektet er

- Nye protokoller som er bedre tilpasset testing av feltinnsamlede lakselus, inkludert beskrivelse av riktig innsamling og gjennomføring av bioassay .
- Kvalitetssikring av fiskehelselaboratorier
- Kurs/ seminar med ringtesting
- Sluttrapport.

2. Kvalitetssikring av fiskehelselaboratorier

Det har blitt utført en runde med kvalitetssikring av de fiskehelselaboratoriene som utfører bioassay i Norge. I perioden oktober 2010 t.o.m. mars 2011 ble totalt 9 laboratorier besøkt og kvalitetssikret ved at minst ett bioassay ble gjennomført og eventuelle avvik fra protokoll ble registrert. To fiskehelsetjenester, Slab på Stord og Havlandet Forskningslaboratorium i Florø er besøkt tidligere, og derfor ikke prioritert i denne omgang. Det ble holdt et seminar i Bergen (22 – 23. september 2010) med representanter fra samtlige fiskehelselaboratorier (unntak: Havlandet). Det ble også utført en ringtesting 28. – 29. mars 2011, der lakselus fra samme anlegg ble testet under like forhold av Slab, AquaLab og AkvaVet Gulen. Ringtesten ble organisert pga. forskjellig resultat for følsomhet for emamektin fra samme anlegg. Resultatene fra ringtesten viste sammenlignbare resultater. Både seminaret og ringtesten ble organisert i samarbeid mellom dette prosjektet og Intervet Norbio som betalte reise og opphold for kursdeltagerne. De gjorde dette fordi det gjaldt testing av følsomhet for emamektin benzoate (Slice). En oversikt over fiskehelselaboratoriene i Norge og oppfølging mhp følsomhetsmålinger er vedlagt (vedlegg 1).

Etter hvert kvalitetssikringsbesøk ble det skrevet en rapport som beskrev hva som var i henhold til protokoll, og eventuelle avvik. Denne rapporten ble kun distribuert til det aktuelle fiskehelselaboratoriet. Et kvalitetssikringsbesøk som dette bygger på tillit, og offentliggjøring av avvik kan redusere tilliten. Fiskehelselaboratoriene har ikke plikt til å delta, og besøket ble derfor utført under forutsetning av at resultatet ikke skulle offentliggjøres.

2.1. Typiske avvik

Under kvalitetssikringsarbeidet ble det registrert at en rekke avvik. De mest typiske var:

- Ikke standardisert vann, dvs. ofte vann fra lokaliteten der det ble samlet inn lakselus. Egenskapene til den aktive substansen i lakselusmiddelet kan variere med salinitet og innhold av organisk materiale i sjøvannet.
- Mye forskjellig utstyr benyttes til fortynning av vann. Det er fortsatt usikkerhet om dette har noe å si mht adhesjon av den aktive substansen til overflaten av ulike typer plast etc.
- Volum under fortynning varierte også, dvs flere benyttet 500 ml som fortynningsvolum. Dette er et direkte avvik, fordi protokollene som har vært benyttet sier at det skal fortynnes i volum på 1 liter.
- Utvelgelse av individer til testing ble hos noen utført ved at én og én gruppe ble fylt opp, noe som kan medføre at de siste gruppene består av lus med variabel kvalitet.

- Responskriteriene for evaluering er for dårlig spesifisert i eksisterende protokoller. Dette har medført at respons er blitt tolket ulikt. Nye og bedre responskriterier er beskrevet i de nye protokollene, vedlegg 2, 3 og 4.

Prof. Einar Horsberg og Kari Olli Helgesen (NVH, Seksjon for Farmakologi og Toksikologi) har undersøkt adhesjon av emamektin til forskjellige materialer. De fant ut at glass og polystyren binder lite emamektin sammenlignet med polypropylen og polyetylen i 24 timers eksponeringer. Det gjenstår å undersøke hvordan dette forholder seg for de andre behandlingsmidlene (pyretroider og organofosfat), og om det er av betydning ved den forholdsvis korte tiden som brukes under fortynning, og eksponering i henholdsvis 30 og 60 min.

3. Følsomheten til ulike stadier

Følsomheten for pyretroider hos aktuelle stadier av laboratorieproduserte lakselus er undersøkt tidligere (Sevatdal 2005), og av Havforskningsinstituttet (Per Gunnar Espedal, pers. med). Det er konkludert med at preadulte hunnlus og adulte hannlus som er like gamle, har lik grad av følsomhet for pyretroider og emamektin benzoat. Hanner utvikler seg noe raskere enn hunner, slik at det i en kultur som er startet med like modne egg vil være utviklet adulte hannlus samtidig som preadult II hunnlus.

For å utvikle bedre protokoller tilpasset lakselus samlet inn i felt, ble det utført en større analyse av eventuelle forskjeller i følsomhet mellom de aktuelle stadiene – preadulte lus (preadult I + preadult II) og adulte hannlus.

Dette ble undersøkt for å finne ut om det var behov for å skille mellom preadulte og adulte hannlus ved testing. Dersom det er vesentlig forskjell i følsomhet bør stadiene ikke blandes i gruppene, men testes hver for seg.

Følsomheten til de ulike stadiene som er benyttet til bioassay med feltinnsamlede lus er undersøkt for pyretroider (AlphaMax - deltametrin, Betamax – cypermetrin), azametifos (Salmosan) og emamektin benzoat (Slice). Undersøkelsen baserer seg på resultater fra felt. Flere fiskehelselaboratorier har spesifisert stadiene (preadult II og adulte hannlus), og hvilke respons disse hadde. Dette gjør det mulig å sammenligne stadiene med et stort datamateriale ved å slå sammen respons til preadulte i mange tester og sammenligne respons med de tilsvarene adulte hannene i de samme testene. Den statistiske sammenligningen er gjort med probit analyse (PoloPlus, http://www.leorasoftware.com) Antallet tester og totalt antall lus er beskrevet i tabell 1.

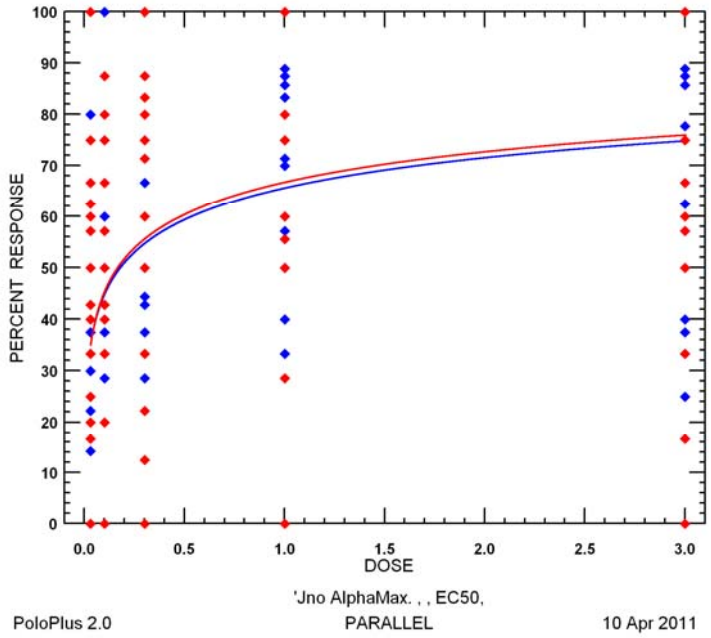
3.1. Resultater

Tabell 1. Antall tester og lus benyttet for sammenligning, og forskjell i følsomheten til preadulte og adulte hannlus.

Substans	Antall tester	Tot. antall lus Pread	Adulte	Sign. forskjell ($p \leq 0.05$)
Deltametrin (AlphaMax)	12	757	645	p = 0.213
Cypermetrin (Betamax)	16	849	965	p = 0.002
Emamektin benzoat (Slice)	10	1081	1333	p = 0.000
Azametifos (Salmosan)	13	675	823	p = 0.069

3.1.1. Deltametrin – AlphaMax

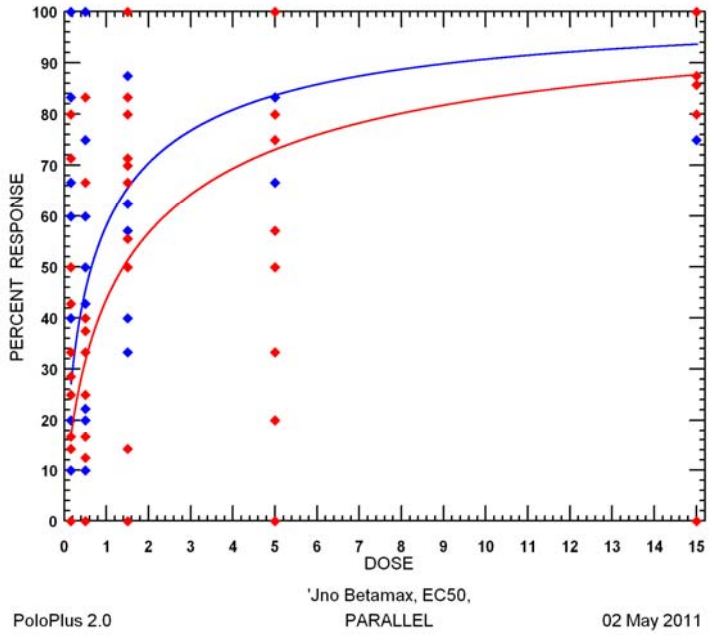
Det ble ikke funnet signifikante forskjeller mellom preadulte og adulte hannlus for deltametrin (tabell1.). Resultatet er vist som figur 1.



