Effect of essential oils on the viability of *Caligus rogercresseyi* using a novel *in vitro* assay based on the inhibition of lice frontal filament formation

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Introduction

- Johnson *et al.* (2004) estimated the economic impact of sea lice infestation in salmon farming in about US$ 100 million.
- In 2007, after the efficacy of the Emamectin benzoate was reduced against *Caligus rogercresseyi* in Chilean salmon farming, the estimated economic impact was about US$ 200 million (Bustos, 2007).
- Consequences of *Caligus* infestation:
  - reduction in fish performance
  - impairment of fish osmoregulation
  - possible vector for fish pathogens like Infectious Salmon Anemia virus (ISAv) and *P. salmonis* (SRS)
  - impact wild fish infestation rates
- To improve the control, other anti-sealice products were allowed by the Chilean authorities (Deltamethrin and Diflubenzuron)
- However, in the long term, we believe the control of sea lice must include management practices and other innovative feed interventions

Introduction

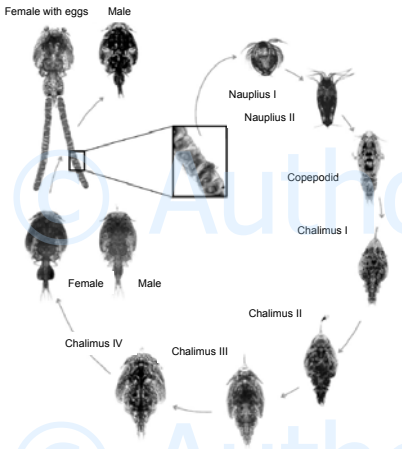

Figure 1. Life cycle of *C. rogercresseyi* (from González & Carvajal, 2003).

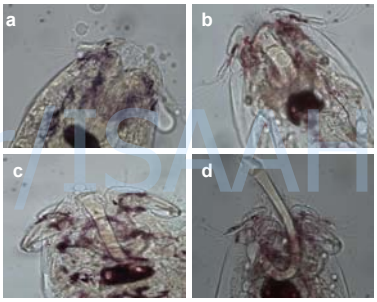

Figure 2. Development of frontal filament in copepodids of *Caligus rogercresseyi*: a) **copepodid** (infestive stage), b) **copepodid a** (with frontal filament into the body), c) **copepodid b** (frontal filament partially everted), d) **copepodid c** (frontal filament completely everted).

Introduction

- **Features**
  - It has been shown that a mixture of essential oils including extracts from neem, cinnamon, thyme, cloves and sesame are able to control pests in agriculture by means of contact, ingestion and as repellents.
  - Products of this characteristics have been developed and successfully marketed for use in programs of organics crops and for the replacement of synthetic pesticides in Mexico and other countries.
  - Due to the chemical characteristics and proved efficacy of these compounds in agriculture, it was interesting to evaluate its effects against sea lice infestation.

Objectives

Determine the LD50 of a mixture of essential oils derived from neem, cinnamon, thyme, cloves and sesame against copepodids and adults of *Caligus rogercresseyi.*

Assess the capacity of copepodids of *Caligus rogercresseyi* to form the frontal filament when exposed to different doses of the essential oils mixture

Material and Methods

- LD50 assay
  - During December 2008 studies were conducted to determine the LD50 of the mixture of essential oils (MEO) against copepodids and adults of *C. rogercresseyi*.
  - Copepodids and adults of sea lice were obtained from a rearing unit in Fundación Chile, Puerto Montt.
  - The MEO was tested at 10 different doses: 0.18, 0.96, 1.75, 9.63, 13.56, 17.50, 52.50, 87.50, 131.25, 175 ppm, with five replicates each.
  - Doses were calculated to achieve a volume of 40 mL per each Petri dish with microfiltrated (5 µm), aerated and sterilized seawater (UV light at 70 µW/cm^3).
  - 20 copepodids and 10 adults were placed in their respectively Petri dish and then incubated in an incubation chamber (14°C) for 48 hrs. The mortality of copepodids was evaluated at 1, 4, 19, 24, 43 and 48 h. The mortality of adults was evaluated at 3, 12, 18, 24, 36 and 48 h.

