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Bioassay Workshop

Azamethiphos and Pyrethroids: current bioassays and best practice

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Introduction

- Bioassays are the classic tool for detecting changes in sensistivity to a given insecticide.
- The typical methodology consists of exposing individuals to a gradient of at least 5 different concentrations plus one control, with the largest concentration being that causing 100% of the expected response (Chapman et al., 1996).
- This methodology was applied to monitor the sensitivity of *L. salmonis* and *C. rogercresseyi* to the different antiparasitics used for their control (Sevatdal & Horsberg, 2003; Search Consortium, 2006).

Introduction

The increasing neccessity to evaluate sensitivity resulted in a larger number of laboratory/research groups performing bioassay

The methodology presents several difficulties (White et al., 2013; Helgesen &Horsberg, 2013)

The methodology presents several stages that may introduce variability

Changes in sensitivity between geographical areas (within a country, among countries)

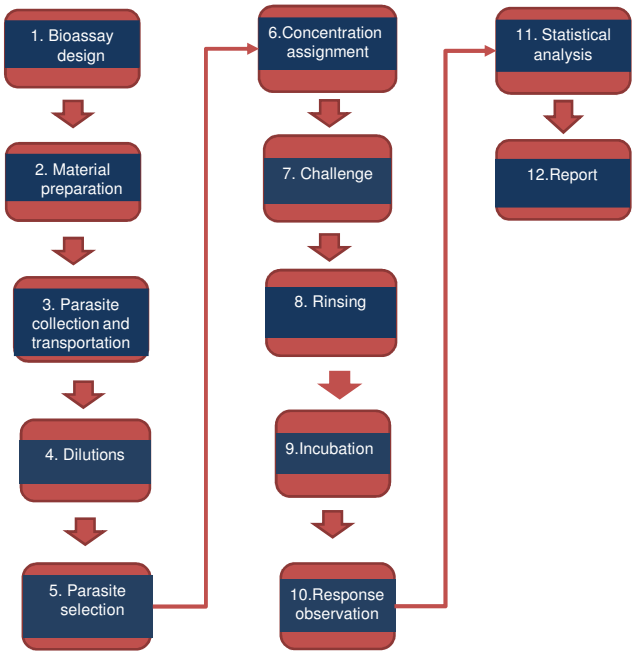
- Differences between protocols
- Difficulties in interpreting the results

Methodology

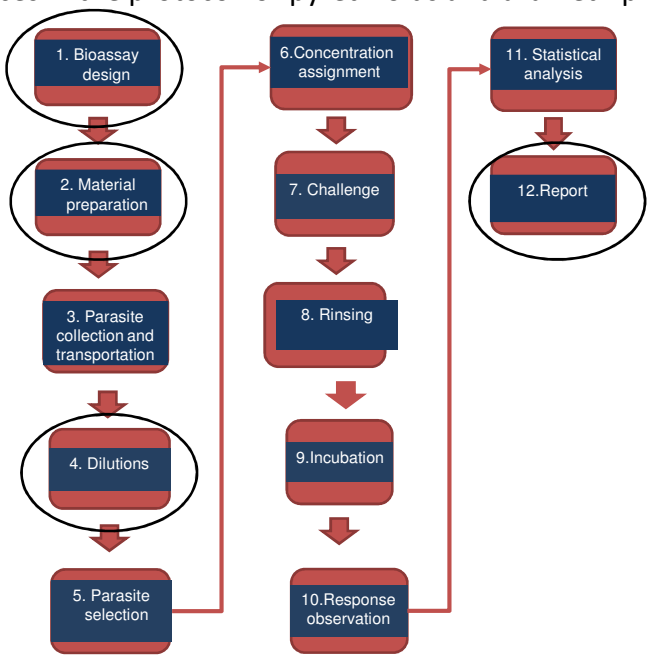
Where to look for best practice in traditional pyrethroid and azamethiphos bioassay methodology?

- Review the current protocols being used in Chilean laboratories for monitoring sensitivity
  - There is no formal sensitivity monitoring program in Chile, all the information that currently exists does so because private companies have requested this analysis from laboratories for their own interests
- Review the available literature regarding sea lice sensitivity bioassays
- Consult people performing bioassays (laboratories, antiparasitic suppliers)

Protocol



Differences in the protocol for pyrethroids and azamethiphos



Where to look for best practice?: traditional bioassay

1. Bioassay design

- ✓ EC_{50} - Concentration gradient: 5 concentrations + control (sea water)
- ✓ Largest concentration must cause 100% of the expected response.
- ✓ It would be useful to include among the 5 concentrations that which the manufacturer recommends for treatments
- ✓ The range of concentrations may need to be adjusted if the maximum concentration does not affect 100% of the exposed individuals
- ✓ Exposure time and concentration: according to the manufacturer's recommendations.
  - ✓ Same active principle have different recommendations or exposure time
  - ✓ Exposure range therefore we should define what is the exposure time for bioassay