Figur 1. Dose respons kurver til preadulte (blå) og adulte hannlus (rød) for deltametrin (AlphaMax). Det er benyttet lineær skala på x-aksen.

3.1.2. Cypermetrin - Betamax

Det ble funnet en signifikant forskjell mellom preadulte og adulte lus mhp cypermetrin (tabell 1). Dette var uventet og ble undersøkt nærmere ved å skille mellom typiske følsomme populasjoner og populasjoner med nedsatt følsomhet. Det viste seg at det var en forskjell i følsomhet mellom stadiene for følsom lakselus, men ikke for nedsatt følsomhet (tabell 2). Dette betyr at det ikke er nødvendig å skille mellom stadiene på samme måte som for emamektin benzoat, ved testing. Resultatene er vist som figur 2, 3A og 3B, og tabell 2.

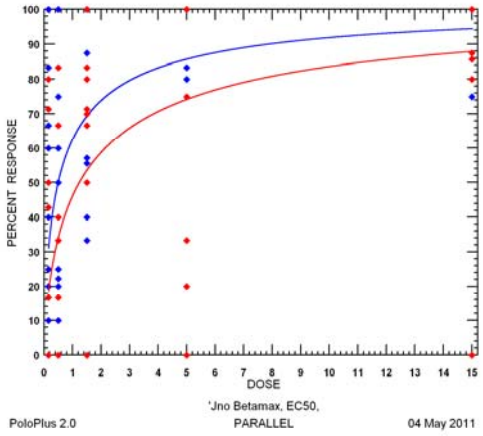


Figur 2. Dose respons kurver til totalt preadulte (blå) og adulte hannlus (rød) for cypermetrin (Betamax). Det er benyttet lineær skala på x-aksen.

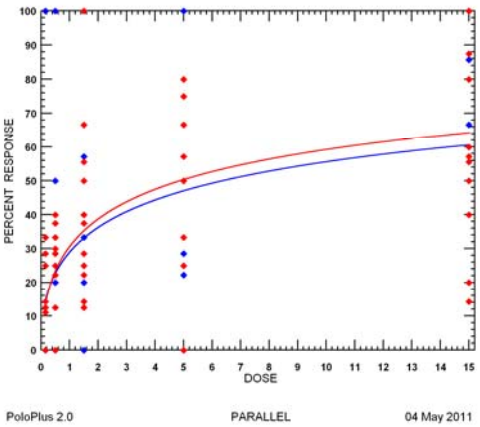
Tabell 2. Antall tester og lus benyttet for sammenligning av følsomheten til preadulte og adulte hannlus for cypermetrin. Følsomme populasjoner er sammenlignet med nedsatt følsomhet.

Type	Ant. tester	Tot. antall lus		EC_{50} (ppb)		Sign. forskj.
		Pread	Adulte	Pread	Adulte	($p \leq 0.05$)
Følsom	8	487	368	0.75	1.32	p = 0.000
Nedsatt følsomhet	8	362	597	6.8	6.3	p = 0.082

A



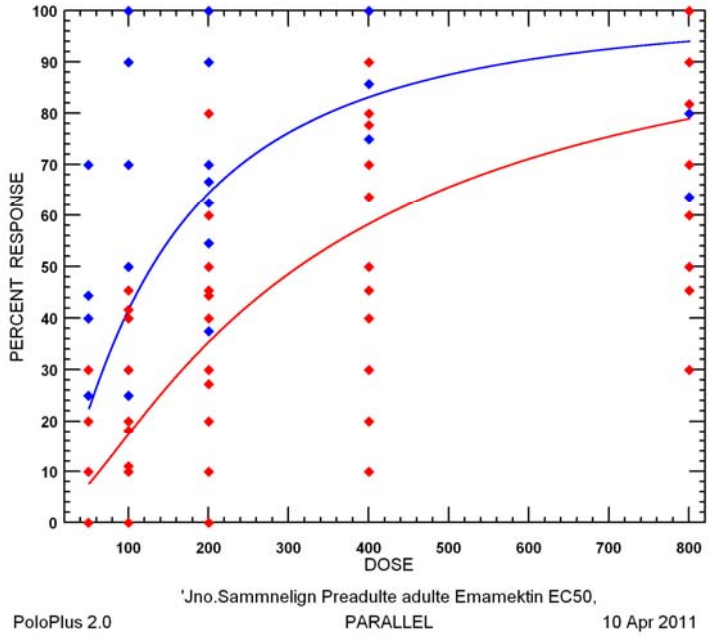
B.



Figur 3. Dose respons kurver til følsomme **A** og nedsatt følsomhet **B** preadulte (blå) og adulte hannlus (rød) for cypermetrin (Betamax). Det er benyttet lineær skala på x-aksen.

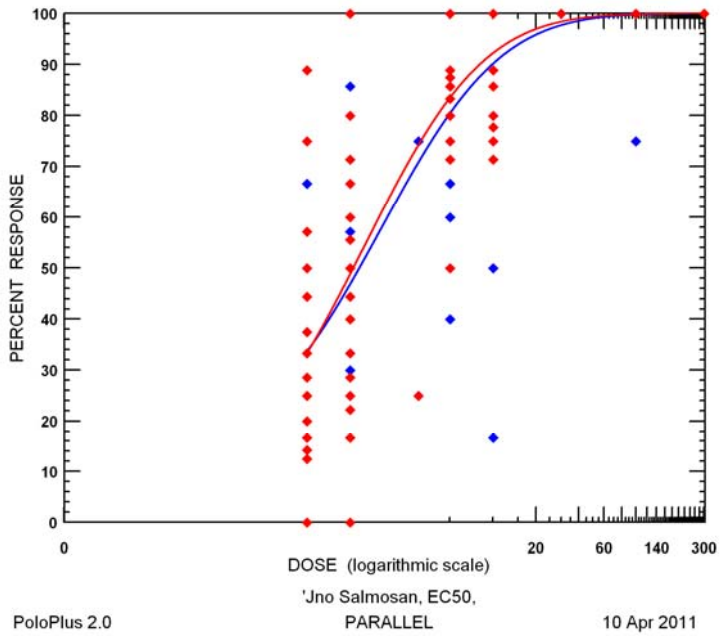
3.1.3. Emamektin benzoat - Slice

Det ble funnet en signifikant forskjell mellom preadulte og adulte lus for emamektin benzoat (tabell 1). Dette var ikke uventet og ble undersøkt nærmere ved å skille mellom typiske følsomme populasjoner og populasjoner med nedsatt følsomhet. Det viste seg at det ikke fantes typiske følsomme populasjoner blant testgruppene. Dette gjør en sammenligning umulig. Testmaterialet totalt representerer derfor en "typisk" nedsatt følsomhet i felten. Det er derfor nødvendig å skille mellom stadiene for emamektin benzoat ved testing. Resultatene er vist som figur 4 og oppsummert i tabell 1.



Figur 4. Dose – respons kurver til preadulte (blå) og adulte hannlus (rød) for emamektin benzoat (Slice). Det er benyttet lineær skala på x-aksen.

3.1.4. Azametifos – Salmosan
Det ble ikke funnet noen forskjell mellom preadulte og adulte hannlus i følsomhet for Salmosan. Resultatet er vist på figur 5.



Figur 5. Dose- respons kurver til preadulte (blå) og adulte hannlus (rød) for Azamethfos (Salmosan). I dette tilfellet er det benyttet logaritmisk skala på x-aksen.

4. Evaluering av EC_{50} verdier og behandlingsresultat.
Det er vanlig at fiskehelsetjenestene tester følsomhet med bioassay før behandling, og behandler deretter med et middel som testen viser god følsomhet for. Det er derfor viktig å sammenligne EC_{50} med behandlingsresultat for å undersøke om bioassay indikerer riktig følsomhet i forhold til en eventuell behandling. Grenseverdiene er bestemt teoretisk med utgangspunkt i hvordan følsomheten var før resistens ble et problem, og etter å ha påvist resistens med bioassay etter en mislykket behandling.

Det er gjennomført en undersøkelse der EC_{50} verdier samlet inn før behandling er sammenlignet med behandlingsresultatet. Et problem med en slik undersøkelse er at det er få høye EC_{50} verdier (over grenseverdien for følsom – nedsatt følsomhet) og tilsvarende EC_{50} verdier. Det er kun i multiresistente tilfeller at det velges å behandle med et middel som har gitt høy EC_{50} verdi.

Å sammenligne EC_{50} verdier med behandlingsresultat kan altså gi svar på om grenseverdiene er riktige, om de eventuelt må justeres og om testing med bioassay gir en riktig indikasjon på følsomhet i forhold til effekt av behandling i felt.