Material and Methods

Material and Methods

- Indirect viability test of copepodids of *Caligus rogercresseyi* exposed to the MEO:
  - The MEO was tested at 4 doses: 0, 0.175, 6.869 and 13.563 µL/L, with three replicates each. Twelve (12) containers of 2.5 L were filled with 2 L of microfiltrated (5 µm), aerated and sterilized seawater (UV light at 70 µW/cm^3).
  - Two Petri dishes, with salmon mucus and for each dose, were placed at the bottom of each container. Mucus was obtained previously from an Atlantic salmon (*S. salar*) brood stock facility.
  - 50 to 100 copepodids were allocated in each container to recognize the mucus attractants and attach to the agar-mucus mix, through the frontal filament.
  - After 48 hrs in an incubation chamber (14°C), the copepodids were extracted and fixed in alcohol solution with distilled water and glycerol. Fixed copepodids were dehydrated at constant temperature of 30° for 1 days.
  - Measurement of the presence/absence of the frontal filament in copepodids was performed with an optical microscope (40x lens).

Material and Methods

Mucus sampling
From ATS

Copepodids

Prepare agar+mucus+MEO

2 Petri dishes/container

48 h
14°C

Remove, fix and dry
Copepodids
(1 day, 30°C)

Logit Analysis

Count presence/absence
of frontal filament

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Results

1) LD50 bioassay of the MEO in copepodids of *C. rogercresseyi*

- The control group and the lowest dose of MEO did not induce any observable mortality of copepodids up to 24 h (Figure 3).
- Doses ≥0,96 µL/L induced different levels of mortality (Figure 3).
- 100% of mortality was obtained with doses ≥87.50 µL/L after 1 h of exposure.

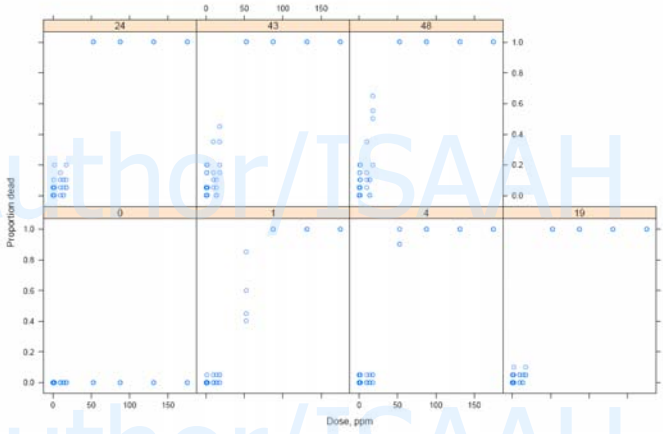

Figure 3. Proportional dead of copepodids of *C. rogercresseyi* over a period (48 h) of exposure to different doses of the MEO.

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Results

1) LD50 bioassay for the MEO in copepodids of *C. rogercresseyi*

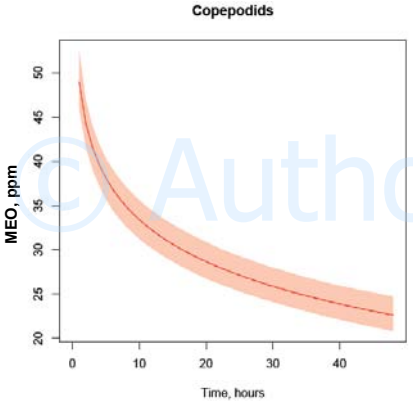

Figure 4. LD50 values predicted from the parametric log-linear time model with 95% credible intervals (shadowed area). Solid line: simulated mean LD50, dashed line: simulated median LD50.

- There is a time-dose response, indicating that as the dose of MEO increased, the LD50 of copepodids occurs earlier (Figure 4).
- After 48 h of exposure, the LD50 was estimated in 21.58 µL/L, with lower and upper confidence limits of 18.5 and 25.8 µL/L, respectively (Table 3).

Table 3: Estimated LD50 values for each exposure time with the MEO over copepodids of *C. rogercresseyi*.

Exposure time	Mean	Median	95% interval
1	49.35	49.36	45.39–53.64
4	35.61	35.47	31.57–40.52
19	30.52	30.29	26.35–36.07
24	29.00	28.71	25.01–34.25
43	25.06	24.83	21.64–29.54
48	21.58	21.32	18.54–25.78

Results

2) LD50 bioassay of MEO in adults of *C. rogercresseyi*

- The control group did not induce any observable mortality of lice up to 48 h (Figure 5).
- Doses ≥9,62 µL/L induced more evident mortality of adults (Figure 5).
- 100% of mortality of adults was obtained only in the highest dose (52.5 µL/L) after 24 h of exposure.