Where to look for best practice: classic bioassay

1. Bioassay design

- ✓ 3 replicates of 10 individuals each
- ✓ Determine the number of parasites needed and add in an additional number so you have options if some of them arrive at the lab in poor condition
- ✓ 50% females and 50% males, or just one gender
  - ✓ if gender differences have been detected with respect to sensitivity, it may be better to do bioassays for the less sensitive gender when the number of parasites is limited,
  - ✓ to separately estimate the EC_{50} for each gender.
- ✓ What to do if there are not enough parasites?: remove one concentration?
- ✓ Time between parasite collection and the bioassay: 12 h, 24 h

Where to look for best practice?: traditional bioassay

2. Preparation of material

- ✓ Material for preparing solutions: glass containers
- ✓ Polystyrene boxes versus glass Petri dish
  - ✓ Boxes make it easier to manage parasites than Petri dishes (cleansing)
  - ✓ Glass Petri dishes facilitate observation of the criteria for classifying parasite condition

✓ Polystyrene boxes versus glass Petri dish?

✓ The temperature in the lab should be set to that at which the parasites have been maintained prior to the bioassay and this same temperature should be maintained post exposure up to the time of observation

Where to look for best practice?: classic bioassay

3. Parasite collection and transportation

- ✓ The water quality must be ensured during the transportation of the parasites to the lab: maintain aeration in the containers, monitor the temperature, take along extra water in case it is necessary to replace some of the water, as well as an ice pack to decrease the temperature if necessary
- ✓ Collect additional information from the farm: previous treatments (3), water temperature, salinity, oxygen, cage number, host species, average abundance of parasites
- ✓ Check and register water parameters (temperature, oxygen and salinity) on arrival at the lab

Where to look for best practice?: classic bioassay

3. Parasite collection and transportation

- ✓ Check and register parasite condition on arrival at the lab: use the same criteria as that used to classify parasite condition after bioassay
- ✓ If at this point there is not enough parasite to do 4 concentrations + the control because parasites are floating, or immobile on the bottom, or not reacting to water movement, the bioassay should be cancelled.

Where to look for best practice?: classic bioassay

4. Dilutions

- ✓ Use instruments and volumetric material that enable you to prepare solutions with the least uncertainty and that allow better accuracy in the estimated concentration
- ✓ Dilutions should be prepared as close as possible to the beginning of the bioassay
- ✓ Ensure the dilution is well-mixed and keep it in a dark, temperature-controlled place until its use
- ✓ Nominal versus achieved concentration: Analyze dilutions to determine the concentration achieved in a given frequency (twice a year, for example, depending on how often bioassays are executed.

Where to look for best practice?: classic bioassay

5. Parasite selection

- ✓ Verify that the parasites are healthy
- ✓ Check to ensure there are enough parasites to carry out the experiment as designed (at least 4 concentrations plus the control?)
- ✓ Decide if the bioassay should be executed

Where to look for best practice?: classic bioassay

6. Concentration assignment

- ✓ Individual parasites should be randomly assigned to a given treatment and replicate
- ✓ But this will depend on the form in which parasite exposure to different treatments/concentrations is designed (simultaneously or deferred)
  - ✓ Time required to observe the parasite response
- ✓ Count again the number of parasites in the Petri dish or box
- ✓ Simultaneously or deferred?

7. Challenge

- ✓ Time of parasite exposure must be registered (to define the end of parasite exposure and to make the response observation

Where to look for best practice: classic bioassay

8. Cleansing

- ✓ The form of doing this will depend on the container in which the parasites were exposed (petri dishes or boxes) but in any case the parasites must be washed using fresh sea water
- ✓ The parasites must be returned to fresh sea water

9. Incubation

- ✓ The parasites should be kept at a controlled temperature for the period defined for the response evaluation
- ✓ Water aeration is possible when using boxes but not Petri dishes

Where to look for best practice: classic bioassay

10. Response observation

- ✓ Response criteria for defining parasite condition after exposure

From Wescott et al. 2008

Table 1. *Lepeophtheirus salmonis*. Bioassay response criteria for sea lice (adapted from Sevatdal & Horsberg 2003)

Response	Criteria
Live	(1) Normal swimming behavior (ability to swim in a straight line) (2) Securely adheres to Petri dish (3) Normal movement of extremities
Moribund	(1) Disabled swimming but capable of weak uncoordinated movement (loop to loop swimming) (2) Inability to firmly adhere to Petri dish (adherence to dish for a period before dropping off) (3) Minimal movement of extremities
Dead	(1) Inability to swim (2) Floating in Petri dish (3) No movement of extremities

Igboeli et al. (2012): live, weak, moribund and dead.