EC_{50} for de ulike midlene er lavere enn behandlingsdose. Dette gjelder bademidler, og ikke emamektin benzoat (Slice) fordi behandlingsdosen er vanskelig å bestemme – den avhenger av dosen som oppnås i fisken, og tiden lusa er eksponert som parasitt på behandlet fisk. Lakselus kan redusere eksponering ved å ikke spise, og ved lav temperatur er aktiviteten lav, da spiser lusene sannsynligvis lite. Det er mye som tyder på at aktiviteten er minimal ved svært lave temperaturer. Grenseverdiene som benyttes i dag er vist i tabell 3.

Tabell 3. Grenseverdier for lakselus i kategoriene følsom, nedsatt følsomhet og resistent.

	Grenseverdier (EC_{50} – ppb)		
Behandlingsmiddel	Følsom	Nedsatt følsomhet	Resistent
Alphamax (deltametrin)	0.1 – 0.29	0.3 – 0.99	> 1
Betamax (cis cypermetrin)	0.5 – 1.4	1.5 – 7.4	> 7.5
Slice (emamektin benzoat)*			
Preadulte	< 100	> 100	> 800
Adulte hannlus	< 200	> 200	> 800
Salmosan (azametifos)	0.5 - 10	10 - 40	> 50

* Tidligere grenseverdi for å skille mellom følsom – nedsatt følsomhet var 200 ppb når andelen hannlus var over 30 % testen

4.1. Resultater

Det har vært vanskelig å få inn resultater fra fiskehelsetjenestene. Det viser seg at disse ikke systematiserer sine data, men var nødt til å gjøre et arbeid i forkant av innsending av data. VESO var nødt til å tilby betaling for datasett for det hele tatt å få inn noe.

Tabell 4. Antall datasett der både EC_{50} og behandlingsresultat er oppgitt.

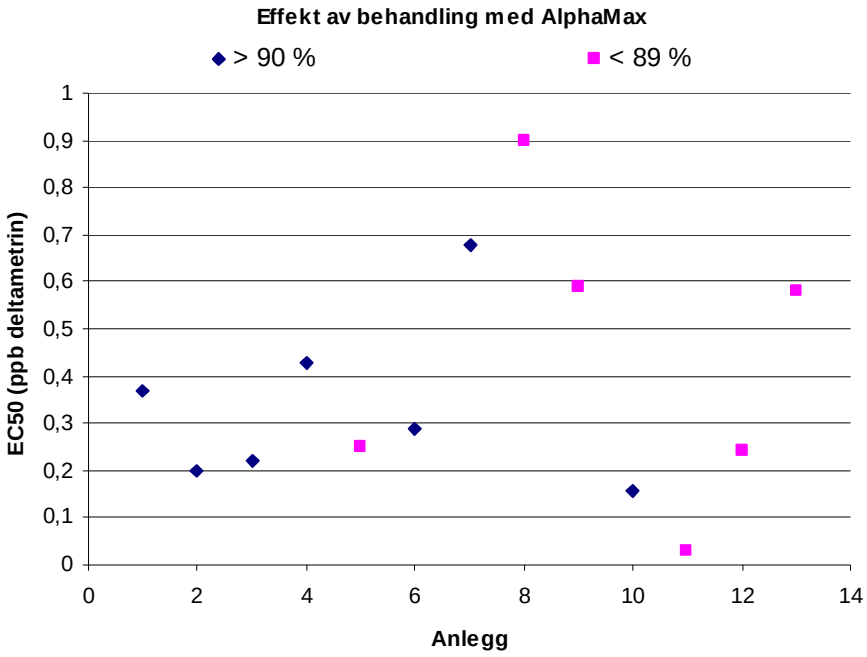
	Antall datasett (EC_{50} + behandlingsresultat)		
Behandlingsmiddel	God effekt (> 90 %)	Redusert effekt (< 89 %)	Merknad
AlphaMax (deltametrin)	7	6	
Betamax (cypermetrin)	5	1	Ikke vist som figur
Slice (emamektin benzoat)	6	12	
Salmosan (azametifos)	24	10	

Det ble ikke alltid sendt inn eksakte tall for behandlingsresultat, og siden det uansett er usikkerhet forbundet med evaluering av effekt, ble de kategorisert på følgende måte:

- God effekt: høyere eller lik 90 %
- Mindre god effekt: lavere eller lik 89 %

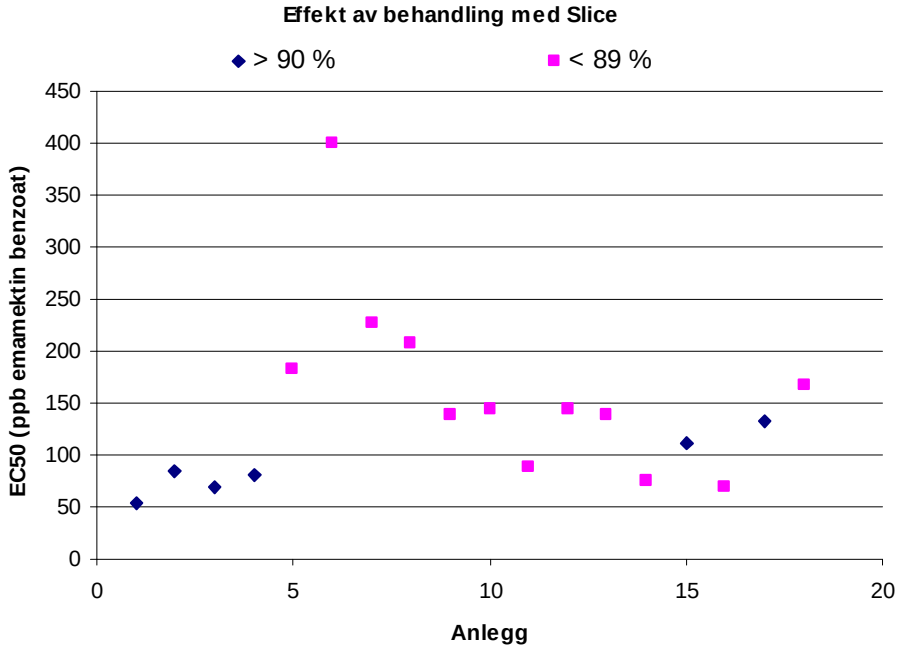
Det ble ikke sendt inn nok data for cypermetrin (Betamax) til å vurdere EC_{50} og behandlingsresultat.

4.1.1. Pyretroider (AlphaMax – deltametrin)



Figur 6. Figuren viser en sammenligning av EC_{50} for deltametrin (AlphaMax) før behandling, og behandlingsresultat klassifisert som: Høyere eller lik 90 % - god effekt, lavere eller lik 89 % - dårlig effekt.

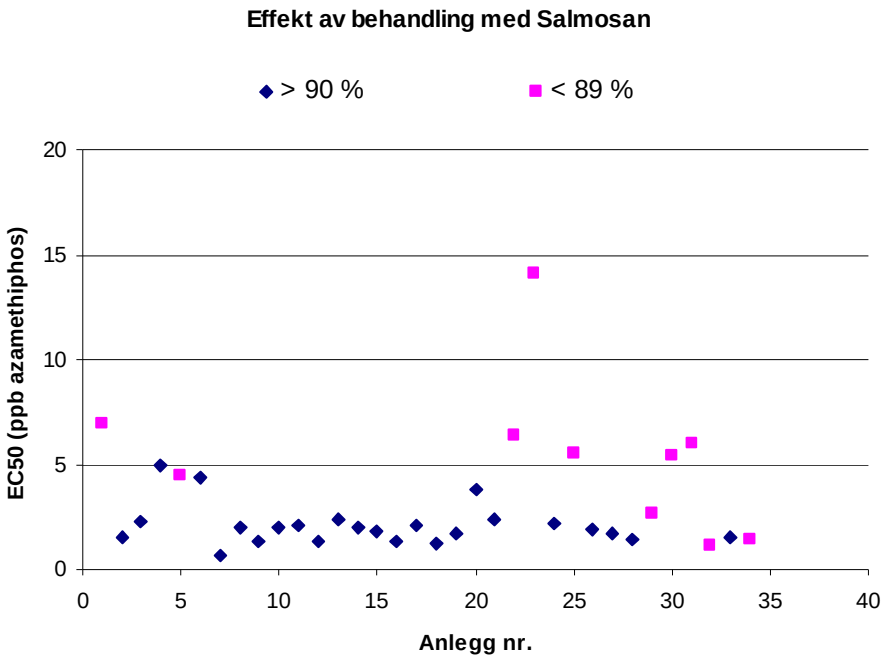
4.1.2. Emamektin benzoat (Slice)



Figur 7. Figuren viser en sammenligning av EC_{50} for emamektin benzoat (Slice) før behandling, og behandlingsresultat klassifisert som: Høyere eller lik 90 % - god effekt, lavere

eller lik 89 % - dårlig effekt. EC50 verdien er beregnet ut fra et samlet materiale, dvs både preadulte og adulte er testet sammen (iht tidligere protokoll).

4.1.3. Azametifos (Salmosan)



Figur 8. Figuren viser en sammenligning av EC_{50} for azametifos (Salmosan) før behandling, og behandlingsresultat klassifisert som: Høyere eller lik 90 % - god effekt, lavere eller lik 89 % - dårlig effekt.