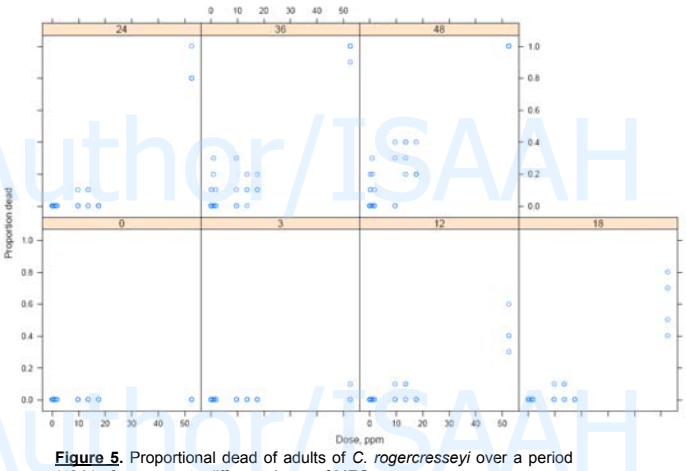

Figure 5. Proportional dead of adults of *C. rogercresseyi* over a period (48 h) of exposure to different doses of MEO.

Results

2) LD50 bioassay of MEO in adults of *C. rogercresseyi*

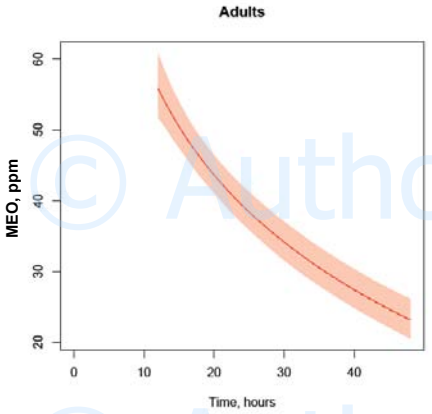

Figure 6. LD50 values predicted from the parametric log-linear time model with 95% credible intervals (shadowed area). Solid line: simulated mean LD50, dashed line: simulated median LD50.

- There is a time-dose response, indicating that as the dose of the MEO increased, the LD50 of adults occurs earlier (Figure 6).
- After 48 h of exposure, the LD50 was estimated in 22.05 µL/L, with lower and upper confidence limits of 18.37 and 27.35 µL/L, respectively (Table 4).

Table 4: Estimated LD50 values for each exposure time with MEO over adults of *C. rogercresseyi*.

Exposure time	Mean	Median	95% interval
12	55.80	55.40	49.67–64.40
18	49.03	48.89	44.06–54.98
24	40.64	40.59	35.83–45.61
36	29.35	29.09	24.61–35.44
48	22.05	21.80	18.37–27.35

Results

3) Frontal filament inhibition in copepodids of *C. rogercresseyi* treated with the MEO

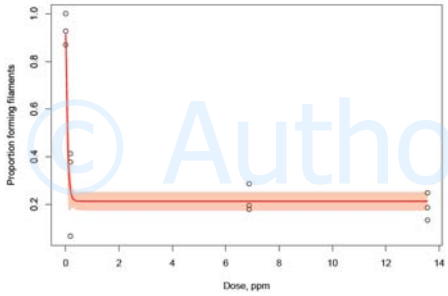

Figure 7. Inhibition in the development of the frontal filament in copepodids of *C. rogercresseyi* with different doses of the MEO. Model fit to the frontal filament development proportions. Shadowed area shows approximate 95% confidence intervals.

- There is minimum level (0.175 µL/L) of MEO that inhibited more than 71% of frontal filaments in copepodids, compared with the control ($p<0.05$) (Table 5).

Table 5: Calculated proportion (%) of inhibition when exposed to different doses of the MEO:

MEO, µl/L / Replication	N° of Parasites with development of FF/Total			
	0	0,175	6,869	13,563
1	28/28	7/17	15/84	9/68
2	73/84	3/46	33/116	12/65
3	89/96	20/53	7/36	18/73
	Inhibition (%) in the development of the FF			
1	0	58.82	82.15	86.77
2	13.10	93.48	71.55	81.54
3	7.29	62.27	80.56	75.34
Average, %	**6.80**	**71.52**	**78,08**	**81,22**

Conclusions

- The MEO tested in these experiments proved to induce high mortality of copepodids and adults of *C. rogercresseyi*, with estimated LD50 (after 48h of exposure) of 21.6 ppm and 22.1 ppm, respectively.

- The MEO has the potential to reduce the attachment of *C. rogercresseyi* to salmon because it showed to inhibit the development of the frontal filaments in copepodids. The dose that inhibited 71% of frontal filament (0.175 ppm) is about one hundredth of the LD50 observed to kill the sea lice after 48 h of exposure.

- Future research should evaluate the toxicity and efficacy of the MEO in salmon conducting *in vivo* studies.