- Weak: refers to parasites that display poor and irregular swimming and are unable to attach to the Petri dish.
- Moribund refers to immotile parasites with twitching appendages

Where to look for best practice?: classic bioassay

10. Response observation

- ✓ This would allow a more precise estimate of the moribund condition
- ✓ The weak category may help to exclude from the EC_{50} estimates those moribund parasites that may recover 24 hours post exposure
- ✓ Should this category be included in a standardized protocol?
- ✓ Bioassays in which a mortality rate of more than 20% of the sea water control is observed after 24 hours should be disregarded

Where to look for best practice: classic bioassay

10. Response observation

- ✓ Time post exposure for observing the response: 24 – 48 hours
- ✓ It has been reported that Deltamethrin EC_{50} stabilizes at 24 hours post exposure, so 24 hours would be the appropriate time for evaluating the sea lice response.
- ✓ Azamethiphos: in the 26% (n=30) of the bioassays 100% mortality 48 hours post exposure was achieved at different concentration than 24 hours post exposure
- ✓ 48 hour observations are useful for monitoring the mortality caused by the exposure as long as the mortality in the control is not statistically different from that observed at 24 hours post exposure.
- ✓ It has been reported that 24 hours post exposure is not enough to evaluate mortality

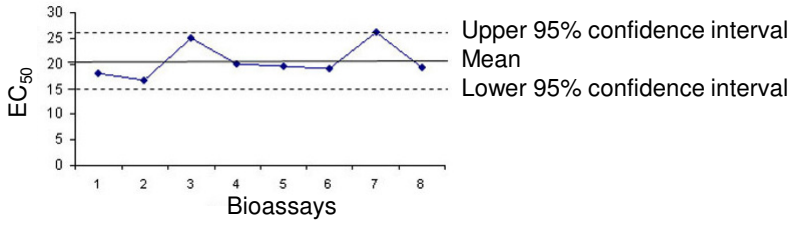
Where to look for best practice: classic bioassay

10. Statistical analysis: EC_{50} estimate

- ✓ Various approaches
- ✓ There are bioassays for which the EC_{50} and its confidence interval can be estimated and seem to be reasonable but the X^2 indicates that the data do not meet model assumptions
- ✓ This happens frequently, should we discharged those bioassay in which X^2 indicates that the data do not meet model assumptions?
- ✓ In the case of azamethiphos, the analysis should be done using the concentration of the active ingredient rather than the commercial product.
- ✓ There is an additional procedure included in the protocol of a Chilean laboratory to intra-laboratory calibration

Control chart

Intra-laboratory precision is described by the mean, standard deviation, and percent coefficient of variation, or CV of the calculated endpoints from the replicated tests.



Usepa. 1993. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. Office of Research and Development, U.S. Environmental Protection Agency, Washington D.C. 206460.

Control chart

- Canadian service of environmental protection (1990): minimal intra-laboratory precisión is a CV = 30%.
  - If CV is larger than 30%, the test should not be included in the control chart.
- If CV is less than 30% but the EC_{50} value is beyond the control limits should be analyzed carefully or considered as provisory.
- When a minimum of 20 data points are available chart should be should be maintained using only the 20 most recent data points

Where to look for best practice?: classic bioassay

11. Report

- ✓Bioassay report: focussed on a farm
  - ✓Farm and company data, date of sampling and bioassay execution, water parameter at the time of arrival at the lab
  - ✓Short description of the methodology, including the range of concentrations
  - ✓Data obtained 24 and 48 hours post exposure
  - ✓EC_{50} and its statistics (95% CI, R^2, X^2 and p)
  - ✓LC_{50} when 100% of mortality is achieved at the maximum concentration

Where to look for best practice?: classic bioassay

11. Report

- ✓Sensitivity surveillance report: Norway National Program is a good initiative that would allow to detect spatial and temporal sensitivity patterns that can not be detected by independent bioassays

Summary of questions

1. What would be the exposure time when manufacturer indication is a range?
2. Should we estimate EC_{50} for each gender separately?
3. What to do if there are not enough parasites to execute the bioassay?
4. What is the appropriate time to execute the bioassay after parasite collection?
5. Were do we prepare antiparasitic solutions, glass containers?
6. Where do we exposure parasites to antiparasitics, polystyrene boxes or glass Petri dish?
7. Should we test nominal concentrations?
8. What are the categories of parasite response after exposure?

Summary of questions

9. What would be the observation time of parasite response, 24 or 48 ?
10. Should we discharged those bioassay in which X^2 indicates that the data do not meet model assumptions?
11. Should we include intra-laboratory calibration?

Acknowledgements

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