4.1.4 Oppsummering
Det er umulig å trekke klare konklusjoner på grunnlag av innsendte data for pyretroider. Av totalt 13 behandlinger var 7 vellykkede, og 6 var mindre vellykket. Av de vellykkede behandlingene hadde 4 lavere EC_{50} verdier enn grenseverdien på 0.3 ppb, mens 3 (50%) av de mindre vellykkede behandlingene hadde EC_{50} høyere enn grenseverdien.

Resultatet for emamektin benzoat viser at samtlige vellykkede behandlinger (6) hadde lavere EC_{50} enn 150 ppb før behandling. Det var 12 mindre vellykkede behandlinger, og av disse hadde 7 lavere EC_{50} enn 150 ppb, mens 5 hadde høyere enn 150 ppb. Dette kan bety at høy EC_{50} medfører økt risiko for redusert effekt, mens lav EC_{50} ikke er noen garanti for god effekt. Det er nødvendig med et større datagrunnlag for å vurdere grenseverdiene i forhold til ny protokoll.

Resultatet for Salmosan viser at alle vellykkede behandlinger (24) hadde en EC_{50} før behandling som var lik eller lavere enn 5 ppb. Det er 9 mindre vellykkede behandlinger, og av disse hadde 4 lavere EC_{50} enn 5, mens 6 hadde høyere. Dette indikerer at en grenseverdi på 10 ppb for Salmosan kan være noe for høy, og at grenseverdien bør justeres til 5 ppb.

5. Andre årsaker til feil resultat - diskusjon

Kvaliteten på lusa er avgjørende for at testen skal gi en riktig indikasjon på følsomhet. Dersom lakselusene er halvdøde i utgangspunktet, vil eksponering med lakselusmidler resultere i at testen viser god følsomhet pga høy respons uansett. Det er derfor viktig at testen

gjennomføres så kort tid etter innsamling som mulig. Det bør ikke gå lenger tid enn 4 timer fra innsamling til gjennomføring av testen. Det er også viktig at lakselusa oppbevares kaldt, spesielt om sommeren. Lufting av vannet under transport kan være aktuelt dersom det går lang tid mellom innsamling og testing.

Riktige prosedyrer er viktige under innsamling, oppbevaring og transport av lakselus. Dette er spesifisert i de nye protokollene for at lakselusa skal ha så god kvalitet som mulig under testing. De nye protokollene spesifiserer også at det bør være en standard vannkvalitet, akklimatisering av lus til riktig temperatur, prosedyrer ved utvelgelse for å unngå å ta med lus av dårlig kvalitet, og evaluering av respons.

Det er viktig å huske på at en test av denne typen gir kun en indikasjon på følsomhet. Et typisk kjennetegn på forandring i følsomhet er at variasjon øker. Følsomme populasjoner har relativt sett mindre variasjon i følsomhet enn populasjoner med nedsatt følsomhet. Protokollene beskriver 120 individer som standard antall som skal benyttes (NB: I protokollen for emamektin testen er det beskrevet opptil 240 individer, 120 av hvert stadium). Dette er et svært lavt tall i forhold til den totale populasjonen i anlegget. Testene hadde blitt sikrere dersom flere individer benyttes, noe det er full adgang til. Antallet, 120 individer, bør betraktes som et minimum. Lakselus med forskjellige egenskaper blir fordelt tilfeldig i gruppene, og skjev fordelig kan oppstå. Dette vil ha innvirkning på resultatet slik at følsomhetsstatus til populasjonen vil bli beregnet feil.

Det er usikkerhet mht. resultat av et bioassay, men også mye usikkerhet forbundet med evaluering av effekt etter behandling. Evalueringen er ikke standardisert mht. tidspunkt etter behandling. Dette er av vesentlig betydning fordi effekten ofte er forsinket ved lav vanntemperatur. En behandling med Salmosan er ikke effektiv mot fastsittende stadier. Dersom det går tid mellom behandling og evaluering vil fastsittende stadier kunne utvikle seg til mobile stadier, og dermed være en stor feilkilde. Vi har valgt å stole på fiskehelse-tjenestenes vurdering av effekt i dette tilfelle, og har tatt med samtlige datasett som er komplette. På grunn av usikkerhet er en slik vurdering avhengig av et stort tallmateriale (> 50 datasett) for hvert behandlingsmiddel, for å kunne vise en reell trend. Antall resultater som kom inn var ikke så stort for hvert behandlingsmiddel (tabell 4).

Det er andre årsaker enn resistens til at en behandling ikke blir 100 % optimal. Ved orale behandlinger som Slice er en avhengig av at fisken spiser normalt, og at foringsregimet under medisinering gjør at alle fisk får en høy nok dose av behandlingsmiddelet. Dette avhenger av at biomassen er beregnet riktig, at fisken er sortert (lik størrelse) og at andelen tapere eller fisk som spiser dårlig er liten. Ved badebehandlinger er det viktig at dosen beregnes korrekt etter volumet. På dette punktet har praksisen vært ulik. Tidspunkt på året for behandling vil være viktig mht hvor mye organisk materiale det er i vannet. Pyretroider bindes lett til organiske overflater, og behandlingsdosen er svært lav, f. eks. 2 ppb (billiontedeler) i lukket system for AlphaMax. Dersom en stor andel av behandlingsmiddelet er bundet til organiske partikler etc., kan det senke konsentrasjonen så mye at deler av vannvolumet ikke inneholder nok medisin.

6. Oppsummering

Følsomheten til de ulike stadiene er undersøkt for pyretroider (deltametrin – AlphaMax og cypermetrin – Betamax), emamektin benzoat (Slice) og azametifos (Salmosan).

Fiskehelselaboratorier er besøkt og evaluert.

Nye protokoller, som er bedre tilpasset felt innsamlede lakselus, er skrevet på bakgrunn av følsomheten til de ulike stadiene og etter behov for spesifisering av prosedyrer avdekket under kvalitetssikringsarbeidet.

Arbeidet med å samle inn EC_{50} og resultat etter behandling bør fortsette. Dette er viktig for å få flere data til å vurdere grenseverdiene. Fiskehelsetjenestene bør sende inn behandlingsresultat i form av rådata, dvs lusetellinger i en periode på 1 – 4 uker etter behandling, for å få et bedre datagrunnlag.

7. Vedlegg

Vedlegg 1. Fiskehelselaboratorier og oppfølging
Vedlegg 2. Protocol emamectin benzoate (Slice)
Vedlegg 3. Protocol pyrethroids (AlphaMax – deltamethrin, Betamax – cypermethrin)
Vedlegg 4. Protocol azametifos (Salmosan)

8. Referanser

PoloPlus, http://www.leorasoftware.com)

Sigmund Sevatdal (2005) Sea lice resistance to chemotherapeutants: Bioassays as diagnostic tools for determination of sensitivity patterns in sea lice (*Lepeophtheirus salmonis* Krøyer). Thesis for the degree of PhD. Norges Veterinærhøyskole.

Vedlegg 1.

Liste over fiskehelselaboratorier som utfører eller planlegger å utføre testing av følsomhet med bioassay (Personer og kontaktinfo i uthevet skrift var på bioassay-kurs 22- 23. september 2010).

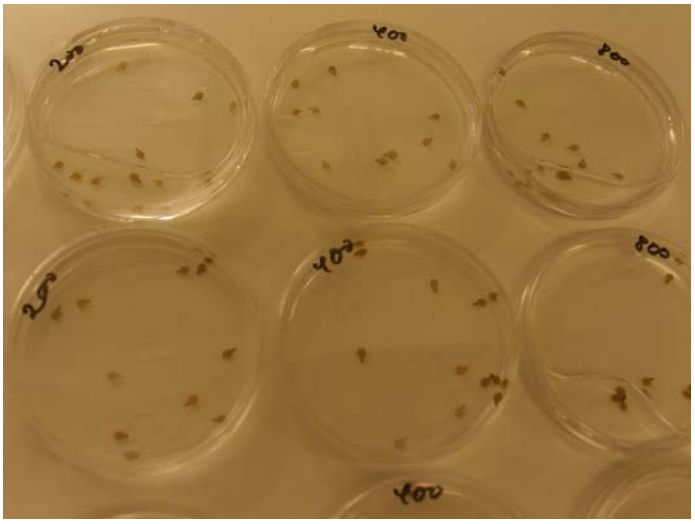
Lab	Adresse	Kontaktperson	Område	Kompetanse (følsomhetstesting)	Opplært/ Besøkt	Kval.sikret
Slab	Slab Borggata 26 5417 Stord	Oddmund Emmerhoff 40 29 68 75 oddmund@slab.no **Nataliia Hansen** **97 51 72 40** **nataliia@slab.no**	Rogaland Hordaland sør	Pyretroider Emamektin benzoat Salmosan	Nov 2009 Sept 2010	Des 2009 Ringtest 28 – 29. mars 2011
FOMAS	Fiskehelse og Miljø (avd. Tysnes) Malkenes 5690 Lundegrend	**Stine Kolstø** **97 60 05 71** **stine@fom-as.no**	Hardanger Bømlo	Utfører ikke bioassay	Sept 2010	
Aqua-Lab	Aqua-Lab AS Eidsvågsveien 150 5105 Eidsvåg i Åsane Bergen Tlf: 55 32 20 15 Fax: 55 31 28 86	Hans Aase fiskehelse@aqualab.no 92 26 16 85 **Kennet Aasen** **55 32 20 15** **fiskehelse@aqualab.no**	Hordaland	Pyretroider Emamektin benzoat Salmosan	Nov 2008 Sept 2010	8 - 9. desember 2010 Ringtest 28 – 29. mars 2011
AkvaVet Gulen	AkvaVet Gulen AS 5961 Brekke Tlf: 577 85 497	Aud Aasheim post@akvavet.no **Silje Sveen** **40 61 60 13** **post@akvavet.no**	Sogn og Fjordane	Pyretroider Emamektin benzoat Salmosan	Nov 2008 Sept 2010	13 -14. januar 2011 Ringtest 28 – 29. mars 2011
Havlandet Forsknings-laboratorium AS	Postboks 5, 6901 FLORØ Besøksadresse: Flora Næringshage, Trolleskjeret i Gunhildvågen Tlf: 90 61 80 71 Fax: 57 74 55 21 E-post laboratorium: hfl.lab@inc.sf.no	Kristin Bergstøl Kristin.bergstol@inc.sf.no	Florø Sunnfjord	Pyretroider Emamektin benzoat Salmosan	Besøk Jan 2010	
Fjord-Lab AS	Fjord-Lab AS Pb. 7 6701 Måløy	**Jan Arne Holm** **97565504** **Jan.arne@fjordlab.no**	Sogn og Fjordane Nordfjord	Pyretroider Emamektin benzoat Salmosan	Nov 2008 Sept 2010	2 – 3. mars 2011

Kystlab	Kystlab AS Eikremsvingen 1 6422 Molde Tlf: 712 50 500 Fax: 712 50 501 www.kystlab.no kystlab@kystlab.no Dragsund 6080 Gurskøy	**Vet. Cecilie Skjengen** **92 89 58 92** **csk@kystlab.no** **Stine Mari Myren** **smm@kystlab.no** **92 47 88 92**	Møre Romsdal Nordmøre	Pyretroider Emamektin benzoat Salmosan	Nov 2008 Sept 2010	16 – 17. februar 2011
Havbruks-tjenesten	Havbrukstjenesten AS Siholmen 7260 Sistranda Tlf: 724 49 377 Fax : 724 49 761	Marianne Halse 99 15 34 45 Marianne@havbrukstjenesten.no **Siri Frafjord Ørstavik** **91 18 43 60** **Siri@havbrukstjenesten.no**	Hitra - Frøya	Pyretroider Emamektin benzoat Salmosan	Des 2008 Sept 2010	21- 22. oktober 2010
Aqua kompetanse	Aqua Kompetanse AS 7770 Flatanger Erstatter Namsos Fiskehelse	**Harriet Romstad** **97 52 24 01** **Fisk-1@online.no**	Flatanger Namdalen	Pyretroider Emamektin benzoat	Nov 2007 Nov 2008 Sept 2010	10 – 11. Nov 2010
Helgeland Fiskehelse	Helgeland Fiskehelse P.boks 100 8827 Dønna Avd. Rørvik	**Bjørn Inge Rikhardsen** **48 14 16 05** **bjorninge@helgelandfiskehelse.no** **Berit Anne Rikhardsen** **95 05 75 54** **beritanne@helgelandfiskehelse.no** Kari Torp **kari@helgelandfiskehelse.no**	Namdalen Ytter Namdalen Sør Helgeland	Pyretroider Emamektin benzoat Salmosan	Mars 2008 Sept 2009 Sept 2010	4 – 5. novembr 2010 10 – 11. november 2011
Helgeland Havbruks-stasjon	Helgeland Havbruks-stasjon Torolv Kveldulvsonsgt. 39 8800 Sandnesjøen Eller: Pb. 21 8808 Sandnesjøen	Kristin Ottesen Kristin.ottesen@fjor-forsk.no **Kristina Birkeland** **99 56 09 16** **kristina@havforsk.com**	Helgeland	Pyretroider Emamektin benzoat	Kurs 2008 Sept 2010	4 – 5. november 2010

Labora	**Labora AS** Notveien 17 8013 Bodø Tlf: 75 56 63 00 Fax: 75 56 63 01	**Eirik Monsen** **95 16 33 61** **Eirik.monsen@laboora.no**	Bodø Nordland	Har vist interesse Sendt bioassay bokser	Sept 2010	
Marin Helse Lyngen		Koen Van Nieuwehove peranton@marinhelse.no			Des 2009	
Vesterålen Fiskehelse-tjeneste	Vesterålen Fiskehelsetjeneste AS Postboks 293 8401 Sortland	Bjarne B. Johansen bjarne@vfh.no **Ørjan Magne Olsen** **99 49 49 36** **oerjan@vfh.no**			Des 2009 Sept 2010	
Fiske-veterinær tjenesten i Alta		Elisbeth Myklebust fiskeveterinaer@alta.kommune.no **Stein Erik Haukanes** **SteinErik.Haukanes@alta.kommune.no**			Des 2009 Sept 2010	

PROTOCOL

Determine the sensitivity to emamectin benzoate (Slice) of field sampled pre-adult and/ or adult male sealice (*Lepeophtheirus salmonis* K.).



Sigmund Sevatdal

May 2011

STUDY DESIGN

This protocol describes determination of the sensitivity for emamectin benzoate (Slice).This bioassay can be performed with pre-adult II (both females and males) and adult males. Evaluation of earlier tests shows a difference in sensitivity between those two categories / stages of salmon lice.

Pre-adults and adult males should be tested in separate petri-dishes, ie one test with pre-adults and one test with adult males according to the evaluation scheme (appendix 2).

PERFORMING THE BIOASSAY – STEP BY STEP

A standardized bioassay should be performed with the following steps in the listed order:

One day prior to sampling

1. Preparing standard seawater by filtration, aeration and temperature adjustment to 12 C.

Day of sampling

2. Sampling of sea lice
3. Transport of sea lice
4. Acclimatization of sea lice to a temp within 11 – 13 C
5. Dilute and prepare exposure solutions (appendix 1)
6. Select and distribute sea lice in groups / petri-dishes with 50 ml exposure solution. Note down group /bioassay box number and times on evaluation scheme (Appendix 2)

Day after sampling

7. Termination and evaluation of response after **24 hours** exposure
8. Estimation of EC50 values and comparing diagnostic EC50 values

IDENTIFICATION OF TEST SUBSTANCES

The test substance is dissolved emamectin benzoate of 100 ppm delivered from Intervet Schering Plough. The batch number must be noted in the evaluation scheme.

IDENTIFICATION OF SALMON LICE

Details regarding the sea lice, as locality, previous treatments, temperature and salinity must be filled in the evaluation scheme.

SAMPLING OF SEA LICE

Collect pre-adult and or adult male salmon lice from Atlantic salmon (*Salmo salar* L.) in seawater. The fish should be anaesthetized [1] or killed by a blow to the head before collection. Collect/ sample lice using forceps or a knife, scraping the salmon skin gently to remove the lice. The tips of the forceps must not be pressed together when handling sea lice during sampling, as this could harm the lice. Place the lice in a container with seawater after sampling. Take care when air temperature is below 0. Even a short time in minus degrees will harm the lice.

TRANSPORT OF SEA LICE

Sea lice are vulnerable to water temperature above 18 ºC and low oxygen concentration. When the air- and/ or water temperature are higher than 10 ºC, the transport sea water should be cooled with ice etc and kept in an isolated box to avoid heating. This water should be kept cold (> 10 ºC) in the period between sampling and performing the bioassay. If the water is cold (< 8 ºC) and air temperature are low, the sea lice can be acclimated to test temperature

[1] AQUI-S must not be used to anaesthetize fish for sea lice sampling

during transport by not using isolation, as the temperature in the car will be around 20 ºC. The container with sea lice should be placed in an isolated box when 12 ºC is reached. The number of sea lice should not excess 50 pr litres of water (approx 500 sea lice in a 10 litre bucket).

ACCLIMATION OF SEA LICE TO TEST CONDITIONS

Sea lice can be used directly if the temperature in the transport water is between 9 – 13 ºC. The sample should be cooled during aeration (aquarium pump) if higher temperature, and heated if lower temperature during aeration by placing the sample in room temperature. The bioassay should be performed within a time period of 6 hours from sampling

SEA WATER AND EXPOSURE CONCENTRATIONS

Seawater of 12 °C with a salinity ranging from 30 – 33 o/oo should be used. The seawater must be absolutely free of organic content and therefore filtered (Coffee-filter) before adding emamectin benzoate and diluting to the exposure concentrations.

The preferred materials for flasks, pipettes, graded gods etc to be used for dilution to avoid adhesion of emamectin benzoate to the walls is unknown to this date.

One litre of each concentration should be made according to appendix 1. Work from dilute solutions to concentrated solutions, ie start with the control group. The temperature must be kept stable at 12 ºC during exposure in an incubator or in a thermo box. The incubator should be controlled by an external thermometer, according to standard laboratory routines[2]. A variation between 11 – 13 ºC can be accepted.

Fill each petri-dish with 50 ml seawater containing emamectin, one concentration in each.

PERFORMING THE BIOASSAY

Pre-adult and male lice with normal behaviour can be used, but exposed in separated petri-dishes according to the evaluation scheme, appendix 2. Select lice with normal behaviour, starting with and completing the control group to avoid contamination. When the control group is fulfilled, select one and one lice to the other concentrations. Do not complete one and one petri-dish with 10 lice, because the total number of lice with normal behaviour is unknown and because overestimation is usual. It is not crucial to have 10 lice in each dish, but one replicate should be complete, ie equal number of lice in all exposure concentrations. Priority according to the evaluation scheme (appendix 2) should be performed if the number of lice of each stage is limited. The lice should be handled with care and they should be active (normal behaviour) at start when they are dropped down in the exposure bath. The selection of lice should be performed during short time, to minimize the period of time at room temperature. Place the test petri-dishes in an incubator at 12 ºC immediately when all available lice are used. Use the time when selecting lice control groups were finished as start of exposure. Note this time on the evaluation scheme (appendix 2).

EVALUATION OF RESPONSE

The response to the pesticide will be evaluated after 24 hours of exposure. Lice can be investigated directly in the petri-dishes. Lice should be classified as live when one of the following criteria is fulfilled:

- fast swimming
- quick reaction if touched

[2] Incubator can be controlled by measure the temperature in 3 – 5 litre of water after 24 h of incubation by a calibrated thermometer.

- capable of sucking to the walls of the dish
- "waging" the tail before sucking to the wall

Typical characteristics for lice to be categorized as inactivated:

- the lice do not move and response to touching with the forceps is slow swimming and in short distances
- the tail has 90 degree angle with rest of the body
- is not able of sucking to the walls of the dish

Note the number of lice in each response category of each exposure group on the evaluation scheme (appendix 2).

STATISTIC AND EVALUATION OF SENSITIVITY

The response data will be analysed by Probit analysis (example: POLO Plus, (http://www.leorasoftware.com). The EC_{50} (- The concentration that immobilize 50 % of the target organism (moribund + dead)) will be estimated, with slope, intercepts and 95 % confidence limits. The result will be compared to diagnostic EC50 values to categorize the sensitivity, according to table 1.

Table 1. Diagnostic EC50 (ppb) values

Sea lice category	Sensitive	Reduced sensitivity	Resistant
Pre-adult II	< 100	> 100 < 200	> 200
Adult males	< 200	> 200 < 400	> 400

TIME-SCHEDULE (DATES)

The dates and time of sampling the test strain of sea lice must be noted on the evaluation scheme (Appendix 3) for each test.

The start time (- the time when selecting lice control groups were finished as start of exposure) must be noted on the evaluation scheme (Appendix 3) for each test.

PERSONS INVOLVED

The person/ persons sampling, performing and/ or evaluating the results must be noted on the evaluation scheme (Appendix 3) for each test.

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APPENDICES

1. List of necessary equipment
2. Evaluation scheme

Appendix 1

List of necessary equipment for and dilution and exposure concentrations

Equipment	**No.**	**Note**
Forceps	2	Curved
Container (5 – 10 l) with lid for transport of lice		
Cooling elements/ ice		For transport
Thermo box, 30 l	1	For transport, the container must fit in the thermo box
Lab. Coat.		
Latex gloves		
Petri-dishes	12 - 24	
Containers, 1.0 L.	6	
Plastic bucket	1	
Bottles 1.0 L.	6	Polypropylene
Pens	2	
Graded cylinder 0.5 L.	2	One for pure seawater (tagged by green), one for emamectin in seawater (tagged by red). Both should be calibrated
Auto pipette or graded	1	1 – 10 ml calibrated
Pippette tips	Min. 100	
Timer	1	
Thermometer	1	calibrated
Incubator 12 ºC	1	
Stock solution with Emamectin benzoate,100 ppm		Delivered by Intervet Scering Plough
Evaluation scheme	1	
Protocol	1	
PC (laptop)	1	
Polo program	1	http://www.leorasoftware.com

Table 1. Dilution and exposure concentrations

Pesticide	**Formu-lation**	**Stock solution**		**Work solution**		**Dilutions**		
		conc. (ppm)	**preparation**	**conc. (ppm)**	**preparation**	**conc. (ppb)**	**ml. work s.**	**ml. seawater**
emamectin benzoate	Ready made stock solution from Intervet Schering Plough	100	5 mg emamectin + 50 ml methanol	1.0	10 ml stock + 990 ml seawater	0	0	1000
						50	50	950
						100	100	900
						200	200	800
						400	400	600
						800	800	200

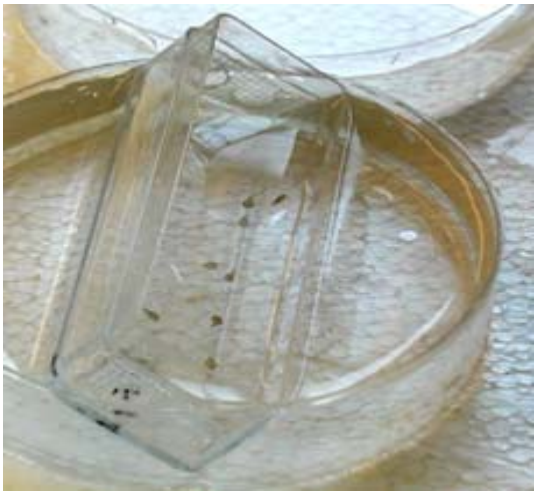
Appendix 2. Evaluation scheme

Date of sampling:				Bioassay performed by:				Previous treatments (type)				
Sampled by:				Laboratory:				Date				
Locality:				Personell:				Results of prev. treatments				
Batch no of emamectin stock solution:												
Stage	Petri-dish no	Dose (ppb)	Priority of replicate*	Time: (24 h exposure)		No of lice in each category		**RESULTS OF BIOASSAY**				
				Start	Evaluation	Live	Inactivated	Estimated EC_{50}	Diagnostic EC_{50} valus			Conclusion
Pre-adult II	1	0	1						Sensitive:	< 100 ppb		
	2	50	1						Red. sensitivity:	> 100 < 200 ppb		
	3	100	1						Resistant:	> 200 ppb		
	4	200	1									
	5	400	1									
	6	800	1									
	7	0	2									
	8	50	2									
	9	100	1									
	10	200	1									
	11	400	2									
	12	800	3									
Adult males	1	0	1						Sensitive:	< 200 ppb		
	2	50	1						Red. sensitivity:	> 200 < 400 ppb		
	3	100	1						Resistant:	> 400 ppb		
	4	200	1									
	5	400	1									
	6	800	1									
	7	0	2									
	8	50	2									
	9	100	1									
	10	200	1									
	11	400	2									
	12	800	3									

* 1- most important, 2 – second most important, 3 – least important

PROTOCOL

Determination of the sensitivity to pyrethroids (AlphaMax – deltamethrin (Pharmaq) and Betamax – cypermethrin (Novartis)) of field sampled sealice (*Lepeophtheirus salmonis* K.).



Sigmund Sevatdal

May 2011

STUDY DESIGN

This protocol describes determination of the sensitivity for AlphaMax and Betamax, performed with one or both substances. Both bioassays can be performed with preadult II (both females and males) and adult males mixed in bioassay-boxes. Both stages have the same degree of sensitivity to pyrethroids.

PERFORMING THE BIOASSAY – STEP BY STEP

A standardized bioassay should be performed with the following steps in the listed order:

One day prior to sampling

1. Preparing standard seawater by filtration, aeration and temperature adjustment to12°C.

Day of sampling

2. Sampling of sea lice
3. Transport of sea lice
4. Acclimatization of sea lice to a temp within 11 – 13°C.
5. Select and distribute sea lice in groups /bioassay-boxes
6. Dilute and prepare exposure solutions (appendix 1)
7. Expose groups and note down group /bioassay-box number and times on evaluation scheme (Appendix 2)
8. Termination of exposure after **30 min**, washing in clean seawater and placing in aerated seawater
9. Observation period of 24 hours

Day after sampling

10. Evaluation of response 24 post end of exposure
11. Estimation of EC_{50} values and comparing diagnostic EC_{50} values

IDENTIFICATION OF TEST SUBSTANCES

The test substance is dissolved AlphaMax or Betamax in seawater (Appendix 1). The batch number should be noted in the evaluation scheme.

IDENTIFICATION OF SALMON LICE

Details regarding the sea lice, as locality, previous treatments, temperature and salinity must be filled in the evaluation scheme.

SAMPLING OF SEA LICE

Collect pre-adult and adult male salmon lice from Atlantic salmon (*Salmo salar* L.) in seawater. The fish should be anaesthetized [1] or killed by a blow to the head before collection. Collect/ sample using forceps or a knife, scraping the salmon skin gently to remove the lice. The tips of the forceps must not be pressed together when handling sea lice during sampling, as this could harm the lice. Place the lice in a container with seawater. Take care when air temperature is below 0, as even a short time in air could harm the lice.

TRANSPORT OF SEA LICE

Sea lice are vulnerable to water temperature above 18 ºC and low oxygen concentration. When the air- and/ or water temperature are higher than 10 ºC, the transport sea water should be cooled with ice etc and kept cold (> 10 ºC) in an isolated box to avoid heating during transport. If the water is cold (< 8 ºC) and air temperature are low, the sea lice can be acclimated to test temperature during transport by not using isolation, as the temperature in

[1] AQUI-S must not be used to anaesthetize fish for sea lice sampling

the car will be around 20 ºC. The container with sea lice should be placed in an isolated box when 12 ºC is reached. The number of sea lice should not excess 50 pr litres of water (approx 500 sea lice in a 10 litre bucket).

ACCLIMATION OF SEA LICE TO TEST CONDITIONS

Sea lice can be used directly if the temperature in the transport water is between 9 – 13 ºC. The sample should be cooled during aeration (aquarium pump) if higher temperature, and heated if lower temperature during aeration by placing the sample in room temperature. The bioassay should be performed within a time period of 6 hours from sampling

SEA WATER AND EXPOSURE CONCENTRATIONS

Seawater of 12 °C with a salinity ranging from 30 – 33 o/oo should be used. The seawater must be free of organic content and therefore filtered (Coffee-filter) before adding pyrethroids and diluting to the exposure concentrations.

The preferred materials for flasks, pipettes, graded gods etc to be used for dilution to avoid adhesion of pyrethroids to the walls is unknown to this date.

One litre of each concentration should be made according to table 1. Work from dilute solutions to concentrated solutions, ie start with the control group. The temperature must be kept stable at 12 ºC during exposure and in the 24 h observation period, in an incubator, water baths or in a thermo box. The temperature should be controlled with a calibrated thermometer during exposure. The incubator should be controlled by an external thermometer, according to standard laboratory routines[2]. A variation between 11 – 13 ºC can be accepted.

PERFORMING THE BIOASSAY

Pre-adult and male lice with normal behaviour can be used. Remove the lids and place all bioassay-boxes in a tray. Fill the boxes with clean seawater. The water level should be at least 0.5 cm. Select and distribute one and one lice to each group. Not complete one and one bioassay-box with 10 lice, because the total number usable lice are unknown and because overestimation is usual. It is not crucial to have 10 lice in each box, but one replicate should be complete, ie equal number of lice in all exposure concentrations. Priority according to the evaluation scheme (appendix 2) should be performed if the number of lice of is limited. Lice should be handled with care and they should be active (normal behaviour) at start when they are dropped down in bioassay-box. Selection and distribution should be performed fast and with routine, to minimize the period at room temperature. Place the bioassay-boxes in well aerated seawater at 12 ºC immediately when all available lice are used.

Prepare the exposure baths as described in appendix 1. Place the bioassay-boxes (without excess water) in the exposure baths, starting with the control. Ensure that all water inside the box is replaced by filling and emptying once with exposure water. Expose from low to high concentration. Note the id (number) of the bioassay-boxes and the exact time on the evaluation scheme (appendix 2). Stop exposure after 30 min by emptying each bioassay-box and wash two times in clean seawater. Place the bioassay-boxes in well aerated seawater of 12 ºC. Note the time of exposure termination on the evaluation scheme. This will be the time for evaluation the following day. The water should be aerated gently the observation period, the next 24 hours. The bioassay-boxes can be tagged with a coloured rubber band if several populations are tested together, one colour for each population

[2] Incubator can be controlled by measure the temperature in 3 – 5 litre of water after 24 h of incubation by a calibrated thermometer.

EVALUATION OF RESPONSE

The response to the pesticide will be evaluated 24 hours after end of exposure. Evaluate one box at the time by placing it in a petri-dish with seawater (as shown on the photo of the front page). The water level should be at least 0.5 cm. Each louse should be inspected individually with a forceps. Lice should be classified as live when one of the following criteria is fulfilled:

- fast swimming
- quick reaction if touched
- capable of sucking to the walls of the dish
- "waging" the tail before sucking to the wall

Typical characteristics for lice to be categorized as inactivated:

- the lice do not move and response to touching with the forceps is slow swimming and in short distances
- the tail has 90 degree angle with rest of the body
- is not able of sucking to the walls of the dish

Note the number of lice in each response category of each exposure group on the evaluation scheme (appendix 2).

STATISTIC AND EVALUATION OF SENSITIVITY

The response data should be analysed by Probit analysis (example: POLO Plus, (http://www.leorasoftware.com). The EC_{50} (- The concentration that immobilize 50 % of the target organism (moribund + dead)) will be estimated, with slope, intercepts and 95 % confidence limits. The result will be compared to diagnostic EC_{50} values to categorize the sensitivity, according to table 1

Table 1. Diagnostic EC_{50} (ppb) values

Substance	Sensitive	Reduced sensitivity	Resistant
AlphaMax	< 0.3	> 0.3 < 1.0	> 1
Betamax	< 1.5	> 1.5 < 7.5	> 7.5

TIME-SCHEDULE (DATES)

The dates and time of sampling the test strain of sea lice must be noted on the evaluation scheme (Appendix 3) for each test.

The start and end time of exposure for each bioassay-box must be noted on the evaluation scheme (Appendix 3) for each test.

PERSONS INVOLVED

The person/ persons sampling, performing and/ or evaluating the results must be noted on the evaluation scheme (Appendix 3) for each test.

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APPENDICES

1. List of necessary equipment and dilution scheme
2. Evaluation scheme

Appendix 1

List of necessary equipment for and dilution and exposure concentrations

Equipment	**No.**	**Note**
Forceps	2	Curved
Container (5 – 10 l) with lid for transport of lice		
Cooling elements/ ice		For transport
Thermo box, 30 l	1	For transport, the container must fit in the thermo box
Lab. Coat.		
Latex gloves		
Bioassay-boxes	12	12 for each bioassay
Containers, 1.0 L.	6	
Plastic bucket	1	
Bottles 1.0 L.	6	
Pens	2	
Graded cylinder 0.5 L.	2	One for pure seawater (tagged by green), one for AlphaMax/ Betamax in seawater (tagged by red). Both should be calibrated
Auto pipette or graded	1	50 – 200 µl calibrated
Auto pipette or graded	1	1 – 10 ml calibrated
Pippette tips	Min. 100	
Timer	1	
Thermometer	1	Calibrated
Incubator 12 ºC	1	
AlphaMax Betamax		deltamethrin/ cypermethrin
Evaluation scheme	1	
Protocol	1	
PC (laptop)	1	
Polo program	1	http://www.leorasoftware.com

Dilution and exposure concentrations

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

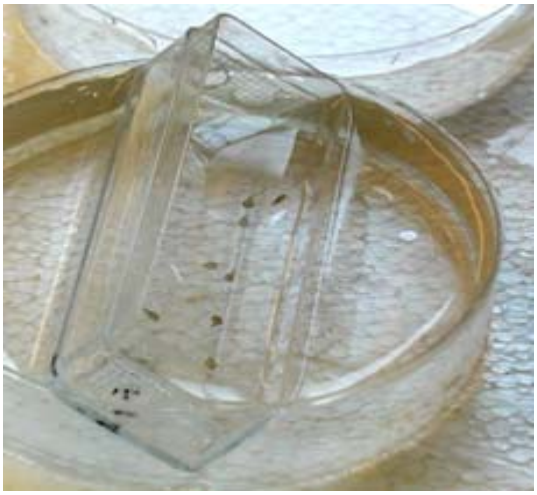
Appendix 2. Evaluation scheme

Date of sampling:				Bioassay performed by:				Previous treatments (type)				
Sampled by:				Laboratory:				Date				
Locality:				Personell:				Results of prev. treatments				
Batch no of AlphaMax/ Betamax:												
Substance	Bioassay-box	Dose (ppb)	Priority of replicate*	Time: (**30 min exposure**)		No of lice in each category		**RESULTS OF BIOASSAY**				
				Start	Evaluation	Live	Inactivated	Estimated EC_{50}	Diagnostic EC_{50} valus			Conclusion
AlphaMax Deltamethrin		0	1						Sensitive: < 0.3 ppb Red. sensitivity: > 0.3 < 1.0 ppb Resistant: > 1.0 ppb			
		0.03	1									
		0.1	1									
		0.3	1									
		1	1									
		3	1									
		0	2									
		0.3	2									
		0.1	1									
		0.3	1									
		1	2									
		3	3									
Betamax Cypermethrin		0	1						Sensitive: < 1.5 ppb Red. sensitivity: > 1.5 < 7.5 ppb Resistant: > 7.5 ppb			
		0.15	1									
		0.5	1									
		1.5	1									
		5	1									
		15	1									
		0	2									
		0.15	2									
		0.5	1									
		1.5	1									
		5	2									
		15	3									

* 1- most important, 2 – second most important, 3 – least important

PROTOCOL

Determination of the sensitivity to azamethiphos (Salmosan - organophosphate) of field sampled sealice (*Lepeophtheirus salmonis* K.).



Sigmund Sevatdal

May 2011

STUDY DESIGN

This protocol describes determination of the sensitivity for the organophosphate azamethiphos (Salmosan). The bioassay can be performed with pre-adult II (both females and males) and adult males mixed in bioassay-boxes. Both stages have the same degree of sensitivity to azamethiphos.

PERFORMING THE BIOASSAY – STEP BY STEP

A standardized bioassay should be performed with the following steps in the listed order:

One day prior to sampling

1. Preparing standard seawater by filtration, aeration and temperature adjustment to 12°C

Day of sampling

2. Sampling of sea lice
3. Transport of sea lice
4. Acclimatization of sea lice to a temp within 11 – 13°C
5. Select and distribute sea lice in groups /bioassay-boxes
6. Dilute and prepare exposure solutions (appendix 1)
7. Expose groups and note down group /bioassay-box number and times on evaluation scheme (Appendix 2)
8. Termination of exposure after **60 min**, washing in clean seawater and placing in aerated seawater
9. Observation period of 24 hours

Day after sampling

10. Evaluation of response 24 post end of exposure
11. Estimation of EC_{50} values and comparing diagnostic EC_{50} values

IDENTIFICATION OF TEST SUBSTANCES

The test substance is dissolved azamethiphos in ethanol (333 ppm stock solution), delivered from VESO (Postboks 300, 0103 Oslo, phone: (+47) 22 96 11 00) (Appendix 1). The batch number should be noted in the evaluation scheme.

IDENTIFICATION OF SALMON LICE

Details regarding the sea lice, as locality, previous treatments, temperature and salinity must be filled in the evaluation scheme.

SAMPLING OF SEA LICE

Collect pre-adult and adult male salmon lice from Atlantic salmon (*Salmo salar* L.) in seawater. The fish should be anaesthetized [1] or killed by a blow to the head before collection. Collect/ sample using forceps or a knife, scraping the salmon skin gently to remove the lice. The tips of the forceps must not be pressed together when handling sea lice during sampling, as this could harm the lice. Place the lice in a container with seawater. Take care when air temperature is below 0, as even a short time in air could harm the lice.

TRANSPORT OF SEA LICE

Sea lice are vulnerable to water temperature above 18 ºC and low oxygen concentration. When the air- and/ or water temperature are higher than 10 ºC, the transport sea water should be cooled with ice etc and kept cold (> 10 ºC) in an isolated box to avoid heating during transport. If the water is cold (< 8 ºC) and air temperature are low, the sea lice can be

[1] AQUI-S must not be used to anaesthetize fish for sea lice sampling

acclimated to test temperature during transport by not using isolation, as the temperature in the car will be around 20 ºC. The container with sea lice should be placed in an isolated box when 12 ºC is reached. The number of sea lice should not excess 50 pr litres of water (approx 500 sea lice in a 10 litre bucket).

ACCLIMATION OF SEA LICE TO TEST CONDITIONS

Sea lice can be used directly if the temperature in the transport water is between 9 – 13 ºC. The sample should be cooled during aeration (aquarium pump) if higher temperature, and heated if lower temperature during aeration by placing the sample in room temperature. The bioassay should be performed within a time period of 6 hours from sampling

SEA WATER AND EXPOSURE CONCENTRATIONS

Seawater of 12 °C with a salinity ranging from 30 – 33 o/oo should be used. The seawater must be free of organic content and therefore filtered (Coffee-filter) before adding azamethiphos and diluting to the exposure concentrations.

The preferred materials for flasks, pipettes, graded gods etc to be used for dilution to avoid adhesion of azamethiphos to the walls is unknown to this date.

One litre of each concentration should be made according to table 1. Work from dilute solutions to concentrated solutions, ie start with the control group. The temperature must be kept stable at 12 ºC during exposure and in the 24 h observation period, in an incubator, water baths or in a thermo box. The temperature should be controlled with a calibrated thermometer during exposure. The incubator should be controlled by an external thermometer, according to standard laboratory routines[2]. A variation between 11 – 13 ºC can be accepted.

PERFORMING THE BIOASSAY

Pre-adult and male lice with normal behaviour can be used. Remove the lids and place all bioassay-boxes in a tray. Fill the boxes with clean seawater. The water level should be at least 0.5 cm. Select and distribute one and one lice to each group. Not complete one and one bioassay-box with 10 lice, because the total number usable lice are unknown and because overestimation is usual. It is not crucial to have 10 lice in each box, but one replicate should be complete, ie equal number of lice in all exposure concentrations. Priority according to the evaluation scheme (appendix 2) should be performed if the number of lice of is limited. Lice should be handled with care and they should be active (normal behaviour) at start when they are dropped down in bioassay-box. Selection and distribution should be performed fast and with routine, to minimize the period at room temperature. Place the bioassay-boxes in well aerated seawater at 12 ºC immediately when all available lice are used.

Prepare the exposure baths as described in appendix 1. Place the bioassay-boxes (without excess water) in the exposure baths, starting with the control. Ensure that all water inside the box is replaced by filling and emptying once with exposure water. Expose from low to high concentration. Note the id (number) of the bioassay-boxes and the exact time on the evaluation scheme (appendix 2). Stop exposure after 60 min by emptying each bioassay-box and wash two times in clean seawater. Place the bioassay-boxes in well aerated seawater of 12 ºC. Note the time of exposure termination on the evaluation scheme. This will be the time for evaluation the following day. The water should be aerated gently the observation period, the

[2] Incubator can be controlled by measure the temperature in 3 – 5 litre of water after 24 h of incubation by a calibrated thermometer.

next 24 hours. The bioassay-boxes can be tagged with a coloured rubber band if several populations are tested together, one colour for each population

EVALUATION OF RESPONSE

The response to the pesticide will be evaluated 24 hours after end of exposure. Evaluate one box at the time by placing it in a petri-dish with seawater (as shown on the photo of the front page). The water level should be at least 0.5 cm. Each louse should be inspected individually with a forceps. Lice should be classified as live when one of the following criteria is fulfilled:

- fast swimming
- quick reaction if touched
- capable of sucking to the walls of the dish
- "waging" the tail before sucking to the wall

Typical characteristics for lice to be categorized as inactivated:

- the lice do not move and response to touching with the forceps is slow swimming and in short distances
- the tail has 90 degree angle with rest of the body
- is not able of sucking to the walls of the dish

Note the number of lice in each response category of each exposure group on the evaluation scheme (appendix 2).

STATISTIC AND EVALUATION OF SENSITIVITY

The response data should be analysed by Probit analysis (example: POLO Plus, (http://www.leorasoftware.com). The EC_{50} (- The concentration that immobilize 50 % of the target organism (moribund + dead)) will be estimated, with slope, intercepts and 95 % confidence limits. The result will be compared to diagnostic EC_{50} values to categorize the sensitivity, according to table 1

Table 1. Diagnostic EC_{50} (ppb) values

Substance	Sensitive	Reduced sensitivity	Resistant
Azamethiphos	< 5	> 5 < 30	> 30

TIME-SCHEDULE (DATES)

The dates and time of sampling the test strain of sea lice must be noted on the evaluation scheme (Appendix 3) for each test.

The start and end time of exposure for each bioassay-box must be noted on the evaluation scheme (Appendix 3) for each test.

PERSONS INVOLVED

The person/ persons sampling, performing and/ or evaluating the results must be noted on the evaluation scheme (Appendix 3) for each test.

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APPENDICES

1. List of necessary equipment and dilution scheme
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Appendix 1

List of necessary equipment for and dilution and exposure concentrations

Equipment	**No.**	**Note**
Forceps	2	Curved
Container (5 – 10 l) with lid for transport of lice		
Cooling elements/ ice		For transport
Thermo box, 30 l	1	For transport, the container must fit in the thermo box
Lab. Coat.		
Latex gloves		
Bioassay-boxes	12	12 for each bioassay
Containers, 1.0 L.	6	
Plastic bucket	1	
Bottles 1.0 L.	6	
Pens	2	
Graded cylinder 0.5 L.	2	One for pure seawater (tagged by green), one for azamethiphos in seawater (tagged by red). Both should be calibrated
Auto pipette or graded	1	1 – 10 ml calibrated
Pippette tips	Min. 100	
Timer	1	
Thermometer	1	Calibrated
Incubator 12 ºC	1	
Stock solution with azamethiphos, 333 ppm		Delivered from VESO AS, Box 300 Sentrum, 0103 Oslo Norway, phone (+47) 22 96 1100
Evaluation scheme	1	
Protocol	1	
PC (laptop)	1	
Polo program	1	http://www.leorasoftware.com

Dilution and exposure concentrations

Stock solution		**Work solution**		**Dilutions**		
conc. (ppm)	**preparation**	**conc. (ppm)**	**preparation**	**conc. (ppb)**	**ml. work s.**	**ml. seawater**
333	5 mg Salmosan + 15 ml ethanol*	1.0	3 ml stock + 997 ml seawater	0	0	1000
				0.5	0.5	999.5
				1	1	999
				5	5	995
				10	10	990
				100	100	900

Appendix 2. Evaluation scheme

Date of sampling:				Bioassay performed by:				Previous treatments (type)				
Sampled by:				Laboratory:				Date				
Locality:				Personell:				Results of prev. treatments				
Batch no of azamethiphos stock solution:												
Substance	Bioassay-box	Dose (ppb)	Priority of replicate*	Time: (**60 min exposure**)		No of lice in each category		**RESULTS OF BIOASSAY**				
				Start	Evaluation	Live	Inactivated	Estimated EC_{50}	Diagnostic EC_{50} valus			Conclusion
Azamethiphos Salmosan		0	1						Sensitive:	< 5 ppb		
		0.5	1						Red. sensitivity:	> 5 < 30 ppb		
		1	1						Resistant:	> 30 ppb		
		5	1									
		10	1									
		100	1									
		0	2									
		0.5	2									
		1	1									
		5	1									
		10	2									
		100	3									

* 1- most important, 2 – second most important, 3 – least important